Frequency of *CYP2C9* (**2*, **3* and *IVS8-109A>T*) allelic variants, and their clinical implications, among Mexican patients with diabetes mellitus type 2 undergoing treatment with glibenclamide and metformin

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Abstract. The majority of Mexican patients with diabetes mellitus type 2 (DMT2) (67.9-85.0%) are prescribed sulphonylureas (SUs), which are metabolized by cytochrome P450 2C9 (abbreviated as *CYP2C9*). SUs are a type of oral anti-diabetic compound which inhibit ATP-sensitive potassium channels, thus inducing glucose-independent insulin release by the β-pancreatic cells. The wide variability reported in SU responses has been attributed to the polymorphisms of *CYP2C9*. The present study aimed to describe *CYP2C9* polymorphisms *(*2*, **3* and *IVS8-109T*) within a sample of Mexican patients with DMT2, while suggesting the potential clinical implications in terms of glibenclamide response variability. From a sample of 248 patients with DMT2 who initially consented to be studied, those ultimately included in the study were treated with glibenclamide (n=11), glibenclamide combined with metformin (n=112) or metformin (n=76), and were subsequently genotyped using a reverse transcription-quantitative polymerase chain reaction (PCR), end-point allelic discrimination and PCR amplifying enzymatic restriction fragment long polymorphism. Clinical data were gathered through medical record revision. The frequencies revealed were as follows: *CYP2C9*1/*1*, 87.5%; **1/*2*, 6.5%; **1/*3*, 5.2%; and *CYP2C9*, *IVS8-109A>T*, 16.1%. Glibenclamide significantly reduced the level of pre-prandial glucose (P<0.01) and the percentage of glycated hemoglobin (%HbA1c; P<0.01) for *IVS8-109A>T* compared with combined glibenclamide and metformin treatment. Concerning the various treatments with respect to the different genotypes, the percentages obtained were as follows: Glibenclamide *A/A*, HbA1c<6.5=33.3%; glibenclamide + metformin *A/A*, HbA1c<6.5=24.6%; glibenclamide *A/T*, HbA1c<6.5=33.3%; glibenclamide + metformin *A/T*, HbA1c<6.5=25%; glibenclamide *T/T*, HbA1c<6.5=100%; and glibenclamide + metformin *T/T*, HbA1c<6.5=12.5%. Altogether, these results revealed that, although genetically customized prescriptions remain a desirable goal to increase the chances of therapeutic success, within the studied population neither allelic variants nor dosages demonstrated a clear association with biomarker levels. A key limitation of the present study was the lack of ability to quantify either the plasma concentrations of SU or their metabolites; therefore, further, precise experimental and observational studies are required.

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Introduction

Diabetes mellitus type 2 (DMT2) is a highly prevalent multifactorial and chronic disease characterized by hyperglycemia, insulin resistance, a decrease in β-cell levels and insulin secretion (1). It may also give rise to micro- and macro-vascular and neuropathic complications, including nephropathy, coronary artery disease, stroke, peripheral vascular disease, retinopathy and neuropathy (2). Globally, DMT2 is one of the most notable premature mortality risk factors, and it currently represents one of the most frequent causes of mortality globally (3). According to a 2017 official survey, this disease is the third most highly ranked cause of mortality in Mexico (15.15%) (4), a country that, since 2013, is ranked sixth highest in its prevalence of DMT2 of all countries and is projected to present 15.7 million cases by 2035 (5).

The incidence of DMT2 is closely associated with demographic and lifestyle factors (including obesity, aging and

physical inactivity), in addition to genetic factors, including polymorphisms encoding the inwardly rectifying potassium channel, Kir6.2 (6,7), insulin receptor substrate-1 (8,9) and the Pro12Ala polymorphism of the peroxisome proliferator-activated receptor-γ gene (10,11). Consequently, alterations in nutrition and exercise regimes constitute the first recommendations to patients diagnosed with DMT2 (12), although eventual pharmacological intervention is unavoidable. The most commonly employed (50-66% of all cases) treatment against DMT2 is based on oral hypoglycemic agents, predominantly sulphonylureas (SUs) (13). Although the Mexican Official Guide of good clinical practice recommends the use of metformin as the initial control drug (14), 67-85% of Mexican patients are treated with SUs (15,16). However, a great variability in drug disposition, glycemic response tolerability and the prevalence of adverse effects to these drugs have been reported. These drawbacks have been predominantly attributed to environmental, pathological and physiological factors, in addition to gene polymorphisms (1,17).

Therefore, pharmacogenomic analysis in association with glycated hemoglobin A1c (HbA1c) monitoring, considered to be the current gold standard for glycemic control, may be applied to individualize dosage regimens of these drugs (18). The gene for cytochrome P450 2C9 (*CYP2C9*) (55 kb) is situated on chromosome 10q23.33, and 60 allelic variants [single nucleotide polymorphisms (SNPs)] have been described within its exon 9-intron 8 structure (19). These variants account for ~40% of the interindividual and interethnic pharmacokinetic differences in responses to SUs (17,20-22). Nevertheless, controversy remains concerning the potential influence of *CYP2C9* polymorphisms (**2* and **3*) on glibenclamide response variability, as the studies that have been performed were designed with different intended doses (23), pursued different objectives (hyperglycemic or hypoglycemic control) (19), analyzed different response markers (1,24) and even included cohorts of patients undergoing varied SU treatment (not glibenclamide alone) (13,24). Additionally, the intronic variant *IVS8-109T* (*CYP2C9*), which has only been reported among healthy volunteers from Asia (37.1-45.6%), Europe (31.8%) and Latin America (21.4% in Ecuadorians and 29% in Mexicans), has been associated with pharmacogenetic differences when CYP2C9 substrates are administered to human subjects (25,26). However, to the best of our knowledge, its association with the pharmacokinetic and/or pharmacodynamic properties of SUs has yet to be analyzed; neither have the frequencies of these *CYP2C9* polymorphisms (**2*, **3* and *IVS8-109T*) been previously studied among patients with DMT2 from Mexico, or from any other American population. Therefore, the aim of the present study was not only to describe these frequencies, in addition to their interindividual and interethnic differences, but also to determine the potential clinical implications in the variability of responses to the administration of glibenclamide among Mexican patients with DMT2.

Materials and methods

Study design and sample description (DMT2 patients). The present study is observational, not a clinical test. Sample collection and clinical record reviewing was accomplished within a cohort of male (32.16%) and female (68.84%) patients (mean age: 62; age range: 49-71) with DMT2 undergoing medical treatment and monitored at first-level public healthcare centers in Mexico City between July 2014 and October 2016. The size of the sample was selected from two previous studies which identified alleles **2* and **3* (slow metabolizers) in a healthy Mexican-Mestizo population of Native American and Spanish heritage (27-29). Inclusion criteria required at least 3 months of pharmacological treatment in order to possess stationary plasmatic levels. HbA1c values were used to estimate the plasmatic glucose levels during the whole trimester prior to their determination. The present study focused on a first level health center where patients' files spanning anywhere from a semester to multiple years may be revised. Determining previous measurements would not have been achievable and the present study was only interested in verifying control over the last trimester. The glibenclamide group was not large; however, statistically the number of patients used was legitimately and properly applied. Glycemic control had been previously reported through measuring fasting glucose levels in the only two previous studies examining *CYP2C9* including glibenclamide-treated patients with DMT2 (20,21). None of them belonged to any Mexican populations, where control was reported through measuring fasting glucose levels (<110 mg/dl) and associated with *CYP2C9* through Xi2 in order to compare genotype frequencies between controlled and uncontrolled patients. Replication and confirmation of these studies is necessary, thus fasting glucose, HbA1c description and investigatory analysis were performed on a Mexican population. The present study took into account the analysis performed by Salam *et al* (21) to compare results from different studies. The participants used in the present study had been previously diagnosed with DMT2 according to World Health Organization and American Diabetes Association criteria (30). All patients were legal adults (18 years or older) with at least three generations of Mexican ancestry, and no kinship between them. Details of consent from the patients and ethical approval of the present study are detailed below.

CYP2C9 genotyping

Genomic DNA isolation. From 200 µl of each patient's venous peripheral blood, genomic DNA was extracted using UltraClean® BloodSpin® DNA isolation reagents (Mo Bio Laboratories; Qiagen, Inc., Valencia, CA, USA).

*CYP2C9 alleles and genotype determination. CYP2C9*2*, **3* and **6* analysis was performed using quantitative polymerase chain reaction (qPCR) with TaqMan® primers (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and allelic discrimination was performed using a Fast 7300 Real-Time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Reactions were performed in a final reaction volume of 10 µl, with 30 ng genomic DNA template, 1X TaqMan® Universal PCR Master mix system (Applied Biosystems; Thermo Fisher Scientific, Inc.), 1X each probe (*CYP2C9*2*: C_25625805_10; *CYP2C9*3*: C_27104892_10 and *CYP2C9*6*: C_32287221_20) and water. The following thermocycling conditions were applied: 10 min for initial denaturation at 95˚C, followed by 40 denaturation cycles of 15 sec at 92˚C and annealing at 60˚C for 1 min. Allelic

Table I. Demographic, anthropometric, clinical, and habitual characteristics of Mexican patients with DMT2.

	Pharmacological treatment (oral hypoglycemiants)			
Characteristics	Glibenclamide (n=11, 5.53%)	Metformin (n=76, 38.19%)	Glibenclamide + Metformin (n=112, 56.28%)	P-value
Age (years)	65.00±5.50	61.03±10.99	59.99±11.24	0.33
Weight (kg)	64.50[a] [61.50-67.50]	71.80[a] [60.51-73.60]	67.50[a] [59.00-75.30]	0.53
Sex, n (%)				
Female	7 (63.60%)	57 (75.00%)	71 (65.10%)	0.54
Male	4 (36.40%)	19 (25.00%)	38 (34.90%)	0.67
DMT2 duration (years)	6.50 [2.00-11.00]	5.00[a] [1.00-9.00]	8.00[a] [2.00-13.00]	0.22
Body mass index	28.27[a] [24.88-31.11]	29.39[a] [22.36-33.20]	27.58[a] [24.97-32.59]	0.14
Overweight (31)	4 (40.00%)	36 (47.40%)	35 (31.50%)	0.54
Obesity type				
I	4 (100.00%)	21 (58.30%)	26 (74.30%)	
II	0	8 (22.20%)	6 (17.10%)	
III	0	7 (19.40%)	3 (8.60%)	
Tobacco	0 (0 %)	10 (26.30%)	22 (33.80%)	0.63
Alcohol	0 (0 %)	11 (31.40%)	24 (38.10%)	0.81
Menopause	4 (36.40%)	17 (22.40%)	11 (10.10%)	0.74
Place of origin	Mexico City, Puebla, Veracruz	Mexico City, Chiapas, State of Mexico, Hidalgo, Michoacan, Oaxaca, Queretaro, Tlaxcala	Mexico City, State of Mexico, Guerrero, Hidalgo, Oaxaca, Puebla, Tlaxcala	

Data are expressed as mean ± standard deviation; [a] = median; [-] = IQR (interquartile range); n (%); P<0.05. DMT2, diabetes mellitus type 2.

discrimination was performed for 1 min at 60˚C (real-time PCR genotyping by TaqMan Probes).

The *CYP2C9 IVS8-109A>T* intronic polymorphism (rs1934969) was analyzed using the PCR amplifying enzymatic restriction fragment long polymorphism (PCR-RFLP) method. PCR was performed on a Mastercycler® 384 (Eppendorf, Hamburg, Germany) to identify its presence. Amplification was performed in 25 μl reaction mixtures containing 0.5 U Eco-*Taq* polymerase (Ecogen SL, Barcelona, Spain), 0.8 μM of each primer (forward, 5'-CTGTTAAGATCTGATATTAGG-3'; and reverse, 5'-TGA AACATAGGAAACTCTCCG-3') (26), 0.2 mM each deoxynucleoside triphosphate (set PCR grade; Roche Diagnostics GmbH, Mannheim, Germany), 2 mM $MgCl_2$ and 1 μl DNA template. The thermocycling conditions were as follows: 8 min of denaturation at 95˚C, with 35 subsequent cycles of 95˚C for 30 sec, 54˚C for 30 sec and 72˚C for 30 sec; and finally, an extension step of 7 min at 72˚C was performed. This PCR reaction generated a fragment of 622 bp.

Subsequently, 20 μl restriction mix containing restriction buffer (New England BioLabs Inc., Ipswitch, MA, USA; cat no. B7204S) and 10 U *Hinf*I restriction enzyme (New England BioLabs Inc.; cat no. RO155S) was added to the 2-μl PCR reaction, followed by overnight incubation at 37˚C to ensure that the digestion was complete. The PCR product was then analyzed directly via 2% agarose gel electrophoresis. DNA was subsequently visualized using ethidium bromide for 5-10 min at room temperature. *CYP2C9 IVS8-109T* alleles were cut into fragments of 468 and 154 bp, whereas the *CYP2C9 IVS8-109A* alleles were uncut (622 bp).

Clinical evaluation. Patients were recruited for the present study according to the following inclusion criteria: i) The patient was undergoing either glibenclamide or metformin treatment, or a combination of the two; ii) the patient had undergone a treatment schedule comprising a stable dose of these drugs for at least 3 months; iii) the precedents and treatment characteristics of each individual may be accessed via their medical record at the corresponding healthcare center, particularly data concerning drug dosage (including hypoglycemic agents) during the aforementioned 3-month period; and iv) the medical file comprised anthropometric parameters (31) and laboratory reports on a number of key biochemical variables (including HbA1c, fasting glucose levels, triglycerides and cholesterol).

The following exclusion criteria were taken into account: Chronic alcoholism, previous pancreatic pathology, renal failure, hypoglycemic treatment with insulin or insulin

Table II. Clinical and biochemical characteristics of Mexican patients with DMT2.

Characteristics	Pharmacological treatment (oral hypoglycemiants)			
	Glibenclamide (n=11, 5.53%)	Metformin (n=76, 38.19%)	Glibenclamide + Metformin (n=112, 56.28%)	P-value
Daily hypoglycaemiant dosage (mg/day)	10.00[a] [3.75-10.00]	1700[a] [850-1750]	Glibenclamide: 15.00[a,b] [10-00-15.00] Metformin: 2550[a] [1700-2550]	<0.05 0.52
Daily hypoglycaemiant dosage [mg/Kg/day]	0.12[a] [0.06-0.15]	34.14[a] [20.42-36.11]	Glibenclamide: 0.18[a,b] [0.13-0.23] Metformin: 32.44[a] [24.55-39.26]	0.01
Comorbidities, n (%)	9 (81.80 %)	72 (94.70%)	96 (89.70%)	0.62
DMT2 complications n (%)				
Nephropathy	0 (0.00%)	1 (1.32%)	6 (5.50%)	
Retinopathy	0 (0.00%)	1 (1.32%)	2 (1.83%)	
Neuropathy	2 (18.20%)	1 (1.32%)	5 (4.59%)	
Diabetic foot, amputations	1 (9.10%)	0 (0.00%)	1 (0.92%)	
Peripheral vascular diseases	0 (0.00%)	5.16 (21.05%)	20 (18.35%)	
Cardiopathies	0 (0.00%)	6.4 (5.26%)	4 (3.67%)	
Polytherapy, n (%)	10 (90.91%)	67 (88.16%)	109 (100.00%)	0.33
CYP2C9 substrates, n (%)	5 (45.45%)	36 (47.37%)	50 (45.87%)	0.86
CYP2C9 inhibitors, n (%)	9 (81.82%)	41 (53.95%)[b]	51 (46.79%)	0.01
Systolic tension, (mmHg)	110.00[a] [110-120]	110.00[a] [110-130]	120.00[a] [110-130]	0.12
Diastolic tension, (mmHg)	80.00[a] [70-80]	70.00[a] [60-80]	80.00[a] [70-80]	0.86
Haemoglobin, % A1c	6.50[a] [5.65-6.90]	7.20[a] [5.96-7.80]	7.70[a,b] [6.75-9.10]	0.01
Fasting glucose, (mg/dl)	127.90[a] [97.50-149.77]	141.30[a] [91.50-165.60]	148.00[a,b] [139.87-175.00]	0.03
Cholesterol, (mg/dl)	212.00[a] [200.40-212.00]	196.00[a] [189.00-232.00]	188.00[a] [167.82-212.86]	0.12
Triglycerides, (mg/dl)	238.00[a] [200-250]	225.00[a] [185-280]	175.00[a] [136-225]	0.05

Values expressed as mean ± sd; [a] = median; [-] = IQR (Interquartile Range); n (%); [b]P<0.05.

analogs, insufficient medical records, DMT1 and voluntary withdrawal.

A database was created to retrieve and analyze the information on the 199 patients included in the study. File revision was performed through random probabilistic sampling.

All patients who agreed to voluntarily be involved in the present study provided written informed consent. The research protocol was ethically approved by the National University of Mexico Faculty of Medicine Research and Ethics Commission, and the study was performed in accordance with the Declaration of Helsinki on ethical principles for medical research involving human subjects.

A database of the collected clinical and biochemical data was compiled that also included each patient's file. Files were thoroughly reviewed in accordance with the study's inclusion and exclusion criteria.

Patients were subsequently grouped in accordance with the designated hypoglycemic agent used for treatment: A glibenclamide group (G); a glibenclamide + metformin group (G+M); and a metformin group (M; the control group).

Statistical analysis. Variables whose distribution was either regular (Shapiro) or homogenous (Levene) were presented as the mean ± standard deviation, while those variables whose distribution was not normal were described as median and interquartile range. *CYP2C9* genotypes were studied on the basis of the following characteristics: i) Dosage [in terms of: a) Daily dose; b) daily dose adjusted to body weight; and

c) the most frequent dosage (5, 10 and 15 mg glibenclamide)]; ii) fasting glucose [concentration (mg/dl) and glycemic control (<110 mg/dl)]; iii) HbA1c [% HbA1c and glycemic control (<7%)]; iv) hypoglycemic event association (fasting glucose <70 mg/dl); v) number of hypoglycemic events (with a fasting level <70 mg/dl); and vi) the metabolic interaction of glibenclamide with *CYP2C9* substrates, inhibitors and inducers.

Comparisons were made using a one-way analysis of variance for the aforementioned variables, upon examination of these parameters. For those variables not following a normal distribution, the median values were determined and compared using a Kruskal-Wallis test. Categorical variables were compared using a χ^2 test. Statistical analyses were performed using SPSS IBM version 24 software (IBM Corp., Armonk, NY, USA).

Results

At the outset, 248 individuals consented to be studied, out of which 199 were clinically analyzed following the application of the exclusion criteria. No significant differences were identified between the treatment groups in terms of their characteristics, as presented in Table I. Within each patient group, women outnumbered men by a factor of 2:1. The disease duration was revealed to be 1.5 years longer in the G + M group compared with the G group. The overweight/obese I condition (31) was more prevalent within the G group, which was also associated with a larger number of menopausal patients. Nevertheless, no significant differences were identified (Table I).

Response biomarker values at stationary states (at least 3 months under the same dosage) were obtained. To account for different prescription periods (8, 12 or 24 h), the total glibenclamide intake (every milligram) over a period of 24 h was used for the estimation (as certain patients were prescribed 5 mg every 8 h, making their total daily dose 15 mg; while others were administered 5 mg every 12 h, for a total of 10 mg per 24 h).

The G group received a significantly lower dose of glibenclamide compared with the G + M group (P<0.05), and a similar level of difference was observed following adjustment for body weight dosage. Furthermore, a number of other DMT2-associated comorbidities, including peripheral vascular diseases, were present within the G + M group. On the other hand, the G group received double the dosage of *CYP2C9* inhibitors compared with the G + M group (81.82 compared with 46.79%; P<0.05).

Measurement of the HbA1c level was germane to the present study, as it primarily identifies the median and interquartile plasma glucose concentration over prolonged periods of time, additionally serving as a marker for mean blood glucose levels over the months prior to measurement (18). These levels should be maintained at <7%, according to the guidelines for DMT2 management as released by the American Diabetes Association in 2009 (18). In this respect, the G group presented a lower HbA1c (6.50 vs. 7.70%; P<0.05), and lower fasting glucose levels (127.90 vs. 148.00 mg/dl, P<0.05) compared with the G + M group. Triglycerides reported no significant differences (P=0.05; Table II).

*CYP2C9 (*2, *3 and IVS8-109A>T) allelic and genotypic frequencies among Mexican patients with DMT2*. The most frequent variant among the 248 patients genotyped was **1/*1* (87.5%), followed by **1/*2* (6.5%) and finally **1/*3* (5.2%). Regarding the *IVS8-109A>T* variants, the most prevalent genotype was *AA* homozygous (71.4%), followed by *AT* heterozygous (25.0%), whereas the *TT* homozygous genotype was substantially less frequent (3.6%). Allele *A* had a prevalence of 83.9%, whereas that of the *T* allele was reported to be only 16.1% (Table III).

Table III. *CYP2C9* genotypic and allelic frequencies calculated among the studied Mexican patients with DMT2.

**2, *3,* and *IVS8-109A>T CYP2C9* polymorphisms		
Genotype	Frequency	N
**1/*1*	0.875	217
**1/*2*	0.065	16
**2/*2*	0.004	1
**1/*3*	0.052	13
**2/*3*	0.004	1
IVS8-109A>T		
A/A	0.714	177
A/T	0.250	62
T/T	0.036	9
CYP2C9 ALLELES		
**1*	0.933	463
**2*	0.038	19
**3*	0.028	14
IVS8-109A>T		
A	0.839	416
T	0.161	80

All of the alleles were on Hardy-Weinberg equilibrium (χ^2, P<0.05). CYP2C9, cytochrome P450 2C9.

*CYP2C9*6* was unsuccessfully tracked within the 248 available samples of Mexican patients with DMT2. Allele **6* is a deletion of a pair of bases in position 818, which produces a premature stop codon and codifies an inactive truncate protein. Its frequency is low among the majority of populations (25).

*Comparison of CYP2C9 (*2 and *3) allelic and genotypic frequencies between patients with DMT2 and healthy volunteers*. Differences between the drug response genes of healthy and diseased populations have been established (regarding efficacy or adverse reactions) (29). In the present study, there were significant differences between the Mestizo patients with DMT2 and healthy individuals from the Mexican population regarding **1/*2* (P<0.05) (data not shown) and the distribution of variants **2* and **3*; but there were also differences with respect to other Caucasian, Asian and Latin American populations regarding *IVS8-109T*, as its association with either slow or rapid *CYP2C9* metabolism remains unclear. Reports from healthy Swedish populations (25) consider its carriers to be lower rate metabolizers, whereas studies on Ecuadorians

Table IV. *CYP2C9* genotypic and allelic frequencies found in samples of patients with DMT2 worldwide.

Reference	Geographic region	Country	N	*CYP2C9* genotype and allele distribution among DMT2 patients								
				**1/*1* (n)	**1/*2* (n)	**2/*2* (n)	**2/*3* (n)	**1/*3* (n)	**3/*3* (n)	**1*	**2*	**3*
Holstein *et al* (32)	Europe	Germany	357	0.66 (237)	0.18 (66)[a]	0.014 (5)[a]	0.017 (6)[a]	0.110 (40)[a]	0.008 (3)	0.81	0.11[a]	0.080[a]
Holstein *et al* (33)			540	0.65 (353)	0.18 (95)[a]	0.011 (6)[a]	0.015 (8)[a]	0.130 (72)[a]	0.011 (6)	0.81	0.11[a]	0.080[a]
Becker *et al* (34)		Holland	475	0.68 (321)	0.19 (92)[a]	0.023 (11)[a]	0.013 (6)[a]	0.090 (43)[a]	0.004 (2)	0.82	0.13[a]	0.060[a]
Zhou *et al* (35)		Scotland	1,073	0.63 (678)	0.21 (221)[a]	0.020 (22)[a]	0.020 (22)[a]	0.120 (125)[a]	0.005 (5)	0.79	0.13[a]	0.070[a]
Klen *et al* (36)		Slovenia	156	0.61 (96)	0.19 (30)[a]	0.017 (3)[a]	0.034 (6)[a]	0.120 (21)[a]		0.78	0.13[a]	0.009[a]
Ragia *et al* 37)		Greece	176	0.68 (120)	0.24 (43)[a]	0.006 (1)	0.006 (1)	0.060 (11)		0.83	0.13[a]	0.003[a]
Hohendorff *et al* (38)		Poland	502	0.80 (402)	0.19 (94)[a]	0.012 (6)[a]				0.90	0.11[a]	
Bhatt *et al* (39)	Asia	India	109	0.73 (80)	0.08 (9)		0.050 (6)[a]	0.130 (14)[a]		0.84	0.07	0.090[a]
Surendiran *et al* (20)		India	80	0.81 (65)				0.190 (15)[a]		0.91		0.090[a]
Zeng *et al* (40)		China	746	0.90 (672)				0.093 (72)[a]	0.007 (5)	0.95		0.050
Suzuki *et al* (41)		Japan	134	0.98 (132)				0.015 (2)[a]		0.99		0.075[a]
Salam *et al* (21)	Africa	Egypt	100	0.53 (53)	0.20 (20)[a]		0.090 (9)[a]	0.180 (18)[a]		0.7	0.15[a]	0.140[a]
	America	Mexico	248	0.87 (217)	0.06 (16)	0.004 (1)	0.004 (1)	0.050 (13)		0.93	0.04	0.030

Values are expressed as (%); [a]P<0.05. DMT2, diabetes mellitus type 2; CYP2C9, cytochrome P450 2C9.

Table V. *CYP2C9* (*IVS8-109A>T*) genotypic and allelic frequencies within samples of healthy volunteers and patients with DMT2 from different regions of the world.

				CYP2C9 (*IVS8-109A>T*) genotype and allele distribution among healthy volunteers				
Reference	Geographic region	Country	N	*A/A* (n)	*A/T* (n)	*T/T* (n)	*A* (n)	*T* (n)
Hatta *et al* (25)	Europe	Sweden	85	0.494 (42)	0.376 (32)[a]	0.129 (11)[a]	0.682 (116)[a]	0.318 (54)[a]
Maekawa *et al* (46)	Asia	Japan	263	0.285 (75)	0.517 (136)[a]	0.198 (52)[a]	0.544 (286)[a]	0.456 (240)[a]
Lee *et al* (47)		Korea	50	0.340 (17)	0.480 (24)[a]	0.180 (9)[a]	0.580 (58)[a]	0.420 (42)[a]
Hatta *et al* (25)		Korea	128	0.406 (52)	0.445 (57)[a]	0.148 (19)[a]	0.629 (161)[a]	0.371 (95)[a]
Dorado *et al* (26)	Americas	Ecuador	187	0.615 (115)	0.342 (64)[a]	0.043 (8)[a]	0.786 (294)[a]	0.214 (80)[a]
Ortega-Vázquez *et al* (29)		Mexico	300	0.500 (150)	0.430 (128)[a]	0.070 (22)[a]	0.710 (428)[a]	0.290 (172)[a]
	DMT2 Mexican-Mestizo patients							
		Mexico DMT2	248	0.714 (177)	0.250 (62)	0.036 (9)	0.839 (416)	0.161 (80)

Values are expressed as (%); [a]P<0.05.

regard them to possess an increased *CYP2C9* hydroxylation capacity (26). When comparing the frequency of variant **1/*2* in the Mexican population from the present study with those from other regions of the world identified in previous studies, significant differences were identified respect to Europe (0.06 vs. 0.18-0.24; P<0.05) and Africa (0.06 vs. 0.20; P<0.05), but not respect to India (21,32-38). As for **1/*3*, statistical differences were identified in the Mexican population when compared with European countries (32-38), India (39), China (40), Japan (41) and Egypt (21) (P<0.05; Table IV).

A statistical comparison revealed that the frequency of *CYP2C9*1/*2* was, overall, 50% lower amongst patients with DMT2 compared with healthy individuals (6.5 vs. 9-16%; P<0.05) (data not shown) (42-45), whereas CYP2C9*2/*2 was 40% lower amongst the patients with DMT2 compared with healthy volunteers from northern Mexico (0.04 vs. 1.0%; P<0.05) (data not shown) (42-45). *CYP2C9*1/*3* was significantly different only amongst populations from Sonora, Mexico City and Guerrero (5 vs. 10%, 1% and 10%; P<0.05) (29,42-46) (data not shown).

IVS8-109T has already been observed amongst healthy volunteers, where it displays substantial interethnic variability (25). When comparing *IVS8-109A>T* variants from the studied Mexican patients with DMT2 with healthy individuals from different countries, including Sweden (25), Japan (46), Korea (25,47), Ecuador (26) and Mexico (29) itself, statistically significant differences were identified regarding the frequencies of *A/T* and *T/T*, and the prevalence of the alleles *A* and *T* in every case (P<0.05; Table V).

Genotype **1/*1* was observed in 90.91% of the G group (n=10), in 86.61% of the G + M group (n=97) and 90.79% of the M group (n=70). However, **1/*2* was absent in the patients receiving glibenclamide monotherapy, whereas it was present in 8.02% of the G + M group, and in 2.63% of the M group. Statistically significant differences were observed between the monotherapy and combined therapy groups regarding the prevalence of *T/T* (9.09 vs. 2.68%; P<0.05), and the allele *T* variants (22.73 vs. 15.00%; P<0.05; Table VI).

Table VII presents the glibenclamide dosage values, including the median and interquartile daily dose and body weight adjustment, for each of the *IVS8-109A>T* allelic variants amongst the Mexican patients with DMT2, where, compared to G group, a significant difference (P<0.05) favoring the G + M group was observed with respect to *A/A* (15 vs. 10 mg) and A/T (13.75 vs. 2.50 mg). *IVS8-109A>T* has been only identified among *CYP2C9*1* carriers. This indicates that *CYP2C9*1/*1* allows the possibility of *AA*, *AT* and *TT*, while *CYP2C9*1/*2* and **1/*3* have either one *A* or *T* allele. Until now, only *CYP2C9*2* and **3* frequencies have been reported among patients with DMT2 (20,23,35-37,39,40). *IVS8-109A>T* frequencies remain unreported (let alone the potential combinations). Note that Table VII displays the data for 97 patients, since the data contained therein corresponded only to *CYP2C9*1/*1* patients who may be *IVS8-109A>T* carriers.

Regarding the biomarkers (including fasting glucose and Hb1Ac levels), lower values were identified for the G group compared with the G + M group (Table VIII). In particular, lower fasting glucose levels were identified among *A/A* carriers (127.65 mg/dl), compared with 160.34 mg/dl calculated for

Table VI. *CYP2C9* genotypes found in DMT2 patients undergoing glibenclamide and/or metformin pharmacological treatment.

	Pharmacological treatment (oral hypoglycemiants)		
CYP2C9 genotypes and alleles	Glibenclamide (n=11, 5.53%)	Metformin (n=76, 38.19%)	Glibenclamide + Metformin (n=112, 56.28%)
**1/*1*, n (%)	10 (90.91%)	70 (90.79%)	97 (86.61%)
**1/*2*, n (%)	0 (0.00%)	2 (2.63%)[a]	9 (8.02%)[a]
**1/*3*, n (%)	1 (9.09%)	3 (3.95%)	5 (4.46%)[a]
**2/*2*, n (%)	0 (0.00%)	1 (1.32%)	0 (0.00%)
**2/*3*, n (%)	0 (0.00%)	0 (0.00%)	1 (0.90%)
**1*, n (%)	21 (95.45%)	138 (90.79%)	104 (92.90%)
**2*, n (%)	0 (0.00%)	4 (2.63%)	5 (4.50%)[a]
**3*, n (%)	1 (4.54%)	3 (1.97%)	3 (2.70%)[a]
IVS8-109A>T,			
A/A, n (%)	7 (63.64%)	48 (63.16%)	84 (75.00%)
A/T, n (%)	3 (27.27%)	23 (30.26%)	23 (20.54%)
T/T, n (%)	1 (9.09%)	4 (5.26%)	4 (1.89%)[a]
A, n (%)	17 (77.27%)	119 (78.29%)	180 (85.00%)
T, n (%)	5 (22.73%)	31 (20.39%)	32 (15.00%)[a]

Data are expressed as n (%); [a]P<0.05. CYP2C9, cytochrome P450 2C9.

Table VII. Daily glibenclamide dosage stratified in accordance with *IVS8-109A>T* (*CYP2C9*) genotypes determined in the present study.

	Glibenclamide daily dosage (mg) and dosage/body weight (mg/kg)		
	CYP2C9 (*IVS8-109A>T*) genotypes		
Oral hypoglycemiant	*A/A* (n)	*A/T* (n)	*T/T* (n)
Glibenclamide (n=10)	10.000 (6)[a] [5.500-15.000]	2.500 (3)[a] [2.500-3.750][b]	10.000 (1)[a]
	0.148 (6)[a] [0.110-0.190]	0.039 (3)[a] [0.038-0.062][b]	0.148 (1)[a]
Glibenclamide (glibenclamide/ metformin group) (n=97)	15.000 (74)[a] [10.000-15.000][c]	13.750 (20)[a] [10.000-15.000][c]	15.000 (3)[a] [12.500-15.000]
	0.193 (74)[a] [0.130-0.230]	0.170 (20)[a] [0.130-0.260][c]	0.180 (3)[a] [0.140-0.240]
Metformin (glibenclamide/metformin group) (n=97)	1,700 (74)[a] [1,275-2,550]	1,700 (20)[a] [850-2,550]	2,550 (3)[a] [1,275-2,550]
	27.370 (74)[a] [19.140-37.780]	31.870 (20)[a] [20.860-36.960]	31.480 (3)[a] [18.050-39.790]

Data are expressed as [a] = median; [-] = IQR (interquartile range); n, number of individuals; [b]P<0.05 (genotypes); [c]P<0.05 (oral hypoglycemiant). CYP2C9, cytochrome P450 2C9.

G + M treatment. Among *A/T* carriers, a value of 107.67 mg/dl was calculated for the G group, whereas 149.97 mg/dl was determined for the G + M group. Lower HbA1c values (5.68%) were identified among *A/A* carriers within the G group compared with among *A/A* homozygous individuals from the G + M group (8.03%). For *A/T* carriers, a value of 6.67% for HbA1c was calculated for the G group, whereas an HbA1c value of 8.11% was identified for the G + M group. Nevertheless, no significant differences were observed for any of the comparisons.

Pharmacogenetic studies aim to identify response variability in order to: i) Reduce adverse effects, whether caused

Table VIII. Glibenclamide response variability among Mexican patients with DMT2 according to *CYP2C9* polymorphisms (**2*, **3* and *IVS8-109A>T*).

	Fasting glucose (mg/dl) and glycemic control (<110 mg/dl)							
	CYP2C9 genotypes							
	**1/*1* (n)			**1/*2* (n)		**1/*3* (n)		**2/*3* (n)
Oral hypoglycemiant (n)	**A/*A*	**A/T*	**T/T*	**A/*A*	**A/T*	**A/*A*	**A/T*	**A/*A*
Glibenclamide (n=11)	127.65 (6)[a] [103.42- 151.88]	107.67 (3)[a] [71.86-143.48]	121.00 (1)[a]			249.00 (1)[a]		
	33.33% (2)	66.67% (2)[b]				0.00% (0)[a]		
Glibenclamide/metformin (n=112)	160.34 (74)[a] [94.61-226.07]	149.97 (20)[a] [88.06-211.88]	130.41 (3)[a] [103.61-157.20]	139.56 (7)[a] [107.74-171.37]	174.58 (2)[a] [103.97-245.20]	169.12 (4)[a] [87.50-250.80]	124.00 (1)[a]	148.00 (1)[a]
	18.00% (13)	25.00% (5)	0.00% (0)[b]	14.29% (1)	0.00% (0)[b]	50.00% (2)[b]	0.00% (0)	0.00% (0)
	HbA1c (%) and glycemic control (HbA1c< 6.5%)							
	CYP2C9 genotypes							
	**1/*1* (n)			**1/*2* (n)		**1/*3* (n)		**2/*3* (n)
Oral hypoglycemiant (n)	**A/*A*	**A/T*	**T/T*	**A/*A*	**A/T*	**A/*A*	**A/T*	**A/*A*
Glibenclamide (n=11)	5.68 (6)[a] [2.81-8.55]	6.67 (3)[a] [5.82-7.52]	6.40 (1)[a]			6.20 (1)[a]		
	33.33% (2)	33.33% (1)	100.00% (1)			100.00% (1)		
Glibenclamide/metformin (n=112)	8.03 (74)[a] [5.71-10.34]	8.11 (20)[a] [5.68-10.55]	9.07 (3)[a] [5.86-12.29]	8.17 (7)[a] [6.93-9.40]	8.46 (2)[a] [7.71-9.22]	8.82 (4)[a] [5.81-11.83]	6.10 (1)[a]	6.40 (1)[a]
	24.64% (18)	25.00% (5)	12.50% (1)[b]	0.00% (0)[b]	0.00% (0)[b]	25.00% (1)	100.00% (1)	100.00% (1)

Values are expressed as [a] = median; IQR [-] = interquartile range; n, number of individuals; [b]P<0.05 (genotypes). CYP2C9, cytochrome P450 2C9.

by polymorphism influence over drug metabolising enzymes, transporters or receptors; and ii) enhance treatment efficacy through dosage adjustment and interaction prevention. In the present study, it was revealed that not every patient was receiving the same dose and this may result in a false positive response. Group comparison highlighted the difference between monotherapy and combined therapy. Genotyping also reported improved glycemic control among *AT* (*IVS8-109*) patients. Since physicians empirically adjust dosages with respect to biomarkers, the present study suggests they may have prescribed a lower dose for *AT* (*IVS8-109*) patients as a result of biomarker control observations, even without knowing genetic factors (including SNPs).

Hypoglycemic events were revealed to be rather uncommon. Only within the G + M group was a significant difference observed (P<0.05) among **1/*3* bearers (intragroup difference, where 40% of these patients reported this reaction (data not shown).

Variability in the response and glycemic control were evident when comparisons between the various DMT2 populations were made. For example, lower fasting glucose levels were reported for Mexican **1/*1* bearers from the G group compared with those from Egypt (156.8 vs. 170 mg/dl); similarly, **1/*2* bearers within the same groups also reported lower levels compared with the same aforementioned populations (137.4 vs. 150 mg/dl; data not shown).

Discussion

The present study is, to the best of our knowledge, the first to describe the main allelic frequencies of *CYP2C9* (**2*, **3* and *IVS8-109T*) among Mexican-Mestizo patients with DMT2. It is also the first report on any Mestizo population globally. Considering the widely heterogeneous ethnic background of Mexican populations (which comprise >65 groups) (48), ideally, it should be only the first of numerous subsequent studies on the influence of this particular genetic heritage in response to SUs.

IVS8-109T was identified in 16.1% of the Mexican patients analyzed in the present study. The effect of this polymorphism on the function of *CYP2C9* has been observed in healthy individuals from Sweden and Ecuador (25,26). With regard to *CYP2C9*1/*1* subjects, contradictory results were observed: A slower rate of metabolism was reported for the subjects (25), whereas the rate of metabolism associated with this genotype was higher in Ecuadorians (26). The three variants were identified in almost one-quarter of the sample population examined in the present study (22.7%).

The *CYP2C9* allele and genotype frequencies described in the present study were compared with those reported by Fricke-Galindo *et al* (49) and Saldaña-Cruz *et al* (45), whose studies involved healthy volunteers from different regions of Mexico. The results of the present study revealed that *CYP2C9* is significantly widely variable between patients from Mexico (P<0.05), a phenomenon that was also observed among the healthy volunteers. These genetic factors differ among populations, and therefore they affect the enzymatic functioning of *CYP2C9* in different manners.

The frequency of *CYP2C9*3* was revealed to be low among healthy volunteers from Mexico City, and significantly different compared with those of patients with DMT2 (0.1 vs. 3.0%; P<0.05). This difference, however, disappeared subsequent to regional adjustment, where healthy volunteers from northern Mexico were reported to have the highest prevalence of *CYP2C9*2*; additionally, this was the only group where a significant difference with respect to patients with DMT2 was identified (10 vs. 4%; P<0.05).

IVS8-109T was identified in 16.1% of the present sample. This variant had not been described previously in any study on DMT2 populations. Only recently was it observed in healthy volunteers (n=300) and patients with epilepsy (n=64), where the T allele was less prevalent among the latter (29 vs. 19%; P=0.02) (29).

In addition to the observed variability regarding *CYP2C9*2* and *CYP2C9*3*, the frequency of *IVS8-109A>T* was ~50% lower when compared with healthy individuals (16.10 vs. 29.00%; P<0.05), suggesting that the T allele may be a relevant factor whose function should be clarified, also considering that its effect on *CYP2C9* substrate metabolism has yet to be elucidated.

The first stage of the present study described the variability and substantial diversity of the distribution of *CYP2C9* within samples from Mexican patients with DMT2 and those from other populations; however, the effect of this genetic factor accounts for only 40% of the glibenclamide response variability (17), and it has only been studied in two other populations (20,21). The function of these polymorphisms in differential glibenclamide dosages, their effect on the variability of the response monitored by HbA1c (considered to be the main efficacy biomarker in the present study) and also on the most relevant adverse glibenclamide reaction (including hypoglycemia), have yet to be reported.

To the best of our knowledge, no studies have yet been published on the functional association of *CYP2C9* polymorphisms with glibenclamide, or with any other tested drug in a healthy Mexican population. Neither has such an association been studied in patients with DMT2 undergoing glibenclamide treatment; nor has the HbA1c biomarker been investigated. Likewise, non-genetic factors (including sex, obesity, menopause or diagnostic period) have yet to be examined. These may be affected by the disease, and may also exert an impact on the pharmacokinetics and pharmacodynamics of glibenclamide due to changes in drug absorption caused by blood flow reduction (in the subcutaneous and muscular tissue), particularly in obese patients (ampicillin absorption reduced by 26%, in contrast with metoclopramide and glipizide) (50-56).

The available information on healthy individuals may differ from the observations of patients with DMT2, in a similar manner to the interaction between genetic, environmental and pathophysiological factors. Therefore, performing studies on the influence of *CYP2C9* polymorphisms among DMT2 samples from different populations (including Mexican populations) is a necessity.

The dosage is clearly a relevant factor to be considered, as previous studies have reported a positive significant association between the daily dose administered of glibenclamide and HbA1c levels (P<0.0001), although this association was not observed in the case of fasting glucose (57-59). In the present study, patients receiving glibenclamide were not prescribed

>7 mg, whereas those receiving glibenclamide and metformin in combination received doses >10 mg, a factor which, combined with the different *CYP2C9* genotypes, may affect the drug response of the patients. Nevertheless, metformin is well tolerated by the majority of patients, and it functions by inhibiting excessive hepatic glucose production (gluconeogenesis), subsequently reducing intestinal glucose absorption and fasting glycemia, and thereby improving insulin sensitivity and peripheral glucose uptake and utilization (60). A couple of published studies have also reported a negative significant association between daily dosage and serum insulin levels (P=0.03) (56-58), in addition to a significant association between daily dosage and stationary levels of active glibenclamide metabolites M1 (4-trans-hydroxyglibenclamide) and M2 (3-cis-hydroxy-glibenclamide).

In comparison, opposite results amongst biomarkers were identified. *CYP2C9*1/*3* and *CYP2C9*1/*1* genotypes were revealed to have no significant differences in terms of fasting glucose levels, whereas HbA1c levels were more closely regulated among **3* heterozygous individuals. Contrasting biomarker results were also reported for *IVS8-109T*, as fasting glucose levels were better regulated among the *AT* genotype, whereas no significant differences regarding HbA1c levels were otherwise identified.

These different factors, considered together with polymorphisms, indicate that it is necessary for heterogeneous dosages to be administered to individual patients, regardless of whether or not they belong to the same population. For example, 20% of patients with DMT2 receiving treatment with SUs experience hypoglycemic events (glucose level <70 mg/ml), whereas DMT2 treatment efficacy is achieved in only 57% of all cases, and the percentage non-adherence rate to treatment may reach 32.5% (24, 59-64).

DMT2 is a major public health concern in Mexico as well as globally. Not only is this due to its high prevalence (11.5 million patients) (16), but it is also due to the low adherence rates for pharmacological treatment (53-80%). These rates may be associated with adverse reactions (hypoglycemia) and therapeutic failure. As far as the latter is concerned, it is also licit to attribute the low adherence rates to an absence of glycemic control, in spite of the pharmacological treatment (a range of 18-75% for HbA1c) (15,65-69). These factors substantially contribute to increasing costs for individuals, communities, and the State in terms of money, quality of life, and lives themselves (61,62,67-70).

As this is an observational study, the number of patients was not able to be modified. The patients were selected from a cohort from healthcare centers in Mexico City using simple random probabilistic sampling. The treating physicians freely determined which drug each patient should receive according to their clinical condition. To avoid bias, the patients were blinded to the genotyping results. All data were obtained from clinical files. A single person collected both the hand written records from physicians and laboratory results.

Altogether, the following points may be concluded on the basis of the present study: i) Genetically customized prescriptions remain a desirable goal to increase the chances of therapeutic success; ii) in the light of the results of the present study, the results regarding the association between *CYP2C9* polymorphisms and SU metabolism appear insufficient to explain the precarious DMT2 control levels achieved through pharmacological treatment in the studied population; and iii) neither allelic variants nor dosages are able to delineate a clear association with biomarker levels. In consequence, it is necessary to perform further studies to investigate these issues, given that *CYP2C9* and its main allelic frequencies have already been genotyped with respect to the prescription of SUs in Mexico (either as monotherapies or as combined drugs). This information would be relevant for physicians in order to improve their patient treatment strategies, working towards the goal of personalizing medicine. Determining the plasma concentrations of these aforementioned drugs may help to establish potential efficacy or failure associations in the population studied in the present study, and further experiments are already being performed in this regard.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PCR, NRR, FDA, FCN, AL and JAMG provided substantial contributions to the conception or design of the work. PCR, NRR, FDA, FCN, AL and JAMG contributed to the acquisition, analysis and interpretation of data of the work. PCR, FDA, AL and JAMG drafted the study. PCR, NRR, FDA, AL and JAMG revised the paper critically for important intellectual content. JAMG had primary responsibility for communication with the journal during the manuscript submission. All authors approved the final version to be published.

Ethics approval and consent to participate

The research protocol was ethically approved by National University of Mexico Faculty of Medicine Research and Ethics Commission, and the present study was performed in accordance with the Declaration of Helsinki on ethical principles for medical research involving human subjects.

Patient consent for publication

All patients who agreed to voluntarily be involved in the present study provided written informed consent. No identifying information, including names, initials, date of birth or hospital numbers, images or statements are included in the manuscript.

Competing interests

The authors declare that they have no competing interests.

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