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Characterization of the CYP2D6 drug metabolizing phenotypes of the Chilean mestizo population through polymorphism analyses

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ABSTRACT

We tested the influence of four polymorphisms and gene duplication in *CYP2D6* on *in vivo* enzyme activity in a Chilean mestizo population in order to identify the most relevant genetic profiles that account for observed phenotypes in this ethnic group.

*CYP2D6**2 (2850C>T), *3 (2549A>del), *4 (1846G>A), *17 (1023C>T) and gene duplication were determined by PCR-RFLP or PCRL in a group of 321 healthy volunteers. Individuals with different variant alleles were phenotyped by determining debrisoquine 4-hydroxylase activity as a metabolic ratio (MR) using a validated HPLC assay.

Minor allele frequencies were 0.41, 0.01, 0.12 and 0.00 for *CYP2D6**2, *3, *4 and *17 variants, respectively, and the duplication frequency was 0.003. Genotype analysis correlated with phenotypes in 18 of 23 subjects (78.3%). 11 subjects were extensive metabolizers (EM), 8 were intermediate metabolizers (IM), 2 were poor metabolizers (PM) and 2 were ultra-rapid metabolizers (UM) which is fairly coincident with expected phenotypes metabolic ratios ranged from 0.11 to 126.41. The influence of *CYP2D6**3 was particularly notable, although only heterozygote carriers were present in our population. Individuals homozygous for *4 were always PM. As expected, the only subject with gene duplication was UM.

In conclusion, there was a clear effect of genotype on observed CYP2D6 activity. Classification of EM, PM and UM through genotyping was useful to characterize CYP2D6 phenotype in the Chilean mestizo population.

1. Introduction

Although expressed at lower levels compared to other human P450, CYP2D6 is one of the most studied and important isoenzymes given the number of metabolic routes of drugs and xenobiotics that it catalyzes. It is estimated that between 20 and 25% of clinically used drugs, some of which have a narrow therapeutic range, are metabolized by this enzyme. *CYP2D6* polymorphisms were discovered in the 1970s [1]. Subsequently, Mahgoub et al. [2] and Eichelbaum et al. [3] independently discovered that debrisoquine and sparteine are metabolized by CYP2D6, demonstrating the polymorphic nature of the drugs. More than 100 drugs have been shown to be substrates of this enzyme [2,3], including antidepressants, neuroleptics, β-blockers and antiarríthmics [4].

The *CYP2D6* gene is located on chromosome 22q13.1 in a gene cluster spanning about 45 kb, including the CYP2D7 and CYP2D8 pseudogenes with 97% and 92% homogeneity, respectively. CYP2D6 includes nine exons and eight introns, with an open reading frame of 1383 bp encoding 461 amino acids. The *CYP2D6* gene locus is complex and highly polymorphic, and variants within this locus affect CYP2D6 activity resulting in a wide range of enzyme activity from absence of activity to ultrarapid metabolism [5,6]. To date, more than 105 CYP2D6 variants and more than 20 SNPs without determined haplotypes have been described [7].

Abbreviations: CYP, cytocrome P450; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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Table 1
Anthropometric characteristics of the studied population. Values represent means ± SD for the number of subjects indicated. The significant differences between mean values (p-value < 0.05), assessed by Student's t test. BMI, body mass index.

Characteristics	Female $n = 188$	Male $n = 133$	Total $n = 321$[a]	p-Value
Age (years)	31 ± 12.3	30 ± 12.2	31 ± 12.0	0.472
Weight (kg)	61.8 ± 9.1	74.9 ± 10.1	66.8 ± 11.4	0.001
Height (m^2)	1.60 ± 0.10	1.73 ± 0.07	1.65 ± 0.09	0.001
BMI (kg/m^2)	24.4 ± 3.2	25 ± 2.9	24.4 ± 3.0	0.087

[a] Amerindian–Caucasian admixture = 18%.

One example of the clinical importance of the CYP2D6 polymorphisms is the tricyclic antidepressant nortriptyline which is cleared by this enzyme: poor metabolizers (PM) need 30–50 mg compared with ultrarapid metabolizers (UM) requiring 500 mg to achieve similar plasma levels. The standard dose of 150 mg is therefore not suitable for PM and UM [8].

The metabolic ratios for CYP2D6 are distributed in a bimodal manner in Caucasian populations, existing individuals with deficient activity (PM) and others with high enzyme activity (UM) [9–12].

CYP2D6 polymorphisms in human populations exhibit large differences in allele frequencies according to ethnicity [13–15]. In Caucasian populations, UMs may have a duplication/multiplication of the gene in about 7% of the population, while single base mutations (e.g. *4, *6, *8, *10, *17) of reduced function [16], partial deletions (*3) or complete deletion of the gene (*5) are nonfunctional alleles [17], all resulting in PM, are found in more than 10% of this population. In Latin America, the frequency of PMs is about 6.6% in Colombia, 3.2% in Mexico, among 2.2% and 4.4% and 3.6% in Panama and Nicaragua and about 10% in Chile [14,18–20].

As the CYP2D6 genotype has been associated with interindividual variability in the activity of the enzyme, there are several *in vivo* assays to evaluate this activity on the basis of the urinary metabolic ratio (MR: drug/urinary metabolite) for sparteine, metoprolol, dextromethorphan and debrisoquine, debrisoquine being the most widely used substrate probe due to its ability to discriminate EM, IM and UM phenotypes. This drug is an antihypertensive agent no longer in clinical use, metabolized by CYP2D6 to 4-hydroxydebrisoquine [1].

Variation in CYP2D6 metabolic activity is one of the most important factors giving rise to inter-individual variation in response to several drugs [21]. Determining CYP2D6 phenotypes, or metabolic capability, promises to help identify individuals for whom a change in drug or drug dosage is appropriate. To achieve this goal, analysis of genotypes is the preferred method when genotypes translate well to observed phenotype in metabolism; however, this is not confirmed in all populations. There can be substantial differences in test interpretation as there is no standardized process of translating CYP2D6 genotype to purported phenotype.

We studied the presence of *CYP2D6**2 (2850C>T, rs1135840, R296C), *3 (2549A>del, rs35742686, frameshift), *4 (1846G>A, rs3892097, splicing defect), *17 (1023C>T, rs28371706, T107I) and the duplication of the gene, with respect to enzyme activity in a Chilean mestizo population in order to identify the most relevant genetic profiles that account for observed CYP2D6 catalytic activity.

2. Materials and methods

2.1. Study population

321 people healthy volunteers (both sexes, 31 ± 12 years old) were recruited from the general population in Santiago, Chile (Table 1). All subjects provided informed consent. The protocol was approved by the Ethics Committee for Human Research at the Faculty of Medicine, University of Chile. The Amerindian–Caucasian admixture was determined to be 18%using ABO blood group distribution (BioClone®, ABO Pharmaceuticals, EE.UU) the Hardy–Weinberg and Bernstein equation [22–24].

A sub-group of 25 individuals, selected according to genotype, were asked to participate in an *in vivo* determination of CYP2D6 enzyme activity, were informed accordingly and consented to participate. Inclusion criteria were: healthy subjects, nonsmokers and without a history of alcohol or drug use. All individuals were screened for suitability by an extensive review of their medical history, physical examination such as blood pressure and pulse, and interpretation of standard biochemical analyses (e.g. glucose, blood urea nitrogen, creatinine, total protein, albumin, total bilirubin, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase, drugs of abuse). Exclusion criteria included: HIV, hepatitis B or hepatitis C infection; consumption of any prescribed or over-the-counter medication 60 days prior to participation; involvement in a clinical trial 90 days prior to participation. For female subjects exclusion criteria included pregnancy, planning pregnancy and breast-feeding.

2.2. DNA extraction and genotyping

Extraction of genomic DNA from whole blood was performed using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics®). DNA samples were quantified and stored at −20 °C until further analysis. Genomic DNA was amplified by PCR using primers for the specific variants studied (Table 2). PCR-RFLP was used to determine *CYP2D6**2, *3, *4, and *17. Amplicons were digested using appropriate restriction enzymes (Table 2). Electrophoresis was performed according to previously reported methods, with minor modifications, in agarose 2% or polyacrylamide16% gels [25]. *CYP2D6* gene duplication was detected using a modified long PCR method (PCRL), described previously [26,27]. The method is based on the detection of a homologous 3.6 kb sequence downstream of *CYP2D6* and *CYP2D7*, which identifies the presence of the duplication. Representative PCR analyses are shown in Fig. 1.

2.3. Evaluation of CYP2D6 enzyme activity

Phenotyping was performed in 25 previously genotyped subjects (26 ± 9.2 years old, body mass index: 22.8 ± 2.4 kg/m^2), according to inclusion and exclusion criteria as describe in Section 2.1. Volunteers were instructed about the procedure for collecting urine at home and were provided with appropriate receptacles and a single tablet of Declinax®, containing 22.5 mg debrisoquine sulfate, equivalent to 20 mg debrisoquine. Flyers were provided instructing not to eat the previous day from 19:00 pm until 00:00 am, and avoid coffee, alcohol and strenuous exercise. Instructions indicated to take a urine sample at 23:00 pm, designated as "control" and proceed to completely empty the bladder, and then ingest the tablet with 250 mL of water. Following 8 h, a subsequent urine sample was to collected, designated as "debrisoquine sample". Samples were processed up to 2 h after completion of the collection of urine samples, and were kept refrigerated (2–6 °C) throughout the process of collection and transportation to the laboratory. 10 mL aliquots of urine samples were stored at −20 °C for subsequent HPLC analyses.

2.4. Substrate determination

Following liquid–liquid urine extraction, chromatographic analysis was performed using a validated method adapted [28,29]. HPLC (Shimadzu LC-20 AT) separation employed to ODS-3 C18 Inertsil column followed by detection with a fluorescence detector

Table 2
Primers used for CYP2D6 genotyping.

Polymorphism	Forward (5′ → 3′)	Reverse (5′ → 3′)	Amplicon size (bp)	Endonuclease
CYP2D6*2 (2850C>T) rs1135840	GCTGGGGCCTGAGACTT	GGCTATCACCAGGTGCTGGTGCT	1029	*HhaI*
CYP2D6*3 (2549A>del) rs35742686	GATGAGCTGCTAACTGAGCCC	CCGAGAGCATACTCGGGAC	270	*MspI*
CYP2D6*4 (1846G>A) rs3892097	GCCTTCGCCAACCACTCCG	AAATCCTGCTCTTCCGAGGC	355	*MvaI*
CYP2D6*17 (1023C>T) rs28371706	TTTTGCACTGTGGGTCCTCGG	CCCGGGTCCCACGGAAATCT	529	*HphI*
CYP2D6xN	TCCCCCACTGACCCAACTCT	CACGTGCAGGGCACCTAGAT	5200	*NA*

NA, Non-applicable.

(Shimadzu RF-10 A XL) to 290 (ex) and 210 nm (em). The mobile phase consisted of NaH_2PO_4 0.1 M acetonitrile (87:13, v/v), pH 6.3, and the flow rate was 1.0 mL/min. The retention times were approximately 6.0 min for 4-hydroxydebrisoquine and 17.5 min for debrisoquine (Fig. 2). Debrisoquine MR was defined as the ratio between the molar concentration of the debrisoquine and 4-hydroxydebrisoquine, equivalent to the area under the curve (AUC) for both chromatograms. We defined phenotypes according MR as follows [25]: ultrarapid metabolizer (UM) <0.2; extensive metabolizer (EM) 0.2–1.0; intermediate metabolizer (IM) 1–12.6; and poor metabolizer (PM) >12.6.

2.5. Statistical analyses

Sample size calculations for genotyped populations were determined using nQuery Advisor 4.0 software considering: (a) 11% expected frequency for CYP2D6*4, from our previous work [14], (b) 95% CI ($1-\alpha$), (c) β value 20%, power 80%. Comparative analysis was performed using Student's t and χ^2 test. Relationships between genotypes and MR are presented descriptively.

3. Results

Allele frequencies were 40.6%, 1.09% and 11.8% for *CYP2D6**2, *3 and *4 variants, respectively, while the frequency of the gene duplication was 0.003 (Table 3). We did not find carriers of the *CYP2D6**17 allele.

Five volunteers of each predominant genotype were selected for phenotyping, with the exception of *4/*4 (four individuals), duplication (one individual) and *3/*3 (not present). Of these, 23 individuals agreed to participate, representing the following groups: four *1/*1, three *1/*3, three *2/*2, five *1/*4, one *4/*4, one *2/*2//duplication, two *2/*2//*1/*4, two *1/*2//*1/*3, one *1/*2//*1/*4 and one *2/*2//*4/*4 (Table 4).

Genotypes correlate with phenotypes in 18 of 23 subjects (78.3%, Table 4). The MR ranged from 0.11 to 126.41. Phenotypes exhibited were: 11 EM, 8 IM, 2 PM and 2 UM, closely aligned with expected phenotypes based on genotyping (7 EM, 13 IM, 2 PM and 1 UM).

As expected, individuals carrying *4/*4 and *4/*4//*2/*2 were PMs, however, heterozygote subjects (*1/*4) did not always correlate with the expected phenotype (two of four individuals were EM where IM was expected). The single subject with the gene duplication was an UM, as expected. Individuals exhibiting haplotype combinations *1/*4//*1/*2, *1/*2//*1/*3 and *1/*4//*2/*2 were IM as expected. Between two subjects carrying the *1/*4//*2/*2 haplotype, one exhibited divergence from the expected phenotype (EM where IM was expected). We were unable to explain the divergent result observed in one subject with *1/*2//*1/*3 and MR of 0.1, exhibiting UM where IM was the expected phenotype.

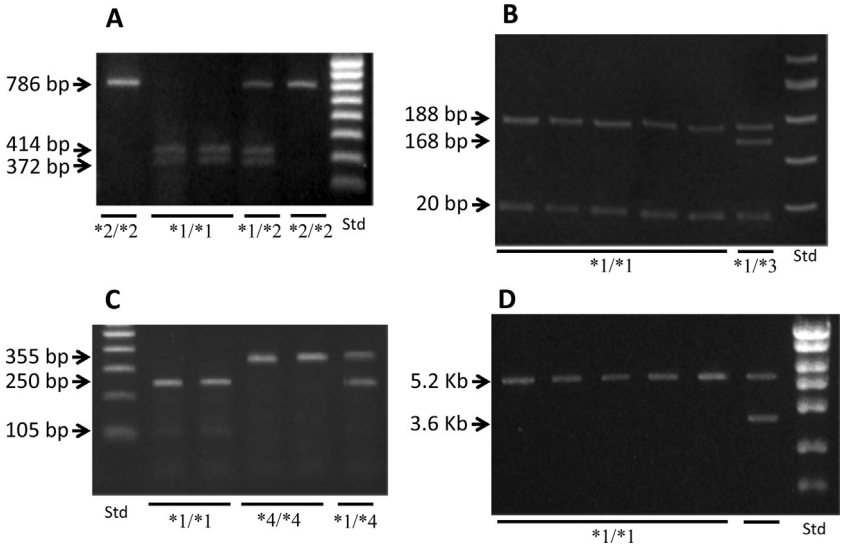

Fig. 1. Representative profiles of PCR-RFLP (A–C) and PCRL (D) analyses for CYP2D6 in Chileans. Different genotypes for CYP2D6 (A) CYP2D6*2, (B) CYP2D6*3, (C) CYP2D6*4 and (D) CYP2D6 duplication are shown.

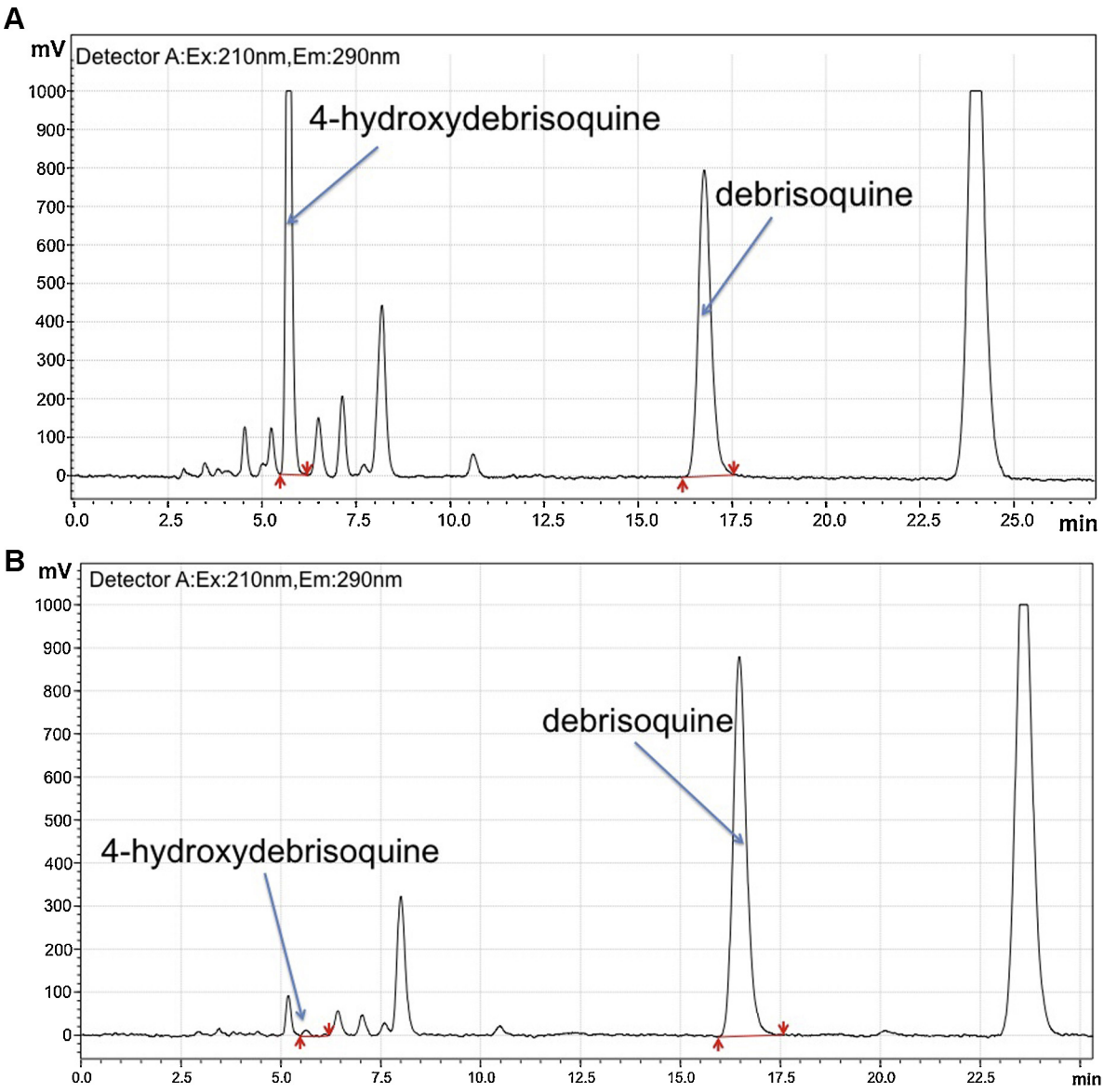

Fig. 2. Representative chromatographic profile of subjects. HPLC-fluorescence analysis (excitation 290 nm, emission 210 nm). (A) Extensive metabolizer and (B) poor metabolizer.

Table 3
Genotype and allele frequencies observed in the studied group.

	Female	Male	Total	*p*-Value
CYP2D6*2				
*1/*1	56 (29.8%)	63 (47.4%)	119 (37.0%)	
*1/*2	85 (45.2%)	58 (43.6%)	143 (44.5%)	
*2/*2	47 (25.0%)	12 (9.0%)	59 (18.4%)	
Allele frequency (*2)	0.476	0.308	0.407	0.003
CYP2D6*3				
*1/*1	183 (97.3%)	131 (98.5%)	314 (97.8%)	
*1/*3	5 (2.7%)	2 (1.5%)	7 (2.2%)	
*3/*3	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Allele frequency (*3)	0.013	0.008	0.011	0.671
CYP2D6*4				
*1/*1	154 (81.9%)	95 (71.4%)	249 (77.6%)	
*1/*4	32 (17.0%)	36 (27.1%)	68 (21.2%)	
*4/*4	2 (1.1%)	2 (1.5%)	4 (1.3%)	
Allele frequency (*4)	0.095	0.150	0.118	0.132
CYP2D6xN				
Present	1 (0.5%)	0 (0.0%)	1 (0.3%)	
Absent	187 (99.5%)	133 (100.0%)	320 (99.7%)	0.400

The significant differences between mean values (p-value < 0.05), assessed by one-way ANOVA and Student's t test.

Table 4
Expected and observed phenotypes according genotypes for 23 volunteers.

ID subject	Genotype	Expected phenotype	Metabolic ratio (MR)[a]	Observed phenotype
Single genotype				
1	*1/*1	EM	0.28	EM
4	*1/*1	EM	0.44	EM
13	*1/*1	EM	0.33	EM
15	*1/*1	EM	0.28	EM
6	*1/*3	IM	1.52	IM
10	*1/*3	IM	1.30	IM
20	*1/*3	IM	2.56	IM
14	*2/*2	EM	0.26	EM
16	*2/*2	EM	0.39	EM
23	*2/*2	EM	0.26	EM
25	*2/*2/xN	UM	0.11	UM
3	*1/*4	IM	1.44	IM
5	*1/*4	IM	0.71	EM
12	*1/*4	IM	0.28	EM
19	*1/*4	IM	1.35	IM
22	*1/*4	IM	0.58	EM
7	*4/*4	PM	126.41	PM
Haplotype				
17	*4/*4, *2/*2	PM	121.38	PM
18	*1/*4, *1/*2	IM	1.05	IM
19	*1/*4, *2/*2	IM	0.68	EM
2	*1/*4, *2/*2	IM	1.46	IM
24	*1/*2, *1/*3	IM	1.75	IM
8	*1/*2, *1/*3	IM	0.11	UM

[a] MR: <0.2 = UM (ultrarapid metabolizer); 0.2–1.0 = EM (extensive metabolizer); 1.0–12.6 = IM (intermediate metabolizer); >12.6 = PM (poor metabolizer).

4. Discussion

In the present work we studied the frequencies of *CYP2D6* alleles (*2, *3, *4, *17) and gene duplication in 321 Chilean healthy volunteers. The frequencies are similar to those of the Spanish population [9,14,27,30], likely attributable to the low Amerindian–Caucasian admixture of the studied group (18%). The lack of the *17 is expected since it is frequent among African populations (34%) but is typically not present among Caucasian populations [31]. While there is African ancestry in the Chilean admixed population, the proportion is very low [24].

In order to analyze potential relationship between genotypes and CYP2D6 phenotype we performed an *in vivo* determination of CYP2D6 (debrisoquine hydroxylase activity) in 23 previously selected volunteers according their genotypes, using the metabolic ratio 4-hydroxydebrisoquine/debrisoquine in urine samples by HPLC analyses. We use the classification previously described [25] to assign a metabolic ratio (MR) to one phenotype.

Following determination of MR, and classification into metabolizer groups [25], we found a coincidence of 78.3% between expected and observed phenotypes. For one extreme divergence, one subject with *1/*2//*1/*3 exhibiting an UM status. This subject has not a duplicated genotype. However, as we did not analyze the promotor polymorphism −1584C>G (rs1080985), previously reported to give rise ultrarapid metabolizers [32], it is possible that the presence of this variant could be responsible of this divergence. In the rest 22 selected volunteers we did not find any other non-explained ultrarapid metabolizer phenotype, giving rise the idea of absence of this variant in this group. On the other hand, the divergence between IM and EM might be explained by the similarity of these phenotypes and limits of the defined ranges.

One limitation of the present work is that we did not analyze all polymorphisms that exhibit ethnic variability [15], for example CYP2D6*5, a well described deletion of the gene. However, a reliable genotyping method for this variant was not available at the time we performed the present study. We were unable to establish a robust PCR method for this variant, coinciding with previous reports of a lack of specificity and potential interferences for published PCR methods [33]. Moreover, several studies evaluating the presence of this polymorphism in American populations have reported allele frequencies about 2% [8,34,35]. Therefore, taking into account this information, i.e. low population frequencies and difficulties to have proper analytic results, we decide not to include CYP2D6*5 polymorphism in this investigation. However, as we cannot completely exclude the influence of this polymorphism in PM of the Chilean population we will continue trying to study this variant in future investigations.

To our knowledge, this is the first report characterizing *in vivo* debrisoquine hydroxylase activity exploring genetic-phenotypic correlation of CYP2D6 in the Chilean population

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