Assessment of biochemical parameters and characterization of *TNFα* -308G/A and *PTPN22* +1858C/T gene polymorphisms in the risk of obesity in adolescents

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Abstract. Obesity is currently considered an inflammatory condition associated with autoimmune diseases, suggesting a common origin. Among other factors, candidate genes may explain the development of this disease. Polymorphisms in the tumor necrosis factor α (*TNFα*) and lymphoid protein tyrosine phosphatase (*PTPN22*) genes lead to an increased risk to development of immune and inflammatory diseases. The aim of the present study was to analyze the biochemical parameters and the effect of the *TNFα* -308G/A and *PTPN22* +1858C/T polymorphisms in the susceptibility of adolescents to obesity. A group of 253 adolescent subjects were recruited and classified as obese, overweight or normal weight according to their nutritional status. Anthropometric measurements, clinical and biochemical data were analyzed. DNA was extracted from peripheral blood samples by the phenol-chloroform method, and *TNFα* -308G/A and *PTPN22* 1858C/T polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism assays. Clinical, genetic and biochemical parameters were analyzed to determine the existence of a possible association with the development of obesity. Statistically significant differences in body mass index, insulin, triglyceride levels and homeostatic model assessment for insulin resistance (HOMA-IR) index were observed among the three groups analyzed (P≤0.05). The studied polymorphisms did not confer a risk for developing obesity in the analyzed population (P>0.05); however, significantly low levels of insulin and decreased rates of HOMA-IR were observed in the 1858 CT genotype carriers of the *PTPN22* gene. In conclusion, no association between the *TNFα* -308G/A and *PTPN22* +1858C/T polymorphisms and the risk to development of obesity in the adolescent population analyzed was observed. However, the 1858 CT genotype of the *PTPN22* gene was associated with variations of certain biochemical parameters analyzed.

Introduction

Obesity is a chronic disease characterized by hyperplasia and/or hypertrophy of adipose tissue as a result of a positive energy balance, which occurs when energy intake exceeds energetic expenditure (1). Although the 'obesogenic environment' contributes to the development of obesity, this does not explain the inter-individual variability that occurs in the susceptibility of this pathology (2). It has been reported that this disease contributes to an increasing number of pathologies, such as diabetes mellitus type 2 (DM2), cardiovascular disease, cancer, hyperlipidemia and metabolic syndrome (3,4), hence the importance of their study. Obesity is established indirectly by the body mass index (BMI), as this corporal value correlates with fat (5).

Obesity is considered a complex and multifactorial disease, as it is the result of an interaction between genetic, behavioral and environmental factors that may influence individual response to diet and daily physical activity (6,7).

Evidence indicates that the accumulation of body fat has a strong genetic background for monogenic and polygenic forms

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of obesity (3). Furthermore, strong evidence suggests the involvement of genes that influence the energy homeostasis and thermogenesis, adipogenesis, leptin-insulin signaling and peptide hormones, which have a role in developing this pathology (4).

Currently, there are >100 genes associated with obesity (4,8). A recent study showed the involvement of the adipose tissue in the regulation of processes, such as inflammation and immunity; in which the major human histocompatibility complex (MHC) has a key role in the activation of these processes in adipocytes (9).

The adipocytes in obese subjects secrete less anti-inflammatory adiponectin and increase the expression of pro-inflammatory factors, which induces a response in immune cells. Tumor necrosis factor α (*TNFα*) has been studied in a wide range of immune diseases in the Mexican population, including the polymorphism located at position -308 of the promoter region of the *TNFα* gene (10-14), however, the association with obesity in this population has not yet been elucidated.

While the MHC locus appears to be a genetic factor that confers susceptibility to autoimmune diseases, variants in certain non-MHC genes share risk factors between different diseases of this nature. Polymorphic variants of the *PTPN22* gene encoding protein tyrosine phosphatase lymphoid, have been associated with various immune diseases (15). The *PTPN22* +1858C/T polymorphism has been associated with susceptibility to diseases, such as diabetes mellitus, Graves' disease and rheumatoid arthritis in different populations, including Mexico (16,17). Although this polymorphism has been associated with the development of diabetes mellitus, it has not been associated with obesity (18).

As it has been suggested that this disease may have an inflammatory and immune origin, the present study examined the possible involvement of the polymorphisms in the *TNFα* -308G/A and *PTPN22* +1858C/T genes with the susceptibility to the development of obesity and its association with clinical biochemical characteristics and the nutritional status of individuals belonging to an adolescent population of the state of Coahuila (Mexico), in order to evaluate their efficacy as markers for early detection of this disease and its complications.

Materials and methods

Subjects. A group of 253 Mexican adolescents from the Coahuila state were classified into 3 groups, according to the nutritional status using data issued by the World Health Organization (WHO; 2007) on the basis of age-specific body mass index (BMI/age): Obese (≥95th), overweight (85th-94.9th) and normal weight (5th-84.9th percentile). Written consent to participate was requested from the parents and adolescents. The study protocols were performed in accordance with the ethical standards and were approved by the University Hospital-Autonomous University of Columnbia Review Board and registered under the code 01-2011.

The study protocol included a background interview, anthropometric measurements, and venous blood sampling for the biochemical and genetic analyses.

Biochemical analysis. To determine the biochemical parameters of glucose, total cholesterol, low-density lipoprotein (LDL),

high-density lipoprotein, triglycerides and insulin, 5 ml of peripheral blood samples were extracted from the participants who fasted for 12 h. These biochemical values were used to calculate the homeostatic model assessment for insulin resistance (HOMA-IR) and the atherogenic index. Biochemical parameters were measured using an SLFIA immunoassay and a TOSOH AIA-600 analyzer (Tosoh Corp., Tokyo, Japan) for insulin, and a Clinical Chemistry Analyzer Diconex InCAA model (Diconex, Buenos Aire, Argentina) for the remaining analyzed parameters.

DNA isolation. Genomic DNA was extracted from peripheral venous blood cells using the phenol-chloroform method, precipitated in ethanol and finally resuspended in Tris-EDTA (pH 7.8) at a concentration of 0.1-1.0 μg/μl.

Genotyping of the TNF -308G/A and the PTPN22 +1858C/T polymorphisms. Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using an MJ Mini PTC1148 thermal cycler (Bio-Rad, Hercules, CA, USA).

For the *TNFα* -308G/A polymorphism, a modified version of the Chen *et al* (19) method was used. PCR was performed in 25 μl using 0.5 μM primers (Integrated DNA Technologies, Coralville, Iowa, USA) (forward, 5'-GGGACACACAAGCAT CAAGG-3' and reverse, 5'-AATAGGTTTTGAGGGCCA TG-3'), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 units Taq DNA polymerase (Promega, Madison, WI, USA) and a total amount of 250 ng of genomic DNA. The PCR program consisted of 35 cycles at 94°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec. The 142 base-pair (bp) PCR amplicons (~1 μg) were digested overnight with *Nco*I (New England Biolabs, Ipswich, MA, USA) at 37°C, and the generated fragments were analyzed by electrophoresis in 3% agarose gels using ethidium bromide staining and were documented in a UVP model 2UV High Performance Transilluminator (UVP, Inc., Upland, CA, USA). The products obtained during this process were 126 bp and 16 bp fragments for the wild-type G allele, and 142 bp (non-digested PCR product) for the variant A allele.

The *PTPN22* +1858C/T polymorphism analysis was performed using a modified method reported by Harrison *et al* (20), using 0.5 μM of each primer (Integrated DNA Technologies) (forward, 5'-ATGTTGCTTCAACGG AATTT-3' and reverse, 5'-CATGCTGCTATTGCTCTG CT-3'), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Promega) and a total amount of 250 ng of genomic DNA. The PCR program consists of 33 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The 400-bp PCR amplicons (~1 μg) were digested overnight with *Xcm*I (New England Biolabs) at 37°C, and the generated fragments were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide and analyzed in a gel documentation system (UVP model 2UV High Performance Transilluminator). The products obtained during this process were 238 bp and 162 bp fragments for the variant T allele, and 400 bp (non-digested PCR product) for the wild-type C allele.

Statistical analysis. For the sample size calculation, it was assumed that the coefficient of variation was 22% for the

Table I. Distribution of adolescent subjects according to gender and nutritional status.

Nutritional status	Female	Male	Total, n (%)
Normal, n	39	31	70 (27.7)
Overweight, n	16	6	22 (8.7)
Obese, n	88	73	161 (63.6)
Total n (%)	143 (56.5)	110 (43.5)	253 (100.0)

BMI. Considering a significance level of 5%, a minimum power of 80%, an Ω of 0.25, and a confidence interval of 90%, a sample size of 15/group is sufficient. To detect any significant differences in the values of the biochemical parameters among the analyzed groups of the nutritional status and genotype, comparisons were made between 2 groups (normal versus overweight, normal versus obese, and overweight versus obese), and Student's t-test or the Mann-Whitney U test were used for parametric or non-parametric distributions, respectively. Differences between >2 groups were assessed by one-way analysis of variance and the Kruskal-Wallis H test for parametric or non-parametric distributions, respectively. Post hoc tests (least significant difference and Tamhane's T2) were used for pairwise comparisons. To confirm the contribution of the genetic factors to the variability of the biochemical parameters, a linear regression analysis was undertaken. Possible associations between genotypes and biological parameters were assessed using contingency tables, χ^2 statistics and Fisher's exact tests. Odds ratios were estimated with 95% confidence intervals. The aforementioned analyses were performed with SPSS for Windows, V.20 (IBM Corp., Armonk, NY, USA). All P-values were two-tailed. The corrected P (Pc) values were adjusted using Bonferroni's correction to exclude spurious associations. P<0.05 was considered to indicate a statistically significant difference. Hardy-Weinberg equilibrium (HWE) was obtained using a goodness-of-fit test.

Results

Subject characteristics. The population studied consisted of 253 adolescent subjects (age range, 11-16 years; mean, 13.49 years), from Saltillo (Coahuila, Mexico), categorized according to the BMI percentile tables of WHO: Obese (161 subjects), overweight (22 subjects) and normal weight (70 subjects) (Table I).

Distribution of adolescent subjects. The distribution of the average levels of glucose, insulin, lipid profile, BMI, HOMA-IR and atherogenic index in the analyzed groups are described in Table II. Statistically significant differences were identified in BMI, insulin levels, HOMA-IR and triglyceride levels among all the three groups analyzed (P≤0.05). There was a significant difference between the normal weight and obese group in all the biochemical parameters analyzed (P≤0.05) (Table II).

Analysis of TNFα -308 G/A and PTPN22 +1858C/T polymorphisms. The molecular analysis of the *TNFα* -308 G/A and *PTPN22* +1858C/T gene polymorphisms by PCR-RFLP showed that the predominant genotype for the polymorphism of the *TNFα* gene was G/G (94.9%; 65 normal weight subjects, 20 overweight and 155 obese), the heterozygous genotype G/A was present in smaller amounts (5.1%; 5 normal weight subjects, 2 overweight and 6 obese), while the homozygous genotype A/A was absent in the studied population. For the *PTPN22* gene, the C/C (94.5%; 63 with normal weight subjects, 21 overweight and 155 obese) polymorphism was the predominant genotype, and the heterozygous genotype C/T was observed in minor amounts (5.5%; 7 normal weight individuals, 1 overweight and 6 with obesity), while the

Table II. Comparison of the biochemical values obtained for each study group, defined according to their nutritional status.

	Nutritional status		
Biochemical parameters	Normal weight, n=70	Overweight, n=22	Obesity, n=161
BMI, kg/m^2	18.88±1.91[a,b]	23.33±1.26[c]	28.99±3.92
Glucose, mg/dl	81.03±9.88[b]	80.18±8.78[c]	89.78±11.68
Insulin, mU/ml	8.34±4.01[a,b]	14.70±9.84[c]	21.90±12.71
HOMA-IR	1.71±0.90[a,b]	2.94±2.07[c]	4.86±3.14
Triglycerides, mg/dl	69.86±34.57[a,b]	89.32±44.38[c]	121.05±58.90
Cholesterol, mg/dl	150.47±22.27[b]	152.68±27.32	162.82±31.40
HDL, mg/dl	64.31±10.98[a,b]	54.09±14.66	51.92±12.71
LDL, mg/dl	80.42±18.94[b]	90.58±25.30	100.79±27.85
Atherogenic index	2.36±0.34[a,b]	3.11±1.14	3.29±0.94

P<0.05 for [a]normal versus overweight; [b]normal versus obese; [c]overweight versus obese. BMI [a]P=1.50x10^{-11}, [b]P=1.66x10^{-33}, [c]P=4.73x10^{-12}; glucose [b]P=8.29x10^{-8}, [c]P=1.55x10^{-4}; insulin [a]P=4.63x10^{-5}, [b]P=4.41x10^{-22}, [c]P=6.51x10^{-4}; HOMA-IR [a]P=5.12x10^{-4}, [b]P=1.05x10^{-20}, [c]P=3.77x10^{-4}; triglycerides [a]P=0.051, [b]P=6.84x10^{-13}, [c]P=0.007; cholesterol [b]P=0.002; HDL [a]P=0.01, [b]P=2.55x10^{-11}; LDL [b]P=2.65x10^{-9}; atherogenic index [a]P=5.77x10^{-4}, [b]P=1.24x10^{-16}. Data shown are mean ± standard deviation; BMI, body mass index; HOMA, homeostasis model assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table III. Association of the *TNFα* -308 G/A and *PTPN22* +1858 C/T polymorphisms with the biochemical variables obtained for the study population.

Biochemical parameters	*TNFα* -308 genotype		*PTPN22* +1858 genotype	
	GG, n=240	GA, n=13	CC, n=239	CT, n=14
Glucose, mg/dl	86.70±11.86	83.31±9.66	86.77±11.76	82.29±11.59[a]
Insulin, mU/ml	17.60±12.08	16.15±16.44	17.96±12.44	9.98±5.90[b]
HOMA-IR	3.84±2.93	3.45±3.76	3.92±3.01	2.08±1.39[c]
Triglycerides, mg/dl	105.26±56.95	83.23±50.72	105.54±57.19	80.07±43.90
Cholesterol, mg/dl	159.32±29.24	143.85±26.76	159.37±29.33	144.00±24.87[d]
HDL, mg/dl	55.58±13.71	54.72±10.33	55.41±13.61	57.72±12.55
LDL, mg/dl	94.66±26.53	86.99±34.05	95.02±26.63	81.47±29.90[e]
Atherogenic index	3.03±0.94	2.75±0.95	3.04±0.94	2.59±0.75

[a]P=0.024, Pr=0.166; [b]P=0.005, Pr=0.018; [c]P=0.006, Pr=0.024; [d]P=0.072, Pr=0.056; [e]P=0.088, Pr=0.067. Data shown are mean ± standard deviation. HOMA, homeostasis model assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Pr, P-value of linear regression analysis.

homozygous T/T genotype was absent in the study population. No statistically significant association was identified between polymorphisms analyzed and the presence of overweight and obese subjects in the population (P>0.05). The two genotypes were in HWE in the population studied (P>0.05).

To determine the possible effect of the genotypes identified for *TNFα* and *PTPN22* with the biochemical values of the studied population, mean comparisons between genotype groups were performed. According to the P-values, carriers of the CT genotype of the *PTPN22* gene exhibited a decrease in glucose levels, insulin levels, and HOMA-IR index (P≤0.05), and a trend towards reduced levels of LDL and cholesterol (Table III). Linear regression analysis using the aforementioned genetic models confirmed that these polymorphisms affected the variability of these biochemical parameters.

Discussion

In recent decades, obesity has been increasing and it is projected to increase across all age groups in Mexico (21). Several clinical and biochemical parameters have been used to assess obesity and associated diseases (22-24). The BMI and biochemical parameters identified in obese children in the present study showed a positive association with this nutritional status; they have been evaluated previously only in other overweight and obese Mexican populations, however, the previous cut-off points of the biochemical parameters are lower than those reported in the present study population (25,26).

Few studies have analyzed the involvement of *TNFα* and *PTPN22* gene polymorphisms and their susceptibility to obesity in several populations. The studies worldwide have reported mixed results, finding associations between polymorphisms described for the *TNFα* gene (27,28), or lack of association with risk of developing obesity (29-31). Currently in Mexico, the association between biochemical parameters in subjects with different nutritional status has been studied,

however, no investigations have focused on determining the association between the genetic component, biochemical parameters and nutritional status of individuals. A clear association was identified between increased body weight and insulin resistance and dyslipidemia in the adolescent population in the present study. Although no association between the presence of polymorphisms of the *PTPN22* and *TNFα* genes and the development of overweight and obesity in this population was found, these results are consistent with those previously described in the Chilean (29), German (30) and Danish populations (31). A tendency of decreased triglycerides and cholesterol levels in the GA genotype carrier population of the *TNFα* gene was observed, and this result is consistent with those previously described for the Mexican population >16 years of age (32). Furthermore, for the *PTPN22* +1858C/T polymorphism there has only been an association with development of rheumatoid arthritis (17) and vitiligo (33); however, in the present study, the *PTPN22* +1858CT genotype showed a marked decrease in insulin and HOMA-IR with a statistical significance (P≤0.05) and a trend towards decreased levels of cholesterol and LDL. Similar results have been described for serum lipid levels for the analyzed polymorphism of the *TNFα* gene (32). For the *PTPN22* gene, it has been reported that the T allele reduces the inflammatory response of macrophages by reducing the production of pro-inflammatory cytokines (34), which could reduce the risk of atherogenic plaque formation at early ages. However, for certain other diseases we must consider that these polymorphisms are described as risk factors.

In conclusion, statistically significant differences in BMI, insulin and triglyceride levels, and HOMA-IR index were observed among the three adolescent groups analyzed with different BMI indices. The *TNFα* -308G/A and *PTPN22* +1858C/T polymorphisms were not associated with obesity; however, the CT genotype of the *PTPN22* gene was associated with lower levels of insulin and a decrease of HOMA-IR (P≤0.05). This genotype was also associated with a trend towards reduced levels of LDL and cholesterol.

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