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Research Article

Genetic Variant A-2518G of the *MCP-1* is not Related to Microvascular Complications of Type 2 Diabetes Mellitus in Mexican-Mestizo Subjects

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Abstract

Background and aims: Vascular complications are the major cause of mortality of patients with Type 2 Diabetes Mellitus (T2DM). Several reports on the role of *Monocyte Chemoattractant Protein-1(MCP-1)* A-2518G gene polymorphism as a risk factor of the microvascular complications of T2DM are controversial for the different studied populations. This work aimed to determine the association of the *MCP-1* A-2518G genetic polymorphism and plasma levels of MCP-1 in Mexican-Mestizo subjects, with type 2 diabetes (T2DM) and microvascular complications.

Methods: Twenty-two subjects with T2DM without microvascular complications, 31 with microvascular complications and 30 healthy subjects, were genotyped by PCR-RFLP using sequence-specific primers for *MCP-1* A-2518G variant and PvuII restriction enzyme. The MCP-1 plasma levels of the subjects were measured by ELISA sandwich.

Results: The G allele was the most frequent in the three groups, but it was equally distributed among them. The heterozygous (AG) was the most frequent in controls (56.7%) and patients (40.9%) without complications, while the genotype GG was the most frequent (48.4%) in patients with microvascular complications. However, the genotype's frequencies did not differ among the studied groups (α=0.05). The risk of microvascular complications only increased with each year of the evolution of the T2DM (OR: 1.08, p=0.039). Mean plasma MCP-1 levels did not differ among the groups of study or by genotypes (α=0.05).

Conclusion: The *MCP-1* A-2518G genetic polymorphism and plasma levels of MCP-1 are not associated with the microvascular complications of T2DM in a sample of Mexican-Mestizo population.

Keywords: Type 2 diabetes mellitus; Microvascular complications; MCP-1; Polymorphism

Introduction

Diabetes Mellitus (DM) is a chronic metabolic disease characterized by a persistent increase in plasma glucose. Type 2 diabetes (T2DM) is the most common form of the disease (90% to 95%). It is accepted that its etiopathogenic key feature is a progressive loss of β-cell insulin secretion frequently on the background of insulin resistance and accordingly defects in insulin action [1]. The prevalence of the disease has increased globally and it was estimated that in 2018 there were more than 500 million prevalent cases of T2DM worldwide [2]. Likewise, an estimated 1.6 million deaths were directly caused by diabetes in 2016 [3]. Another 2.2 million deaths were attributable to blood glucose levels higher than those reported in the population in 2012 [3]. Mexico is not exempt from the problem and at least 9.4% of the population was diagnosed with DM in 2016 [4] and during 2019 about 337173 new cases have been reported [5]. Besides, the long-term vascular complications of DM are a leading cause of heart disease, stroke, blindness, kidney failure and lower limb loss [6].

It is well-recognized hyperglycemia may induce a chronic inflammatory state in the vessel walls, via the generation of oxidative stress and non-enzymatic glycation of proteins and lipids which provokes the formation of Advanced Glycation End Products (AGEs) [6,7]. These events accelerate the development of macro and microvascular complications of DM, which are the major cause of death in patients with diabetes [8]. AGEs promote the pro-inflammatory response interacting with their endothelial cell receptors (RAGE) [9]. The interaction AGEs/RAGE triggers signaling cascades recruiting multiple downstream pathways, including p21ras,

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the mitogen-activated protein kinases, nuclear translocation of nuclear factor-kB, phosphatidylinositol 3-kinase, Cdc42/Rac, and the Jak/stat pathway [7,10]. This process induces the expression of effectors as cytokines, chemokines, and oxygen reactive species that favors the formation of AGEs and the up regulation of RAGE, as well as the production of effectors as metalloproteases [4,7]. All these processes provoke besides inflammation, thrombosis, atherosclerosis, angiogenesis, vascular remodeling and cell proliferation eliciting hypertrophy and vascular dysfunction that leads to a broad spectrum of diseases [11,12].

One of the chemokines induced in this process is the Monocyte Chemoattractant Protein-1 (MCP-1) which increases adhesion molecule expression on monocytes and produces superoxide anions [13,14]. It has been suggested; the hyperglycemia induction of MCP-1 production in vascular endothelial cells activates and recruits monocytes and macrophages to the macro and microvasculature via regulation of adhesion molecule expressions, which provokes chronic vascular inflammation [8].

Several reports have evidenced that serum levels and biological activity of the MCP-1 protein may be regulated by a biallelic single nucleotide polymorphism occurring at position-2518 of the *MCP-1* gene promoter (rs1024611) [15,16]. However, the alleles and genotypes distribution of this polymorphism differ depending on the ethnic group [17,18]. Nevertheless, the evidence supports that *MCP-1* gene A-2518G polymorphism confers an increased risk of vascular complications in patients with T2DM [14,17,19]. Compared with healthy individuals, the frequencies of the G/G genotype and G allele of the *MCP-1* A-2518G polymorphism has been reported significantly higher in Asian patients with T2DM and micro and macrovascular complications [20]. Also, the frequencies of the GG genotype and G allele were higher in Asian patients with T2DM and microvascular complications of the disease than in patients with macrovascular complications, and in turn, in the latter, the frequencies of such genotype and allele were higher than in the patients with T2DM without vascular complications [19]. On the other hand, it has been reported the association of the A-2518G *MCP-1* variant with T2DM [21-23]. Even so, these results have not been replicated in all studies or the reports are very discordant [20,24]. For example, according to a meta-analysis of the published results until 2013 the AA and GG genotypes were not associated with Diabetic Nephropathy (DN) risk among Asians, but the GA genotype did [17]. Nevertheless, the AA genotype was associated with the DN risk using as control the patients with DM [25]. G allele has also been associated with increased diabetic foot ulcer susceptibility in patients with DM [16], but the results reported about the association of *MCP-1* gene A-2518G polymorphism with Diabetic Retinopathy (DR) are controversial [26-31]. However, a meta-analysis of the reported data suggested that *MCP-1* A-2518G polymorphism affected the risk of presence and progression of DR in subjects with T2DM [20].

The *MCP-1* A-2518G polymorphism has been studied in Mexican-Mestizo subjects with tuberculosis, bladder carcinoma, atherosclerosis, cardiovascular disease, obesity, and insulin resistance without DM [32-36]. In this work, we explored this polymorphism as well as the plasma levels of MCP-1, in a sample of the Mexican-Mestizo population with microvascular complications of T2DM, considering the evidence of its association with vascular complications of this disease and the discordance in the reports for other populations.

Materials and Methods

Study population

This study was conducted in 83 unrelated Mexican-Mestizo subjects, older than 18 years of age (39 males and 44 females), from them, 30 were metabolic healthy controls and 53 patients diagnosed with T2DM. From the last, 31 had at least one of the following microvascular complications: nephropathy, diabetic foot, or neuropathy. The subjects were recruited at the General Hospital ´Dr. Juan Ramon de la Fuente´ in Iztapalapa, the Health Center 'Metropolitana´ in Netzahualcoyotl, and the Medical Services of Autonomous University of Mexico City (UACM), between October 2017 and August 2018.

Diagnosis of T2DM and its microvascular complications were in line with the diagnostic and classification criteria established by the World Health Organization and it was performed by the corresponding head doctor in the mentioned institutions.

Exclusion criteria were T1DM, an infectious disease in 4 weeks before the study enrollment, autoimmune diseases, cancer or neurodegenerative diseases, blood transfusion in 4 months before the blood sampling, antecedent of stroke, myocardial infarction or ischemic heart disease.

The control sample was composed of healthy subjects without any condition of metabolic syndrome, such as high blood sugar, hypertension, obesity or dyslipidemia, and without cardiovascular, liver, kidney or endocrine diseases or first degree relatives with the diagnosis of DM.

Ethics statement

The research protocol was approved by the Ethics and Research Committee of Health Secretary of Mexico City, and it was conformed according to the standards of the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to the study.

Clinical data and blood sample collection

Data provided by all subjects were collected using a standard data collection form, which includes the name, age, gender, and years after the onset of the disease, blood pressure, height, weight, and family history of T2DM. Hypertension diagnosis was established using the Guides for Clinical Practices of Secretary of Health of Mexico. These include a systolic blood pressure equal to or above 140 mm Hg or a diastolic blood pressure equal to or above 90 mm Hg. Body Mass Index (BMI) was calculated according to the formula BMI=weight (kg) / [(height (m)]^2 and the subjects classified according to the four categories established by WHO, underweight (15 to 19.9), normal (20 to 24.9), overweight (25 to 29.9), and obese (30 to 35 kg/m^2 or greater).

Blood samples, for plasma levels of lipids and glucose detection, MCP-1 protein assay and DNA extraction, were collected from all subjects after fasting 12 hours. Leukocytes from fresh blood were used for DNA extraction and the plasma was separated after blood coagulation and stored at -80°C before use.

Detection of plasma levels of lipids and glucose

Total Cholesterol (TC), High-Density Lipoprotein Cholesterol (HDL-c), and Triglycerides (TG) were quantified enzymatically with commercially available reagents following the manufacturer's protocol (Spinreact, Spain) using a Spin 120 equipment (Spinreact, Spain). Low-density lipoprotein cholesterol (LDL-c) was estimated by using the Friedewald formula: LDL-c = TC-[HDL-c + TG/5]. Fasting plasma glucose was measured using the glucose oxidase method (Spinreact, Spain).

Detection of plasma MCP-1 level

A commercial sandwich ELISA kit (Human MCP-1/CCL2 ELISA Max deluxe, Bio Legend, USA), based on a capture antibody immobilized on ELISA plates and a biotinylated detection antibody that is recognized by the conjugate Avidin-Horseradish peroxidase was used for MCP-1 measuring from plasma samples, according to the manufacturer´s instructions. MCP-1 recombinant protein was twofold diluted from 500 pg/ml to 7.8 pg/ml for building the standard curve. The intra and inter-assay coefficients of variation were 16% and 13.5%, respectively.

Genotype determination for *MCP-1* A-2518G polymorphism

DNA molecules isolated from fresh blood samples using Gentra Puregene Blood Kit (QIAGEN, Germany) were used for detecting the *MCP-1* polymorphism A-2518G (dbSNP: rs1024611), by polymerase chain reaction-restriction fragment length polymorphism as it was previously reported [15]. Briefly, the fragment of 930 bp distal regulatory region of the *MCP-1* gene was amplified using the oligonucleotide primers: forward 5'-CCG AGA TGT TCC CAG CAC AG-3' and reverse 5'-CTG CTT TGC TTG TGC CTC TT-3'. The PCR reaction, containing 50 ng of genomic DNA, 2.5 pmol of each primer and 1X PCR Master Mix (Roche, Germany) in a final volume of 25 µl, was subjected to an initial denaturation temperature of 94°C for 5 minutes followed by 40 cycles of: denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 45 seconds, with a final extension at 72°C for 7 minutes. Ten micro liters of the PCR reaction mixture ware incubated with 5 units of Pvull restriction enzyme, for 4 hours at 37°C and subjected to 2% agarose gel electrophoresis for *MCP-1* A-2518G genotyping and visualized under the UV transilluminator (ChemiDoc MP, BioRad, USA). The amplified fragment of the *MCP-1* A-2518G polymorphism region was 930 bp, and after the PvuII restriction, the GG genotype had two fragments of 708 and 222 bp; AG genotype had three fragments of 930, 708, and 222 bp; and AA genotype had a fragment of 930 bp. Aleatory samples and doubtful results were checked by DNA sequencing in the Sequencing Unit of Postgraduate Program of Genomic Sciences of UACM, using a 3130 Genetic Analyzer and BigDye Terminator 3.1 Chemistry (Applied Bio systems, USA).

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was tested for *MCP-1* A-2518G genotypes in the control group with χ^2 test. The analysis of normality was performed by the Shapiro-Wilk test. Categorical data were presented as the percentage of total and tested by Pearson's χ^2-test. Continuous data were presented as mean ± Standard Error of the Mean (SEM) and compared by ANOVA test; or as percentile range $P_{10\text{-}90}$ and compared by Kruskal-Wallis or Mann-Whitney U tests. Differential effects of diagnosis or GG genotype allele on plasmatic MCP-1 levels were screened using covariance analysis, adjusting the values by diabetes onset in years, age and BMI according to the general linear model:

$$y_{ij} = \mu + a + bX^{\text{years of onset}} + b_1X^{age} + b_2X^{BMI} + \varepsilon_{ij}$$

The risk of the T2DM or microvascular complications of the disease was estimated by calculating the odds ratio values using logistic regression and considering age, genotype G, BMI, presence of dyslipidemia and years of onset of the disease as independent variables. 95% confidence intervals (CI 95%) were calculated too.

All of the statistical analyses were performed with Graph Pad Prism data analysis software system version 6.0 (Graph Pad Software, La Jolla, CA, USA) at a significance level of 0.05. Imputation of missing data in lipids values was performed replacing them with the mean of that variable for all other cases.

Results

Clinical and demographic variables in the participants of the study

In this study, we determined the A-2518G polymorphism of the *monocyte chemoattractant protein 1 (MCP-1)* gene and the plasmatic levels of this chemokine in 83 individuals with the following characteristics: a group of 22 patients with T2DM, another one of 31 patients with microvascular complications of the disease (T2DM-MC) and a control group with 30 subjects. Most of the clinical and demographic data of the patients shared by groups did not differ between them (Table 1). The age of all subjects was between 20 and 85 years, with a median of 50 years, but the control group´s age was significantly different from the rest (α=0.05, Table 1). Probably, it was influenced by the exclusion criteria used for setting the study, which comprised the absent of obesity, hypertension, first-degree family with T2DM or other signs of metabolic diseases. Considering the prevalence of these risk factors in the Mexican population [37] and the age of onset of T2DM (>40 years), it is expected the age range of metabolically healthy subjects without risk factors was significantly lower than in the group of patients. On the other hand, the evolution time of the disease was longer in patients with microvascular complications of the T2DM (Mann Whitney U=185.5, p=0.0044; Table 1). However, the fasting glucose, BMI and triglycerides were different with respect to the control group but did not differ between the two groups of patients (α=0.05, Table 1).

Table 1: Comparison of clinical and demographic characteristics between subjects with diabetes and healthy controls.

Variables	Subjects			p-value
	Controls	T2DM	T2DM-MC	
	n=30	n=22	n=31	
Gender (F/M, %)	53.3/46.7	50.0/50.0	54.8/45.2	0.9400[a]
Age (years)*	34.5 ± 13.5	56 ± 17	60 ± 14	0.0000[b]
Fasting glucose (mg/dl)**	91.6 ± 1.8	211.76 ± 23.7	196.8 ± 18.7	0.0000[b]
BMI (kg/m^2)*	24.8 ± 3.5	27.6 ± 5.2	29.6 ± 7.2	0.0021[b]
Hypertension (%)	N.A.	40.9	32.3	0.7288[a]
Diabetes onset (years)*	N.A.	10.8 ± 9.3	16.0 ± 9.0	0.0044[c]
First-degree family history of T2DM (%)	N.A.	50	64.5	0.2906[a]
TC**	141.5 ± 5.0	159.0 ± 4.5	145.5 ± 6.5	0.0908[b]
HDLc**	44.3 ± 1.5	47.2 ± 4.9	40.3 ± 2.3	0.2507[d]
LDLc**	73.8 ± 4.2	78.7 ± 4.2	74.8 ± 4.8	0.1414[d]
TG**	117.0 ± 7.9	165.8 ± 9.5	150.4 ± 8.3	0.0005[d]

*Percentile range $P_{10\text{-}90}$; **mean ± SEM; T2MD and T2MD-MC: Subjects with diabetes in the group without and with microvascular complications, respectively. Statistical significance of the results of Pearson's χ^2[a], Kruskal-Wallis[b], U-Mann Whitney[c] or ANOVA[d] tests, NA: Not Applicable

Table 2: Allele and genotype frequencies of A-2518G *MCP-1* polymorphism of the participants in the study.

Subjects	Allele's frequency (%)		Genotypes frequency (%)		
	A	G	AA	AG	GG
Controls n=30	45	55	16.7	56.7	26.7
T2DM n=22	47.7	52.3	27.3	40.9	31.8
T2DM-MC n=31	35.5	64.5	19.3	32.3	48.4

T2DM and T2DM-MC denote the subjects with diabetes in the group without and with microvascular complications, respectively.

A-2518G *MCP-1* polymorphism allele and genotype frequencies in controls and patients with T2DM with and without microvascular complications

The A-2518G genotype and allele frequencies for patients and controls are shown in Table 2. The sample was in Hardy-Weinberg equilibrium (p=0.4278). The allele G was the most frequent in the three groups under study, but its frequency did not differ among them ($X^2_{2, 166}$=1.9, p=0.3852, Table 2). The heterozygous (AG) for the polymorphism was the most frequent in controls and patients without complications (T2DM), while the genotype GG was the most frequent in the group of patients with microvascular complications (T2DM-MC, Table 2). However, the frequencies of the genotypes did not differ among the studied groups ($X^2_{4, 83}$=5.01, p=0.2864, Table 2) nor any of the genotypes increased the risk of diabetic microvascular complications (p=0.05), but a tendency was observed for GG genotype (p=0.076). Notwithstanding, the use of the T2DM patient's group without microvascular complications as control did not improve the significance of the test. We neither detected an association between this polymorphism nor type 2 diabetes in the Mexico-Mestizo population sample, nor an increase of the risk for the disease (p=0.05). However, the risk of microvascular complications increased by 0.08% (OR: 1.08, 95% CI: 1.004-1.171, p=0.039, n=53, Binomial Log it model) for each year of the evolution of the T2DM. Similarly, the risk of T2DM increased by 0.16% (OR: 1.16, 95% CI: 1.09-1.24, p=0.0002, n=83, Binomial Log it model) for each year of life. On the other side, only the total cholesterol (F=7.13 for 2 and 80 degrees of freedom, p=0.0014) and the LDL plasma levels (H [2, N=83]=8.25, p=0.0161) differed among genotype subgroups. Although these metabolites fell into the normal range their levels were significantly higher in genotype GG with respect to AG (p=0.005), which suggests that they might be independent risk factors for T2DM-MC.

MCP-1 plasmatic levels in controls and patients with T2DM with and without microvascular complications

The quantification of plasma concentrations of MCP-1 in the 83 subjects and their analysis by groups based on diagnostic, revealed no differences among them (F(2,80)=0.6056, p=0.5582, Figure 1A). The comparison of plasma concentration of MCP-1 in all subjects shared by their genotypes neither revealed differences among them (p=0.05, Figure 1B). On the other side, the analysis of the plasma levels of MCP-1 by groups of study and genotype did not show any differences among them (p=0.05). We neither find differences between the plasmatic concentration of MCP-1 of the subjects shared in two groups, with diabetes and without diabetes (p=0.05). In addition, the adjustment of plasma levels of MCP-1 by groups of study (diagnosis), GG genotype, age, years of diabetes onset, and BMI did not reveal differences between the levels of the chemokine (p=0.05).

Discussion

The first report of the *MCP-1* A-2518G polymorphism was performed by Rovin et al. [15] studying Caucasic, African American, Asian and Mexican individuals. These authors found that the G allele frequency in Asian and Mexican population was higher than in Caucasic individuals. On the contrary, the allele frequencies did not differ between Caucasic and African American individuals [15]. After that, a myriad of association studies of this polymorphism with cancer, tuberculosis, cardiovascular diseases, insulin resistance, diabetes mellitus, and vascular complications of this disease have been performed in some of the mentioned populations [17,19,21,32-36]. However, the association of this polymorphism with the diverse pathologies studied has varied as the allele and genotype distribution do in different ethnic groups [17,18]. For example, according to the reported by Guzman-Ornelas [35], the distribution of the *MCP-1* A-2518G polymorphism in the Mexican-Mestizo population differed from the Caucasian and Asian population and even from the reported in another previous study for Mexican population [33]. Likely, it is related to the differences in sample size used in both studies.

In this study, the G allele was the most frequent (greater than 50%, Table 2) in the control group as it was reported by the majority of the studies of *MCP-1* G-2518A polymorphism in Mexican-Mestizo

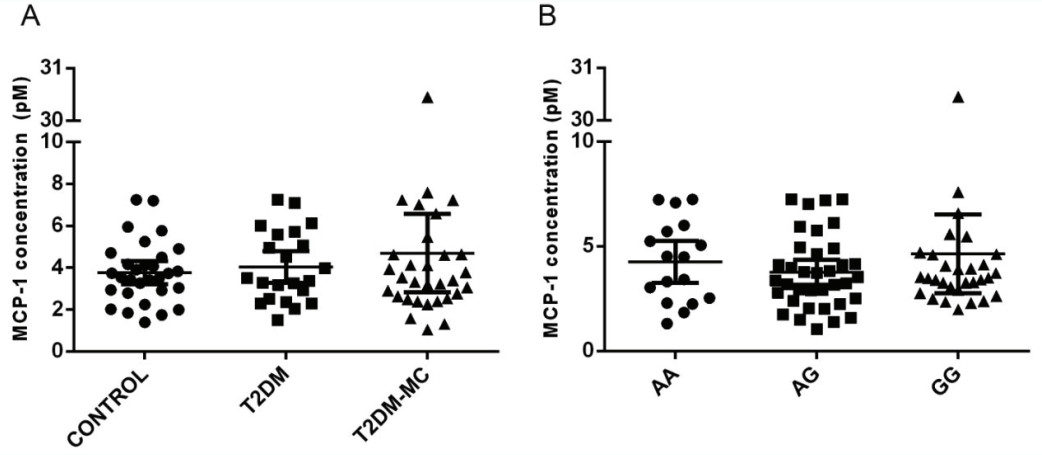

Figure 1: Plasma concentration of MCP-1 for the groups of study (A) or A-2518G genotypes of the *MCP-1* gene (B). The means and 95% confidence interval (I), and the raw data (•, ■, ▲) are shown. T2DM and T2DM-MC denote subjects with diabetes in the group without and with microvascular complications, respectively.

population [15,32,33,36]. Likewise, we also found that heterozygous genotype (AG) was the most frequent in our control group followed by the homozygous GG, which is similar to the previously reported for Mexican-Mestizo population [15,32,33,36].

Although, it has been suggested a potential role of the *MCP-1* gene A-2518G polymorphism as a risk factor for macro and microvascular complications in Asiatic populations with T2DM [17,20,30] controversial results have been reported. In some studies, the risk genotype is the GG [26-28,30,38], in others the genotypes AA [25,39] or GA [17,29] and in other studies, any association has been found [31,40-42]. Maybe these results obey to the ethnic differences reflected in the allele frequency divergences already discussed. In the Mexican-Mestizo population had not been studied the association of this polymorphism with microvascular complications of T2DM, but the GA genotype has been associated with insulin resistance in overweight subjects without T2DM [35]. We found that the distribution of the alleles and genotypes was homogeneous among the groups studied and any of the genotypes increased the risk of microvascular complication of T2DM (Table 2, p=0.05). However, the genotype GG was more frequent in patients with T2DM than in controls and although this genotype did not increase the risk of the complications of the disease (p=0.076) a tendency to be significant was observed.

In our sample, the plasma levels of MCP-1 did not differ among the three groups of subjects or by genotypes (Figure 1), neither between patients with T2DM with or without complications (p=0.05) nor between patients with diabetes and controls (p=0.05). The mentioned result did not vary even when the plasma MCP-1 concentrations were adjusted by age, BMI and years of onset of the disease. This observation was consistent with the one reported in a study of the Chinese population where the MCP-1 serum levels in healthy controls and patients with T2DM without and with microvascular complications did not differ among them [19]. Also, the G allele of *MCP-1* A-2518G gene polymorphism was significantly and negatively correlated with plasma MCP-1 levels and the prevalence of insulin resistance and T2DM in a large cohort of Caucasians [21].

Other authors have reported an increase in the serum levels of MCP-1 in patients with T2DM and microvascular complications [43,44], but the increase of this chemokine is mainly reported in the macrovascular complications of the disease [19,45]. However, it is known that the secretion of MCP-1 is affected by age, body mass index, exercise, increased serum triglyceride concentration, statins, etc., [13], which could be influencing this result. In our work, some of these confounding factors did not differ among the two groups of patients (Table 1), but total cholesterol and LDL plasmatic levels differed by genotype. Also, it has been postulated that the increased MCP-1 levels in patients with T2DM are due to supplementation with n-3 PUFAs [46], which is a variable that we did not consider. On the other hand, there is evidence that *MCP-1* promoter hypomethylation correlates to serum MCP-1 in patients with T2DM by means of epigenetic modifications on the *MCP-1* promoter mediated by HbA1c, fasting blood glucose, and triglyceride levels [47]. However, we cannot rule out other epigenetic factors coming from the diet or other environmental factors contributing to the hypermethylation of *MCP-1* promoter in the Mexican-Mestizo population and consequently to the diminish of the MCP-1 plasma levels. Additionally, we determined MCP-1 plasma levels instead of in endothelial cells or urine, which could be a bias factor for our results.

Conclusion

The results of our study suggested that the *MCP-1* A-2518G polymorphism does not increase the risk of microvascular complications of T2DM in the Mexican-Mestizo population. In addition, the MCP-1 plasma levels neither could be used as a marker of the disease. The present design has a limitation related to a small sample number that could be contributing to increasing the type 2 error of the study. Also, we did not determine the Hb1Ac plasmatic levels in the participants, which could give a measure of the control of the disease. However, it has been commonly found elevated levels of HbA1c in the Mexican population without diabetes, which is probably not mediated by hyperglycemia [48]. Further studies involving the whole contribution of *MCP-1* and another pro-inflammatory gene should be also considered.

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