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ACE I/D (Rs1799752), MTHFR C677T (Rs1801133), and CCR5 D32 (Rs333) Genes and their Association with Hypertension and Diabetic Nephropathy in Urban Areas of Costa Rica, Nicaragua, and Mexico

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Additional information is available at the end of the chapter

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Abstract

Aim: Type 2 diabetes mellitus (T2DM) is a procoagulant state because it is associated with increased risk of atherosclerosis. The purpose of this study was to investigate the prevalence in thrombotic markers that lead to hypercoagulability and its association with hypertension and diabetic nephropathy (DN).

Objective: To determine the association of molecular markers: (insertion/deletion) of the ACE gene Rs1799752, CCR5 D32 Rs333, and MTHFR C677T Rs1801133 with hypertension and DN in T2DM patients of urban areas in Costa Rica, Nicaragua, and Mexico.

Materials and methods: A total of 521 samples of diabetic patients were collected: 132 from Costa Rica, 192 from Nicaragua, and 197 from Mexico.

Results: A high prevalence of ACE genotype D/D ($p < 0.001$) and CCR5 ($p < 0.001$) in T2DM patients from the three different countries was found. The CCR5 D32/D32 genotype was seen in the population of Costa Rica. The prevalence of hypertension was 57.7% and nephropathy was 34% in overall T2DM patients. A statistical association was found; ACE polymorphism has significance with cardiovascular disease (CVD) $p = 0.02$, CCR5 D32 with dyslipidemia ($p = 0.008$), and hypertension ($p = 0.022$).

Conclusion: It is important to know the role of ACE and CCR5 molecular markers and their possible association with the development of microvascular complications in our populations.

Keywords: diabetes mellitus, diabetic nephropathy, urban areas, I/D ACE, C677TMTHFR, CCR5 D32, hypertension

1. Introduction

Diabetes is a disease of high human, economic, and social cost whose incidence has increased considerably. An estimation of seven million people develop the disease each year, which is equivalent to a person acquiring the disease every 5 s worldwide [1]. Clearly, diabetes is a disease in remarkable growth and is expected that by 2025 about 350 million people will have the disease, equivalent to 7.1% of the world population [2, 3]. The United Nations General Assembly adopted a resolution which recognizes diabetes as a threat and global epidemic due to its a chronic, debilitating, and costly nature associated with severe complications, which poses severe risks for families, member states, and the world [4].

Diabetes mellitus (DM) is one of the most common causes of morbidity and medical consultation in Mexico, Costa Rica, and Nicaragua [5]. DM patients, in Costa Rica, consume an enormous amount of medical resources, and their cost occupies the first place in all public hospitals and clinics [5, 6] due to its chronic complications such as lower extremity amputations, blindness, nephropathy, and heart disease, which in turn have devastating and irreversible effects on the health of people. All this has a major economic impact on people and the Health Social Security System [6, 7]. Therefore, with increasing number of diabetic patients, there is an increased demand for services and medical costs.

The DM prevalence reported in Nicaragua and Costa Rica is 11.5% [6, 8]. However, there are still many gaps in knowledge regarding risk factors and epidemiology of DM and the effectiveness of prevention guides which would allow to control this health problem and its complications in both countries.

In a multiregional study of adult populations in urban areas, there was evidence of high DM prevalence in Guatemala (8%) and Nicaragua (9%) [8]. In 2002, DM accounted for 24% (4273) of all hospital charges for chronic diseases (CDs) in Nicaragua. During the period between 1993 and 2002, mortality from diabetes increased rapidly, especially, in people aged above 50 years. The described Nicaraguan trend for DM is that the disease is affecting younger ages (15–34 years) and productive ages (35–49 years), which will result in lost life-years, as well as business days. The lack of optimal diabetes care favors the development of complications.

The estimated prevalence of DM in 1998 in Costa Rica was 4.8% in those aged 20 years and above. In 2006, the percentage of diabetic patients raised to 5.3% in the same age range population [6]. If the population is divided by age groups, the prevalence for people above 40 years is 9.4% and for older adults is 23.4% [6]. In Mexico, chronic disease (CDs) is a public

health problem, as well as in other Latin American countries, which is the result of change in the epidemiological behavior and cardiovascular diseases (CVDs). They are conditions that prevail among adults and constitute the main causes of overall mortality [9, 10]. Heart disease, diabetes, dyslipidemia, and hypertension stand among these conditions because of their high prevalence and serious complications, such as neoplasms, cerebrovascular disease, and renal disease. DM, as part of cardiovascular risk factors, is one of the most important points of the health agenda of Mexico, because of the increased prevalence and complications in addition to high costs (direct and indirect) for Mexican society. The prevalence of DM in Mexico, according to ENSANUT 2012, is 9.2% in individuals aged 20 years or more [11]. This prevalence varied with the age of the individuals. The highest prevalence is found in subjects between 60 and 69 years (25.3%). DM prevalence in Yucatan reported in ENSANUT 2012 is 9.2%, ranking eighth nationally [11].

The ADVANCE study showed that in people with DM after eight years of evolution, when hemoglobin A1c (HbA1c) level was reduced from 7.3 to 6.5, it decreased the development of nephropathy in 20–30% of microvascular complications [12]. However, even with metabolic control, there are still environmental factors that enhance the progression of organ damage; in many cases, renal deterioration is inevitable, demonstrating the involvement of genetic factors. Participation of these factors is evident in the results of family studies, linkage, and association with genetic variants in populations unrelated, conducted in different regions of the world [13]. The genesis and progression of early stages of the latest clinical stages of nephropathy are influenced by own genetic variation, age, sex, metabolic factors, such as hyperglycemia, elevated levels of triglycerides and cholesterol, high body mass index, high blood pressure (HBP), and metabolic control [10, 14].

1.1. Diabetic nephropathy and genetic susceptibility

Diabetic nephropathy (DN) is a complex multifactorial disease; the development of this complication depends on the additive effect of the variation in the genes and the interaction with environmental factors, such that it may have mutations or genetic variants that predispose to the progression to the disease.

Genetic susceptibility plays an important role in the pathogenesis of DN, and several genetic approaches, including association studies of candidate genes and genomics, have identified susceptibility genes for DN [15]. In addition, it has been reported that the inflammatory mechanisms contribute to the development and progression of DN. However, these mechanisms underlying the regulation of cytokines in the kidneys of patients with DM remain unclear. Genetic variations in genes encoding inflammatory cytokines can confer susceptibility to DN by altering functions or expressions [16]. Polymorphisms in the CCR5 receptor and MMP9 have also been associated with increased risk of nephropathy following these mechanisms [15, 17]. Another gene associated with risk of DN is the angiotensin-converting enzyme gene (ACE). This gene modulates the generation of angiotensin II, which increases intraglomerular pressure, leading to glomerulopathy. The course of DN can be considerably improved by treatment with ACE inhibitors, in patients with type 1 diabetes [18].

The DN is considered a complex polygenic disorder in some studies, in which the association of polymorphisms in individual genes can be small and sometimes not informative, while specific combinations of specific genotypes may generate significant changes [15, 19, 20].

Polymorphism /SNP	Mutation	Chromosome	Genotype	Symbol	Phenotype Effect	Pathologic Association	References
MTHFR Gene MTHFR 677C>T Rs 1801133	Ala >Val 677C>T	Chromosome 1 (1p36.3)	CC (wild type) CT (heterozygous) TT (homozygous)	CC CT TT	Homocysteine level normal normal mild increased	Associated with hyperhomocysteinemia.	19 ; 37; 41
ACE Gene I/D ACE Rs1799752	alu repeat insertion	Chromosome 17 (17q23) Intron 16	II (insertion) ID (insertion/deletion) DD (deletion)	II ID DD	ACE enzyme normal mild increased increased	Associated with DN in three different european population*	21; 39
CCR5 <i>delta</i> 32 Rs333	chemokine (C- C motif) receptor 5	Chromosome 3 (3p21:31)	II (insertion) ID (insertion/deletion) DD (deletion)	wt/wt wt/ D32 D32/D32	Normal level of CRP Low levels of CRP,	The chemokine receptor gene CCR5 plays an important role in many immune-related processes. D32 rs333, designating the CCR5- D32deletion of 32 nucleotides from within the gene, is perhaps the most famous allele of CCR5	19;25

* Unknown effect.

Table 1. Characteristics of the different polymorphisms analyzed associated with diabetic nephropathy.

This chapter presented the results about the prevalence of known risk factors and some genetic variants, such as ACE I/D (Rs1799752), MTHFR C677T (Rs1801133) and CCR5 D32 (Rs333), see **Table 1**, associated with DN in DM patients belonging to urban areas of Costa Rica, Nicaragua, and Mexico.

1.2. Genetic mutations associated with DN

1.2.1. ACE gene (Rs1799752)

ACE gene is located on chromosome 17q23.3, and one molecular variant contains an insert (I) or a deletion (D) of 287 bp in intron 16 [21]. The DD genotype has been associated with higher levels of ACE and an activity four times higher than the II genotype, in addition to higher levels of blood pressure, obesity, and increased cardiovascular risk [21, 22]. Other studies suggest that polymorphism ID is an aggressive factor for developing kidney damage in type 1 diabetes [22, 23].

The renin-angiotensin-aldosterone system (RAAS) is a cascade of interactions culminating in the production of angiotensin II (Ang II), which is the peptide responsible for the effects of this physiological axis. ACE is a protein that may have pleiotropic effects and play a role in various diseases and not just in hypertension [24]. ACE is a regulatory enzyme in RAAS. Due to the activation of ACE, conversion of angiotensin I to angiotensin II is given, which is a vasocon-

strictor. ACE is also known to inactivate bradykinin, and kallikrein, vasodilator molecules. For this purpose, ACE is an enzyme that increases blood pressure [22, 23]. It is considered one of hemodynamic and vascular factors like other genes such as AGT1q42-q43, NOS37q36, NPR11q21-q22, among others [19].

1.2.2. CCR5 gene (Rs333)

Recently, there have been identified variants in CC chemokine receptor (CCR) and its importance in infectious and autoimmune disorders, which are significantly linked to diabetes and its complications. These variants interact with other genes associated with the inflammatory cascade, including CCR5 3p21.31, CCR2 3p21.31, IL6 7p21, TNF 6p21.3, and SELL 1q23-q25 [16, 17, 19].

The CCR5 variant, in particular, is a CC, which recruits immune receptor sites of infection, inflammation, and injury including renal disease cells. CCR5 is expressed on dendritic cells derived from peripheral blood, macrophages, lymphocytes, and vascular endothelial cells and its activity average ligands RANTES, eotaxin smooth muscle, and macrophage inflammatory proteins (MIP-1, MIP-1) [19]. Moreover, CCR5 and their ligands, for example, MIP-1 and RANTES [25, 26], have been detected in muscle cells smooth and macrophages of the atherosclerotic plaque.

The CCR5 gene is located on chromosome 3p21.31 [19]. Previous studies have shown that a 32-bp deletion leads to loss of CCR5 expression and function, resulting in a truncated protein, which is not expressed on the cell surface. CCR5 mediates monocyte recruitment and differentiation of macrophages in the glomerulus and intestine. It has been associated to have a role in the development of fibrosis and glomerulosclerosis in DN [26]. The CCR5 D32 variant has been associated with low levels of CRP, decreased intima media thickness, and risk of cardiovascular disease [26].

Furthermore, it has been reported an association of this mutation with risk of myocardial infarction [26]. These studies are consistent with the hypothesis that CCR5 receptor is involved in mediating systemic low-grade inflammation and can participate in diabetes, atherosclerosis, and cardiovascular disease (CVD).

1.2.3. The MTHFR C677T (Rs1801133) and hyperhomocysteinemia

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme encoded by a gene located on chromosome 1p36.3 in position. This sequence has a size of 2.2 kilobases (kb) and consists of 11 exons [27]. In humans, the product of this gene is a protein of 77 kilodaltons (kDa) that catalyses the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulating form of folate [28, 29]. This form of folate participates in the transfer of one carbon atom during the nucleotide synthesis, the synthesis of S-adenosylmethionine and methylation of DNA, proteins, neurotransmitters, and phospholipids [28]. Furthermore, it also acts as a donor of methyl groups on primary methylation of homocysteine to methionine, catalyzed by the enzyme methionine synthase (MS) [28].

The MTHFR enzyme activity helps maintain reserves of 5-methyltetrahydrofolate and methionine and negatively regulates circulating plasma homocysteine concentration [28]. Homocysteine levels can increase due to environmental factors such as smoking, low intake of folate, and vitamin B12 and related genetic polymorphisms in genes encoding enzymes or transport proteins [30].

Defects in the MTHFR enzyme have also been linked to venous thrombosis [29], Alzheimer's disease [31], some types of cancer [32], pregnancy complications [33], and neural tube defects (NTDs) [34]. The latter condition has been one of the most studied [28] and is one of the most common birth defects worldwide [35].

The C677T allele is in a 23.7–37% of the Caucasian population in Europe, 30.5–47.5% of the Hispanic population, 8.3–14.6% of African American population, in a homozygous state in 11% of the Australian population [36], and 26.8% in a Costa Rican population [37].

The main aim of this research was to determine the prevalence of some polymorphisms as ACE (I/D) Rs1799752; CCR5 D32 Rs333, and MTHFR (C677T) Rs1801133 and known risk factors associated with DN in DM patients belonging to urban areas of Costa Rica, Nicaragua, and Mexico.

2. Materials and methods

This research is descriptive and transversal, held from September to December 2012 in San Jose, Costa Rica; Leon, Nicaragua; and Yucatan, Mexico. The samples were from individuals who attended clinic centers from urban areas of Costa Rica, Nicaragua, and Mexico.

The Costa Rican samples were obtained from the DNA bank Research Center in Hematology and related disorders (CIHATA) of the University of Costa Rica. These samples were from DM patients who live in the areas covered by the basic care teams (EBAIS) within the program of comprehensive health care (PAIS)-CCSS-UCR region of Montes de Oca, Curridabat, and Tres Rios.

DNA samples were obtained from diabetic patients from Leon, Nicaragua, attending an attention program at different health territories, which are as follows: Maria Perla Health Center Norori, Sutiava, and Mantica. In the case of the Regional Research Center in Yucatan, Mexico, the samples were taken from patients attending consultation at the University Social Integration Unit of San José Tecoh. The samples were recollected anonymously with main clinical information and personal history of the individuals with the diagnosis. All participants gave informed consent according to a protocol approved by Bioethics on Human Subjects Research Committee and participating Universities.

2.1. Criteria samples of T2 DM patients

1. Adults between ages 20 and 80 years.
2. Any gender.

3. DM diagnosis, according to the criteria of the American Diabetes Association (ADA) or as directed by the attending physician on the ballot application of clinical laboratory diagnostic tests.
4. Clinical data, including cardiovascular and DN disease history.
5. Agreement to participate with signed written consent.

2.2. DNA analysis

DNA isolation was obtained following the standard NaCl precipitation method [38].

2.3. Mutation I/D ACE gene (Rs1799752)

Mutation study I/D ACE gene developed by polymerase chain (PCR) of intron 16 of the ACE gene, as primers the following sequence: 5'-3'-CTGGAGACCACTCCCATCCTTTCT 3'-5'-GATGTGGCCATCACATTTCGTCAGAT [21], obtaining a 190 bp for DD genotype and a fragment of 490 bp in the presence of the corresponding insertion genotype II; heterozygous individuals have both bands (I/D). To avoid false-positive DD genotype, a second amplification [39] was performed, attempting to obtain a band of 300 bp for the heterozygous genotype (I/D) and the homozygous deletion allele (D/D) a band of 200 bp. The bands obtained were analyzed in agarose gels by electrophoresis.

2.4. CCR5 D32 mutation (Rs333)

CCR5 gene was performed by PCR with oligonucleotides 5'-CTTCATTACACCTG-CAGCTCTC CCR5 F-3' and CCR5 R 5'-CTCACAGCCCAGTGCGACTTCTTCT-3', which flank the deletion of 32 bp [40]. The reaction conditions were as follows: 10 mM Tris, 2.5 mM MgCl₂, dNTPs 0.2 mM c/u, 0.25 μM oligonucleotide c/u, 1.5 GoTaq U (Promega). Amplification was performed with the following program: 94°C 2 min (1 cycle); 94°C 20 s, 55°C 20 s, 72 s 30 (35 cycles); 72°C 7 min (1 cycle). The products were detected by gel electrophoresis 8% polyacrylamide, stained with silver nitrate. The genotyping was performed according to the size of the amplification products (CCR5 wt/wt) a band of 184 bp was observed for the homozygous, deletion of 32 bp (D32/D32); the expected product was 152 bp and heterozygotes (wt/D32) 2 bands: 184 and 152 bp.

2.5. MTHFR C677T (Rs1801133)

RFLP-PCR method [41] was used. The region of interest was amplified with the following oligonucleotides: 5'-AGGACGGTGCGGTGAGAGTG 1F-3' and 2R-5'-TGAAGGA-GAAGGTGTCTGCGGGA-3' (37). Amplification after digestion proceeded to the post-separation by agarose gel electrophoresis amplicon and 175-pb fragments which were obtained 23 pb and mutant homozygotes (T/T), a single fragment of 198 pb in wild homozygotes (CC) and the three fragments in heterozygous (C/T).

2.6. Statistical analysis

Statistical analysis was performed using SPSS version 16.1 program (SPSS Inc., USA). To determine the association between participating countries were compared, Pearson χ^2 value. The associations were tested for statistical significance; qualitative variables were assessed by OR, equivalent to relative risk, confidence interval, χ^2 value, and a value of $p < 0.05$ was considered. Yates correction for values in cells smaller than 5 was used. Later, analyses were performed to assess the possible association between the risk factors and the presence or absence of nephropathy or some of the complications listed in the questionnaire.

The association for quantitative variables was evaluated by Student's t-test for parametric data, previously applying the Levene's test of homogeneity of variances. Data were captured and analyzed in a database in Excel created for the case for each country and then were analyzed together and separately. Finally, allele frequencies of each polymorphism were determined according to the Hardy-Weinberg law, with χ^2 -test. Statistically significant differences were set as $p < 0.05$.

3. Results

3.1. Demographic and clinical characteristics of the study cohort

A total of 521 DNA samples from DM patients were selected, 132 from San Jose, Costa Rica; 192 from Leon, Nicaragua; and 197 from Yucatan, Mexico. Demographic and clinical characteristics and the prevalence of risk factors are summarized in **Table 2**. The average age of the total group studied was 58.4 years, and it was not found a significant difference ($p > 0.05$), in terms of age distribution. Gender was a variant with statistically significant between the DM patients, there were more women (68.2%) than men (31.8%), $p = 0.005$, and the presence of DN was found with statistical significance, $p = 0.021$. The presence of HTA among all the studied patients was found as a risk factor with significant difference (OR of 25.1; $p < 0.000$). The same result was observed in the presence (+)/absence of dyslipidemia and history of CVD. The prevalence of DN is present in a 33.9% of the total 501 DM patient group studied (**Table 2**).

3.2. Prevalence of the genetic polymorphisms and DN risk factors association

The prevalence of polymorphisms and their distribution can be seen in **Table 3**. All polymorphisms were in Hardy-Weinberg equilibrium, to give an adequate composition and distribution of the polymorphisms analyzed in the populations remain in balance and natural selection.

The results of comparison of prevalence of genetic variants in the populations studied can be seen in **Table 3**, wherein the ACE I/D ($p < 0.001$) and CCR5 ($p < 0.001$) show differences between countries. Genotype I/I ACE is less prevalent in the Mexican population, compared to others ($p = 0.01$). A difference was also found in one homozygote Costa Rican patient in the CCR5 D32 polymorphism, which was not found in the other groups ($p = 0.001$). MTHFR C677T polymorphism, in the total group studied showed a higher frequency of the 677TT phenotype, but there was no significant difference ($p = 0.146$) between any of the groups.

The ACE polymorphism had a significance in the total group of DM patients with a history of CVD, $p = 0.02$, CCR5 with a history of dyslipidemia ($p = 0.008$) and presence of the HTA ($p = 0.022$).

Another analysis was performed between the presence of DN in patients with a history of CVD, and it was found a statistical association, $p < 0.001$ OR 3.1 (CI, 1.6–5.8).

The presence or absence of HTA with any of the polymorphisms analyzed was compared, obtaining statistical significance ($p = 0.042$) for Nicaraguan DM patients. In the presence or absence of dyslipidemia, there was a significant difference ($p = 0.031$) in the group of Nicaraguan patients and I/D polymorphism of the ACE gene.

The presence or absence of CVD in DM patients between the countries was compared, a significant difference ($p = 0.030$) was obtained in the group of Nicaraguan patients with CVD and MTHFR C677T gene, and also statistical difference was obtained ($p < 0.001$).

In the results of the interpopulation comparison, a significant difference ($p = 0.000$) between the onset of HTA in DM patients against Nicaraguans and similar behavior was found in the DM patients in Costa Rica ($p < 0.001$).

Variable/risk factor n (%)	Costa Rican (n = 132)	Nicaraguan (n = 192)	Mexican (n = 197)	p
Age, years	60.8 + 5.6	58.8 + 11.9	57.7 + 11.9	–
Gender	132	192	197	0.439
Male	38 (28.8)	61 (31.8)	69 (35.0)	
Female	94 (71.2)	131 (68.2)	128 (65.0)	
DN	132	192	197	0.009
DN+	54 (40.9)	71 (37.0)	50 (25.4)	
DN–	78 (59.1)	121 (63.0)	147 (74.6)	
Hypertension (HTA) (%)	132	192	104*	0.000
HTA+	104 (78.8)	106 (55.2)	37 (35.6)	
HTA–	28 (21.2)	86 (44.8)	67 (64.4)	
Dyslipidemia	132	192	101*	0.000
Yes	54 (40.9)	165 (85.9)	24 (23.8)	
No	78 (59.1)	27 (14.1)	77 (76.2)	
Cardiovascular disease	132	192	102*	0.000
Yes	45 (34.1)	187 (97.4)	06 (5.9)	
No	87 (65.9)	05 (2.6)	96 (94.1)	

DN, Diabetes nephropathy.

*Some data were not available in the Mexican cases. Age, mean + standard deviation. In the other rows, the values denote numbers of cases followed by percentage of the total group; p is significant <0.05

Table 2. Demographic and clinical characteristics of the study cohort.

The CR cases reported the use of glycemic control of DM, 10.9% of Nicaraguan cases reported that are not using any anti-glycemic drug, which is a significant difference ($p < 0.05$) between these Costa Rican and Nicaraguan populations. It was not possible obtain this information for the Mexican group.

The comparison was done according to genetic polymorphism and cases of each country. The following results were obtained: in the I/D ACE gene, there is a greater number of Nicaraguan cases with the heterozygous genotype ACE I/D, without this becomes significant, while a DM patient of Costa Rican cases showed a similar behavior. In relation to MTHFR C677T polymorphism, both Costa Rican and Nicaraguan DM patients showed a higher frequency of the C677T polymorphism, showing no significant difference ($p > 0.05$) between any of the groups. The CCR5 D32/D32 had very few cases with homozygous, and only one case was found in the Costa Rican group.

Polymorphisms	Costa Rica	Nicaragua	México	p**
ACE	132	192	128*	
II (%)	41 (31.0)	39 (20.3)	12 (9.4)	0.000
ID (%)	55 (41.7)	97 (50.5)	80 (62.5)	
DD (%)	36 (27.3)	56 (29.2)	36 (28.1)	
<i>Allelic frequency</i>				
I	137 (0.52)	175 (0.46)	104 (0.41)	0.000
D	127 (0.48)	209 (0.54)	152 (0.59)	
MTHFR	132	192	197	
CC677 (%)	40 (30.3)	53 (27.6)	53 (26.9)	0.146
C677T (%)	67 (50.8)	83 (43.2)	82 (41.6)	
677TT (%)	25 (18.9)	56 (29.2)	62 (31.5)	
<i>Allelic frequency</i>				
C	147 (0.56)	189 (0.49)	188 (0.48)	0.122
T	117 (0.44)	195 (0.51)	206 (0.52)	
CCR5	132	192	197	
wt/wt (%)	123 (93.2)	183 (95.3)	161 (81)	–
wt/D32 (%)	8 (6.0)	9 (4.7)	36 (18.3)	0.000
D32/D32(%)	1 (0.8)	0	0	
<i>Allelic frequency</i>				
wt	254 (0.96)	375 (0.98)	358 (0.91)	–
D32	10 (0.04)	9 (0.002)		36 (0.09)

*Some Mexican DNA samples were without results for this polymorphism.

**p values were calculated by χ^2 -test, p significant <0.05 .

– Data not applicable.

Table 3. Prevalence of genotypes and allele frequency of the polymorphisms studied in type 2 DM patients from urban areas of San José, Costa Rica; León, Nicaragua; and Yucatán, Mexico.

4. Discussion

In this multicenter study from three Mesoamerican populations, we combined a metabolic disease as DM and a three genetic variants analysis to study their prevalence with the presence of DN. We found that DN in T2-DM patients was associated with the studied polymorphisms principally with ACE I/D and CCR5 d32 as well with HTA and dyslipidemia as risk factors. Our results related to I/D polymorphism are consistent with those reported in other studies [18], one meta-analysis [42], and with prospective follow-up studies [43]. We found that both, the ACE D allele and the CCR5 D 32 polymorphism, are prevalent in our type 2 DM patients, with statistical significance in the total studied group.

In an analysis of the total studied population, according to sex distribution, there was a prevalence of 67.6% (n = 339) females. This is a finding that could be explained given that in women, especially those who are near to menopausal age or those who live than this period, aging processes are complicated by hormonal, metabolic, and psychological changes that accompany them [44]. The fact that women are the majority population who assists to a primary health center in these countries; after menopause, pancreatic insulin secretion decreases and insulin resistance increases, and a further estrogen deficiency occurs. This deficiency also affects blood flow to the muscles, limiting the already reduced glucose uptake.

In the case of HTA variable, a significant difference ($p < 0.001$) with an OR of 25.1 was detected, demonstrating a strong association between the Costa Rican and Nicaraguan DM. It is clear that altering blood pressure contributes to the development and progression of chronic complications of this disease. In individuals with DM, HTA may be present in elemental diagnosis even before developing hyperglycemia and is often part of a syndrome that includes glucose intolerance, insulin resistance, obesity, dyslipidemia and coronary artery disease, constituting metabolic syndrome [45]. It is known that a strict control of blood pressure of 130/80 mmHg reduces cardiovascular morbidity and mortality, and renal complications than the control of other complications, hence the importance of maintaining strict control over the value of hypertension in DM patients [46].

On the other hand, 34.1% of Costa Ricans and 97.1% Nicaraguan had a history of some type of CVD, compared with 59% of Mexican patients, a difference that was statistically significant ($p = 0.001$), **Table 2**. These latter variables show similar behavior in other studies since it is known that the higher the value of blood pressure, the greater the probability of having a heart attack, heart failure, stroke, and/or kidney disease [47]. This applies especially for individuals aged between 40 and 70 years, since each increase of 20 mmHg in systolic blood pressure or 10 mmHg in diastolic in this population doubles in the risk of CVD over the range from 115/75 to 185/115 mmHg [48].

The 85.9% of DM Nicaraguan patients had dyslipidemia, it is also statistically significant ($p < 0.05$) compared with the full studied group, and this proportion is maintained by the value obtained for the OR of this study (OR = 9.54). This finding is consistent with other studies and is the association between DM and the presence of dyslipidemia [49]. The vasodilator action of insulin would frequently alter in the presence of insulin resistance; in these situations, by

capillary recruitment, typical insulin target tissues (skeletal muscle) would be decreased. Insulin has different effects on the vascular tissues because it stimulates the activity of nitric oxide synthase enzyme endothelial and the endothelium-dependent vasodilatation. In theory, the alteration of the latter mechanism, insulin resistance as in the DM, contributes to endothelial vasomotor dysfunction and would lead to hypertension and atherogenesis [50]. All these variables, including the presence or absence of DM, tend to operate as a single entity, referred to as metabolic syndrome (MS), which is a complex entity, including risk factors predictors of CVD.

These associations found between hypertension, CVD, and dyslipidemia with DM confirm that the most important element in a strict treatment and followed program for DM patients is to avoid the complications as DN and CVD. If we discard the genetic components and rather focus solely on traditional risk factors, which can be prevented or improved, such as healthy lifestyle, together with existing drug therapy, they positively affect the quality of life of patients [51].

However, we found a higher prevalence of I/D ACE polymorphism in our DM patients and some association with DN, **Table 3**. It is important to know that DN is considered as a multicausal complication. Renal complications due to T2 DM owing to the multifactorial causes are a comprehensive expression of conditioning phenotype with the additive effect of multiple loci, and several environmental factors inherent to each population. More genetic regions are associated through sequencing techniques with the predisposition of developing diabetic complications. Around 69 genetic loci are described now, whereas binding studies have described more than 24 genes; therefore, more reports are required, given the genetic heterogeneity influences nephropathy, studies with more population are necessary to assume with greater relevance the interaction between multiple genetic loci and to get an association with a particular phenotype [19].

Comparing Costa Ricans against Nicaraguan and Mexican patients, a significant difference in some of the polymorphisms between these populations was found. One of those differences was CCR5 D32, with a significant difference ($p = 0.042$). This observation is consistent with that described in the literature where it is stated that there is an interaction in pathological states, including some classic risk factors (such as DM and overall metabolic syndrome) and some immunomodulatory factors (such as CCR5). However, the mechanism by which this process occurs had not been well described. An explanation is related to the obesity-associated insulin resistance, which is characterized by a chronic inflammation of tissues, including visceral adipose tissue, which recruits a large number of macrophages, T lymphocytes, and B lymphocytes; the interaction of these cells generates multiple cytokines secretions and autoantigens that predispose to the development of diabetic complications mainly of cardiometabolic type. The exact mechanism of how these interactions occur has not yet been elucidated nor the effects that are associated with this interaction [52].

For the variable of DN presence or absence of disease in Nicaraguan DM patients, two of the three polymorphisms showed statistical significance: MTHFR gene C677T genotype ($p = 0.030$) and genotype I/D ACE gene ($p < 0.001$). MTHFR C677T genotype literature

does not describe associations between disease and this genotype, but rather a protective 677TT genotype of MTHFR gene [53] was documented effect. The differences obtained could be due to a small sample size or bias on the part of the information provided by the patient.

The genotype I/D ACE gene was found with significant difference in the distribution among Costa Rican and Nicaraguan populations and in association with the presence of DN. This finding is novel, because even that greater presence of individuals with the D/D genotype is described in the literature, the found difference is significant, therefore, and to confirm this finding, it is necessary to increase the sample size [24, 54].

A significant difference ($p=0.031$) in the genotype I/D ACE gene was found among Nicaraguan patients with and without dyslipidemia. Similar to the comments on this genotype and an association with this risk factor was also found in the Nicaraguan population. This is an unreported (with statistical significance) finding; however, it had higher frequency of individuals with this polymorphism described in the studies [24].

Another important finding was the significant difference ($p < 0.001$) between the use or not of DM medicament by Nicaraguan patients, since 10.9% of the Nicaraguan population reported not using such medications to control serum glucose level. This finding is explained by a deficiency in the coverage of the Nicaraguan health system, which is a risk factor to take into account in the development of complications in the diabetic population.

In summary, the HTA and dyslipidemia are classic risk factors strongly linked to the DN disease to the T2 DM of the urban areas of San José, Costa Rica; Leon, Nicaragua; and Yucatan, Mexico. The molecular analysis of the genotype I/D ACE was the most important in T2 DM between the patients, principally in Nicaragua and Costa Rica. The polymorphism of the gene CCR5 was significant, with differences in its prevalence between the analyzed countries.

The highlights of the present study are that this is the first report of DN risk factors and three molecular variants in Mesoamerican T2 DM patients. Further studies are necessary to determine the exact impact of the genetics and environment on the risk and to define possible interaction with other candidates involved in the pathogenesis of DN in type 2 DM. These data prove the importance of continuation of this kind of research in order to consider the possibility of offering molecular analysis to type 2 DM population as a potential preventive diagnosis for DM patients with risk of DN.

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