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ORIGINAL ARTICLE

Glutathione Peroxidase 3 Serum Levels and *GPX3* Gene Polymorphisms in Subjects with Metabolic Syndrome

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Background and Aims. Glutathione peroxidase 3 (GPx3) plays a main role in removing hydro- and lipoperoxides from the body. Changes in concentration and several single-nucleotide polymorphisms (SNP) at the *GPX3* gene have been associated with vascular diseases, but the relationship of GPx3 with metabolic syndrome (MetS) remains unexplored. We undertook this study to determine the association of GPx3 serum levels and several *GPX3* SNPs with the presence of MetS in Mexican subjects.

Methods. Clinical, biochemical, and anthropometric evaluation were conducted in 426 subjects assigned to three groups: control ($n = 42$); risk group (RG, $n = 200$), and MetS group ($n = 184$). Insulin sensitivity (IS) and cardiovascular risk were determined by the QUICKI and TG/HDL-C index, respectively. Serum GPx3 was determined by enzyme immunoassay and polymorphisms within *GPX3* gene were identified by nucleotide sequencing.

Results. MetS group showed low IS and increased cardiovascular risk with respect to controls as well as higher GPx3 serum levels (172.9 ± 32.2 vs. 145.6 ± 24.8 ng/dL; $p < 0.05$). Only three of the ten *GPX3* SNPs screened were polymorphic with two haplotypes observed (CCT and TTA—rs8177404, rs8177406, and rs8177409), indicating tight linkage disequilibrium in this genetic region. No differences for either genotype or allele frequencies among groups were observed, but rs8177409 (allele T) was associated with cardiovascular risk (odds ratio [OR], 4.5; $p = 0.0125$).

Conclusion. This study shows that serum levels of GPx3 are increased in subjects with MetS and that rs8177409 SNP was associated with cardiovascular risk in a Mexican population.

Key Words: Metabolic syndrome, Glutathione peroxidase 3, Cardiovascular risk, *GPX3*·gene polymorphisms.

Introduction

Metabolic syndrome (MetS) is considered a public health problem worldwide, especially in ''westernized'' countries (1). The parallel increase in the prevalence of overweight/obesity and MetS is a global phenomenon and Mexico is

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not an exception, with a prevalence of 71.28% for overweight/obesity and of 39.7% for MetS (2,3).

MetS is a risk factor for developing type 2 diabetes (T2D), ischemic heart disease, and arteriosclerosis-associated stroke (1,4), which are the main causes of mortality and jointly account for about 30% of the total deaths in Mexico (5).

Recent studies have suggested that MetS may be the result of different but interrelated pathophysiological mechanisms such as insulin resistance, endothelial dysfunction, low-intensity inflammatory process, visceral obesity, oxidative stress (OS), and genetic factors (4,6,7). Different studies have found that individuals with MetS have altered antioxidant protection (8,9) as well as an increase in oxidative damage (10).

Among these antioxidant processes, glutathione peroxidase 3 (GPx3) exerts a main role and is the only extracellular member of theGPx family that scavenges the reactive oxygen species (ROS) produced during normal metabolism or after an oxidative insult (11). As a major antioxidant enzyme in plasma, GPx3 maintains the bioavailability of nitric oxide (NO) in the vasculature (12). Deficiencies of the cellular and plasma isoforms of GPx have been associated with cardiovascular disease in several clinical reports (13−15).

In a previous study by our research group (16), we found that Mexican subjects with overweight/obesity exhibited increased cardiovascular risk as well as higher serum levels of the antioxidant enzyme GPx3, compared with lean subjects. It has been reported that GPx3 shows changes both in gene expression and in protein concentration in animal models of T2D and obesity (17,18), but the variability of GPx3 levels in different human populations and under different health conditions is currently unclear.

There are a few studies that have evaluated the influence of single-nucleotide polymorphisms (SNP) in the *GPX3* gene promoter region in several clinical settings; thus, it has been established that individuals who are carriers for certain *GPX3* allele combinations (CCTCCTTC −942, −927, −861, −568, −518 −302,−284, and −65) have a higher risk of ischemic stroke (19) and the occurrence of cerebral venous thrombosis compared with subjects not exhibiting these polymorphisms (20,21). *GPX3* allele diversity in several populations including Brazilian, Caucasian, African, and Asian (19) has been analyzed, but in Latin American admixed populations, this information is unknown.

To our knowledge, there are no studies assessing the influence of SNP on the *GPX3* gene or circulating GPx3 serum levels on MetS that allow comprehensive understanding of the role of this antioxidant enzyme in fat and glucose metabolism. Therefore, this research was aimed to evaluate the involvement of GPx3 levels and *GPX3* SNPs in Mexican subjects with MetS.

Materials and Methods

Subjects and Setting

This study is part of the Mexican Diabetes Prevention Study (MexDiab Study), which had the participation of 10,589 adults and 5,623 children from 11 states of the Mexican Republic. The subjects of the present study (n = 426) were Mexican Mestizos who resided in the city of Puebla, of both genders (excluding women who were pregnant or breastfeeding), aged between 18 and 60 years, and with or without a family history of diabetes. Subjects with incomplete clinical history or who had ongoing chronic inflammatory (arthritis, rhinitis, and trauma), endocrine (hyperthyroidism and hypothyroidism), or any chronic disease (except hypertension and hyperlipidemia) were excluded from the study. Use of medications, alternative treatments, smoking, and alcoholism were also considered exclusion criteria as well as subjects with a previous diagnosis of T2D.

The study was approved by the Scientific Research and Ethics Committee of the Instituto Mexicano del Seguro Social (IMSS). MetS was defined according to the criteria of Third Report from the Adult Treatment Panel (ATP III) of the National Cholesterol Education Program (NCEP) criteria (22) with waist circumference (WC) values adjusted to Mexican population (NCEP-ATP IIIm) (23). Diagnosis of MetS was established if three or more of the following risk factors were present: blood pressure (BP) ≥130/85 mmHg; fasting glucose ≥100 mg/dL; triglycerides (TG); ≥150 mg/dL; high-density lipoprotein-cholesterol (HDL-C) <40 in males and <50 mg/dL in females, and WC ≥90 cm in males and ≥80 cm in females.

Subjects were classified into three study groups: (1) control group (n = 42) comprised of subjects without overweight or obesity (body mass index [BMI] <25 kg/m^2), fasting glucose <100 mg/dL, TG <150 mg/dL, HDL-C, ≥40 mg/dL and BP <130/85 mmHg (24); (2) risk group (n = 200) comprised of subjects without MetS with one or two risk factors, and (3) MetS group (n = 184) comprised of subjects with MetS according to NCEP ATP-IIIm criteria.

Clinical Characterization

Anthropometric measurements such as height, weight, and percentage of body fat (%BF) were determined using an electronic digital scale (Tanita Body Composition Analyzer, Model TBF-215, Tokyo, Japan) (scale capacity, 200 kg). Waist circumference was measured at the midpoint between the highest point of the iliac crest and the lowest point of the costal margin at the mid-axillary line using a non-stretching anthropometric measuring tape. Body mass index was calculated using the Quetelet formula (25).

Blood pressure was determined in a sitting position and after 5 min of rest according to the Mexican Official

Standard (24) for the prevention, treatment, and control of hypertension, using a Baumanometer (Microlife AG, Heerbrugg, Switzerland) and a stethoscope (3M LITTMAN Classic II; Neuss, Germany).

Biochemical Characterization

Following an overnight fast (10−12 h) by the study participants, blood samples were obtained by venipuncture. Glucose, insulin, total cholesterol (TC), TG, and HDL-C levels were measured according to conventional laboratory protocols using the periodic end-point method. Plasma glucose levels and lipid profile were determined using the Synchron CX5 Analyzer System (Beckman Coulter, Fullerton, CA). Insulin concentration was determined by chemiluminescence in a sandwich enzyme immunoassay utilizing anti-insulin mouse monoclonal antibodies with alkaline phosphatase (ALP) (Beckman Coulter Access System).

Insulin sensitivity (IS) was calculated according to the quantitative insulin sensitivity check index (QUICKI) with the following formula: QUICKI = 1/(log Insulin + log glucose mg/dL); values <0.357 are representative of low IS, based on what was reported by Hrebıcek et al. (26), whereas this cut-off point correlated more strongly than the cut-off calculated for study population (0.329, data not shown) with MetS-related variables.

Normal values of TC levels <200 mg/dL, TG levels <150 mg/dL, and HDL-C levels ≥40 mg/dL were considered according to the NCEP-ATP III (22). Low-density lipoprotein-cholesterol (LDL-C) was determined using the formula cited in NOM-037-SSA2-2002 for the prevention, treatment, and control of dyslipidemia (27) in which very-low-density lipoprotein-cholesterol (VLDL-C) = TG/5 and LDL-C = TC − (VLDL-C + HDL-C). The TG/HDL-C index was calculated by dividing the concentration of TG by HDL-C. The cut-off point for cardiovascular risk was TG/HDL-C >3 as determined by Boizel et al. (28) and taking into account a recent study reports (29) that this index (used cutoff point of 3) is associated with the presence of insulin resistance in apparently healthy Mexican subjects.

Glutathione Peroxidase Analysis

For determination of the GPx3 serum concentration, blood collection was carried out in dry tubes, allowed to stand for 15−20 min, and then extraction of the serum fraction was performed. Serum determination of GPx3 was carried out using the glutathione peroxidase 3 (GPx3) EIA kit (ALPCO Diagnostics, Salem, NH) following the supplier's directions. All measurements were performed by duplicate, and inter- and intra-assay coefficients of variation were 4.0 and 1.1%, respectively.

Identification of GPX3 *SNP*

GPX3 polymorphism analysis was conducted in three study groups: 1) MetS group, subjects with MetS; 2) non-MetS group, subjects with 0, 1 or 2 diagnostic criteria of MetS, and 3) general population group; this latter group was used only to establish the Hardy-Weinberg Equilibrium (HWE).

Genomic DNA was purified from 5.0 mL of peripheral blood using a conventional method (30). For genotyping, primers (GPx3-F 5′-cttgctgggtttcccactcagttt-3′ and GPx3-R 5′-tcccttcctcttttggctccaaag-3′) flanking 581 base pairs (bp) of the promoter region of the *GPX3* gene (from position −642 to −61 according to the sequence reported at Gene Bank (accession number: NM_002084.3) were designed with FastPCR software (31). The expected fragment included ten known SNPs rs8177403, rs8177404, rs8177405, rs181590329, rs61381043, rs8177406, rs8177407, rs8177408, rs8177409, and rs6888961.

Polymerase chain reaction (PCR) was performed in a final volume of 50 µL containing 1X of 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mMKCl) (Invitrogen Corp., Carlsbad, CA), 1.5 mM $MgCl_2$ (Promega Corp., Madison, WI), and 5 mM of GPx3-F and GPx3R primers, 0.2 mMdNTP, and 1.25 U of Taq DNA polymerase (Invitrogen Corp.).

Amplification was carried out in a thermocycler (GeneAmp PCR System 9700; Applied Biosystems) and at a temperature of 95°C, with 2 min of initial denaturing followed by 30 cycles including 95°C for 30 sec, 56°C for 40 sec, and 72°C for 30 sec. Finally, extension temperature was 72°C for 7 min.

Amplicon products were purified using the GF-1 Gel DNA Recovery kit (Vivantis, Inc., Oceanside, CA) according to the manufacturer's specifications.

The purified PCR product (250 ng) was sequenced using either of the following sequencing primers: F: 5′-ccactcacatttctgcgcatttcc-3′ or R: 5′-gcagacacagcggtctccattac-3′, with the GenomeLab Dye Terminator Cycle Sequencing kit (Beckman Coulter, Inc.) and a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems) under conventional cycle sequencing.

Sequencing reaction products were purified with the CleanSeqAgencourt Dye-Terminator Removal kit (Agencourt Bioscience, Beverly, MA) according to the manufacturer's specifications. Sequencing was performed in the CEQ 8800 Genetic Analysis System (Beckman Coulter).

Restriction Nuclease Analysis

In addition to sequencing, the rs8177409 polymorphism (formerly known as −302A>T), localized 149 bp upstream of the *GPX3* gene transcription start site, was detected by means of a nuclease digestion approach. Approximately 0.5 µg of 581-bp amplified DNA was incubated at 37°C for 1 h with 2.5 units of *Bfa*I restriction endonuclease (New England BioLabs, Ipswich, MA) in

a reaction volume of 10 μL. Digestion products was electrophoresed on 6.0% polyacrylamide gel for 30 min at 180 V. Samples carrying the A allele showed two restriction fragments (88 bp and 493 bp), whereas in T-allele carriers this recognition site was missing, leaving the whole amplicon. All sequenced amplicons were re-genotyped by this approach and no mismatches were observed using these two strategies. Because data on *GPX3* gene polymorphisms in Mexican population was not available at time of this study, a sample consisting of individuals from the same locality (general population) was genotyped by DNA sequencing ($n = 93$) and *Bfa*I-restriction analysis ($n = 106$) to evaluate this population's usefulness for genetic studies of the considered rs8177409 polymorphism.

Statistical Analysis

The Kurtosis Normality of Residuals test was used to determine the normality of data distribution. Continuous variables with normality and equal variances were analyzed using one-way analysis of variance (ANOVA). When not normality but equal variances were observed, a Kruskal-Wallis test was used. Nonparametric continuous variables were analyzed using the Mann-Whitney *U* test, and to establish their proportions, Fisher exact test was utilized. Spearman correlation test was used for variables. Allele frequencies were obtained by the method of counting from observed genotypes. Allele and genotype frequency comparisons among the study groups, as well as for determining for the HWE, were performed using the conventional χ^2 test, with the Fisher correction when necessary. To analyze the association between SNP and metabolic variables, we utilized multinomial logistic regression. Data were analyzed with SPSS software (v.12.0 for Windows; SPSS, Inc., Chicago, IL). Differences between groups were considered significant at $p < 0.05$.

Results

Anthropometric characteristics of study subjects including GPx3 serum levels are depicted in Table 1. Significant differences ($p < 0.01$) among groups for variables such as age, weight, WC, BMI, and %BF were observed. Subjects with MetS as compared with risk- and control-group subjects had the highest levels of systolic blood pressure (SBP) (113.9 ± 14.5 vs. 103.3 ± 11.1 and 96.6 ± 11.0 mmHg, respectively) and of diastolic blood pressure (DBP) (76.6 ± 9.7 vs. 69.5 ± 7.8 and 66.1 ± 8.5 mmHg, respectively ($p < 0.01$).

A significant elevatedcoronary heart disease risk (5.7 ± 2.9, 2.5 ± 0.9 vs. 1.45 ± 0.7; $p < 0.01$) was observed in MetS and risk-group individuals with respect to controls. On the other hand, MetS- and risk-group subjects presented low IS in contrast with the control group (0.334 ± 0.02 and 0.365 ± 0.03 vs. 0.398 ± 0.02 ($p < 0.01$), respectively. To determine whether increases in plasma GPx3 protein correlated with low insulin sensitivity or with cardiovascular risk, correlation analysis using these variables were performed. This analysis showed a significant relationship between GPx3 serum levels and IS (rho = 0.1344; $p < 0.05$) and the TG/HDL-C index (rho = 0.1290; $p < 0.05$). No significant correlation was observed with other variables related to MetS.

Table 1. Characteristics of study subjects

	Control group $n = 42$	Risk group $n = 200$	MetS group $n = 184$
Gender (M/F)[d]	14/28*	24/176**	46/138*
Age (years)[c]	30.9 ± 10.7*	36.6 ± 11.2**	41.2 ± 10.8***
Weight (kg)[c]	56.3 ± 8.5*	67.1 ± 13.0**	76.2 ± 14.2***
WC (cm)[c]	76.7 ± 5.2*	90.4 ± 11.3**	99.6 ± 11.2***
BMI (kg/m^2)[c]	22.1 ± 1.8*	27.3 ± 4.6**	30.6 ± 4.7***
TBF (%)[a]	21.8 ± 6.3*	33.2 ± 7.5**	36.7 ± 6.8***
FG (mg/dL)[c]	86.4 ± 5.1*	88.9 ± 6.8**	99.7 ± 10.3***
FI (μU/mL)[c]	4.1 ± 1.5*	7.0 ± 3.3**	11.7 ± 5.7***
TG (mg/dL)[c]	82.8 ± 30.9*	105.7 ± 37.5**	198.8 ± 84.4***
HDL-C (mg/dL)[b]	59.9 ± 13.3*	45.3 ± 11.0**	37.3 ± 8.6***
GPx3 (ng/dL)[a]	145.6 ± 24.8*	173.1 ± 34.7**	172.9 ± 32.2**

MetS, metabolic syndrome; M, masculine; F, feminine; WC, waist circumference; BMI, body mass index; TBF, total body fat; FG, fasting glucose; FI, fasting insulin; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; GPx3, glutathione peroxidase 3.
Results are expressed as mean ± standard deviation (SD).
[a]Comparison performed using one-way analysis of variance (ANOVA).
[b]Comparison performed using Kruskal-Wallis one-way ANOVA.
[c]Comparison performed using Mann-Whitney *U* test.
[d]Comparison performed using F-test for two proportions. Different literal (*, ** or ***) in row indicates significant difference ($p \leq 0.05$) among groups.

Sequencing of 581 bp of the promoter region of *GPX3* gene, which includes 10 known SNP (rs8177403, rs8177404, rs8177405, rs181590329, rs61381043, rs8177406, rs8177407, rs8177408, rs8177409, and rs6888961), revealed low nucleotide variability of this gene fragment in Mexican population. Only rs8177409, rs8177406, and rs8177404 SNPs were polymorphic and their genotype and allele frequencies are shown in Table 2. As previously mentioned, these polymorphisms were alternatively screened by restriction analysis in a sample of the general population ($n = 106$) from the same locality as the study groups and in MetS ($n = 98$) and in non-MetS ($n = 85$) randomly selected subjects. The genotype frequencies are consistent with expectations under HWE ($p > 0.05$) and no differences in allele frequencies among groups were observed. Complete linkage disequilibrium ($D' = 1.00$) among the three polymorphic loci was detected; thus, only two haplotypes were observed (CTCGCTCCAT and CCCGCCCCTT) in which presence of a given allele in one of the three polymorphic sites predicts the presence of specific alleles in whichever other positions. For example, allele A at rs8177409 always runs with alleles T and C at rs8177406 and rs8177404, respectively. Therefore, further

Table 2. Genotype, allele, and haplotype frequencies of SNP rs8177404, rs8177406, and rs8177409 of the *GPX3* gene

SNP	GP group *n* (%)	Non-MetS group *n* (%)	MetS group *n* (%)	*p*, HWE[b]
rs8177404				
Genotype				
TT	86 (92.5)	78 (90.7)	56 (88.9)	0.1547
TC	6 (6.4)	8 (9.3)	7 (11.1)	
CC	1 (1.1)	0	0	
Allele				
T	178 (95.7)	164 (95.3)	119 (94.4)	
C	8 (4.3)	8 (4.7)	7 (5.6)	
rs8177406				
Genotype				
TT	86 (92.5)	78 (90.7)	56 (88.9)	0.1547
TC	6 (6.4)	8 (9.3)	7 (11.1)	
CC	1 (1.1)	0	0	
Allele				
T	178 (95.7)	164 (95.3)	119 (94.4)	
C	8 (4.3)	8 (4.7)	7 (5.6)	
rs8177409[a]				
Genotype				
AA	98 (92.5)	89 (90.8)	75 (88.2)	0.1353
AT	7 (6.6)	9 (9.2)	10 (11.8)	
TT	1 (0.9)	0	0	
Allele				
A	203 (95.8)	187 (95.4)	160 (94.1)	
T	9 (4.2)	9 (4.6)	10 (5.9)	
Haplotype				
CTCGCTCCAT	203 (95.8)	187 (95.4)	160 (94.1)	
CCCGCCCCTT	9 (4.2)	9 (4.6)	10 (5.9)	

SNP, single-nucleotide polymorphism; HWE, Hardy-Weinberg Equilibrium; GP, general population; MetS, subjects with metabolic syndrome; non-MetS, subjects without diagnosis of metabolic syndrome.
Haplotype formed by 10 known (SNP) (rs8177403, rs8177404, rs8177405, rs181590329, rs61381043, rs8177406, rs8177407, rs8177408, rs8177409, and rs6888961). Only rs8177409, rs8177406, and rs8177404 SNP were polymorphic, and these are underlined in the table.
[a]rs8177409 (−302 A/T) by *Bfa*I-restriction analysis for GP ($n = 106$), non-MetS ($n = 98$), MetS ($n = 85$).
[b]HWE analysis done for GP sample ($p \geq 0.05$) χ^2 test.

analysis among *GPX3* SNPs only considered data for rs8177409. Because neither allelic nor genotypic differences between the non-MetS and MetS group were found, it was decided to analyze the association between the presence of SNP rs8177409 and cardiovascular risk measured by the TG/HDL-C index, regardless of the presence or absence of MetS. A significant association of this SNP (allele T) with cardiovascular risk was observed (Table 3).

Discussion

In a previous study we found that patients with overweight or obesity had increased serum levels of antioxidant enzyme GPx3 compared with control subjects (16). In continuity, the influences of other variables including SNP on *GPX3* gene and GPx3 serum level analysis that could impact MetS were evaluated.

The main finding of the present study was that subjects with MetS showed a higher concentration of serum GPx3 than control subjects, and these concentrations correlated with cardiovascular risk (TG/HDL-C index) and with IS (QUICKI index). According to recent studies, it has been suggested that GPx3 concentration changes depending on the disease stage (18,32) or BMI (16,33). The difference in GPx3 concentration between the study groups can be explained with that reported by Chung et al. (18). They found that the expression of GPx3 is induced by activation of the peroxisome proliferator activated receptor gamma (PPAR-γ). Different studies have reported that both expression of PPAR-γ and GPx3 are increased in the human adipose tissue preadipocyte (34) and are involved in its differentiation to the adipocyte (17,35−37). Moreover, previous studies have shown that GPx3 expression may decrease in adipose tissue due to an increment in prooxidant conditions, an increase in tumor necrosis factor-alpha (TNF-α) concentration, and hypoxia (17,33). According to the above, Montague et al. (33) reported that GPx3 levels could be regulated by the TNF-α concentration in the organism. The production of this pro-inflammatory cytokine is decreased in subjects with a BMI <35 kg/m^2 in which there is greater differentiation of adipocytes and where GPx3 expression is not being inhibited (32). In our study, subjects in the risk- and MetS-group had a BMI 27.3 ± 4.6 kg/m^2 and 30.6 ± 4.7 kg/m^2, respectively, suggesting that the concentration of GPx3 is not being inhibited. On the other hand, levels of TNF-α were not determined. We need to measure this inflammatory cytokine to verify this proposal.

A deficiency in GPx3 has been associated with increases in extracellular peroxide-related oxidants and inhibition of the insulin pathway (18). This decreased in terms of bioavailable NO, hypothesized to contribute to promoting platelet activation, endothelial dysfunction, platelet-dependent thrombosis, and vascular thrombotic disease (38). Thus, plasma GPx may be a key factor in determining

Table 3. Relationship between rs8177409 SNP of the *GPX3* gene and cardiovascular risk

	OR_{crude}, (95% CI)	P_{crude}	$OR_{adjusted}$, (95% CI)[a]	$P_{adjusted}$[a]
A allele	0.2628 (−2.4749 to −0.1976)	0.0214	0.2216 (−2.6845 to −0.3242)	0.0125
T allele	3.8049 (0.1976−2.4749)	0.0214	4.5123 (0.3242−2.6895)	0.0125

SNP, single-nucleotide polymorphisms; OR, odds ratio; 95% CI, 95% confidence interval.
[a]Adjusted for age, gender, and body mass index (BMI); $n = 183$, no-MetS ($n = 98$) plus MetS ($n = 85$) groups, considered as one under a dominant inheritance genetic.

when disease processes activating platelets result in arterial thrombosis (39).

MetS is a heterogeneous clinical condition of multifactorial etiology in which environmental as well as genetic factors contribute to its development (4). Genetic association studies on *GPX3* gene SNP have been conducted and an influence of certain *GPX3* risk alleles for vascular diseases, a main MetS complication, such as cerebral venous thrombosis (21) and arterial ischemic infarction (20), have been proposed for Brazilian, Caucasian, African and Asian population. However, it is unknown whether, in populations with a different genetic background such as Mexican, these observations remain both in these and in other clinical settings.

In association studies, it is necessary to first determine some population genetic parameters for marker-of-interest such as allele frequencies and HWE, among others, from the same region where patients/subjects reside to discard bias due mainly to population genetic substructure. Therefore, and due to absence of data for *GPX3* polymorphisms in Mexican population, in the present study we genotyped individuals from the general population of the city of Puebla, Mexico and from the same ethnic background (Mestizos). No deviations of genotype frequencies from those expected according HWE were observed either in the general population sample or any of the other groups analyzed, which make us confident to discard sampling bias due to population substructure.

Sequence analysis revealed only three polymorphic sites for the Mexican population (rs8177404, rs8177406, and rs8177409) which, in turn, exhibited complete linkage disequilibrium. Therefore, information obtained from any of these sites could be applied to the remainder of these. Specifically for SNP rs8177409, which was initially denominated −302 A>T (20,21) and for a recognition site for the restriction nuclease *Bfa*I present, a simple PCR-restriction fragment length polymorphism (RFLP)-based protocol for genotyping was designed that, in turn, permitted us to validate our genotype data obtained from DNA sequencing and to provide an alternate cost-effective genotyping approach compared with nucleotide sequencing. Minor allele (−302T) for this polymorphism accounts for an average of roughly 5% among all groups, similar to that reported for other populations of different ethnic origin including African, Asian, and Caucasian (20). Although allele frequencies were not statistically different among the three study groups (non-MetS group, MetS group, and general population group), there was a slight increase in the T allele in the Met Sgroup with respect to subjects without MetS and the general population group.

Previous studies have found the presence of eight *GPX3* haplotypes (−942A>C, −927T>C, −861A>T, −568T>C, −518T>C, −302A>T, −284T>A, and −65T>C), of which H2 consisting of CCTCCTTC (same loci order) conferred a risk for developing arterial ischemic infarction (20) and cerebral venous thrombosis (21). It also has been found that, specifically, sites −568, −518, and −302 conform a unique block strongly linked with and bordered by recombination sites between these positions and −861 and −284. Opposed to that is our finding of only three polymorphic sites (−568, −518, and −302) and only two haplotypes, suggesting that the length of the unrecombination block could be greater in Mexicans than that reported for other populations. This could imply that, in this population, it will be sufficient to analyze only the −302 site in order to search for any association with a given phenotype. Future studies are warranted in other Latin American populations known to be genetically related to the Mexican population in order to corroborate or discard our suggestion.

In previous studies (20,21), the association was reported of SNP in the promoter region of the *GPX3* gene with cardiovascular disease such as cerebral venous thrombosis and arterial ischemic stroke. However, although in the present study the SNPs −568, −518, and −302 of *GPX3* were not associated with MetS (cardiovascular disease risk), the TG/HDL-C index was associated with the presence of the T allele of the −302 A>T polymorphism (rs8177409), even when adjusted for age, gender, and BMI (Table 3).

In patients with T2D and MetS, the fact that LDL particles may be small has not been ruled out. Although techniques for analyzing LDL subfractions are not likely to be used in clinical practice, a prediction of LDL size based on a regular lipid profile may be useful for assessment of cardiovascular risk, as the TG/HDL index is (28). This index was validated in subjects with T2D (28) and has been widely used in patients with metabolic disorders such as obesity (16), insulin resistance (29) and MetS (23). A recent study suggested that this index is adequate for MetS diagnosis to identify insulin-resistant subjects (40), and it has been proposed as the sole predictor of angiographically defined coronary artery disease (41).

The TG/HDL-C index may be related with the processes involved in LDL-size pathophysiology and relevant with regard to risk for clinical vascular disease. It may be suitable for selection of patients requiring earlier and aggressive treatment of lipid abnormalities (28), and SNP rs8177409 may serve as a biomarker of cardiovascular risk.

Some limitations of this study merit mention. First, the small sample size of the control group was due to difficulty in finding persons who complied with the parameters for consideration in metabolic balance and who did not have overweight/obesity, a situation commonly faced in this type of study. Second, longitudinal studies are needed to establish the role of GPx3 in the development of MetS and T2D as well as to determine the variation of GPx3 activity and of other antioxidant enzymes over time as well as variation in the levels of oxidation markers. Finally, Mexican subjects have different genetic backgrounds; therefore,

further studies in patients with T2D or diagnosed cardiovascular disease from other regions of the country are needed to establish whether *GPX3* gene polymorphisms could entertain some relationship with the evolution of the disease in a Mexican population.

In conclusion, to our knowledge, this study shows for the first time that serum levels of *GPX3* are increased in Mexican subjects with MetS and that the increase in *GPX3* correlated with the TG/HDL-C index and with IS. Also, the promoter region of *GPX3* shows strong linkage disequilibrium and only three sites (−568, −518, and −302) could be considered as usable genetic markers in a Mexican population for future genetic association studies.

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