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Cystathionine β-synthase and methylenetetrahydrofolate reductase mutations in Mexican individuals with hyperhomocysteinemia

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Abstract

Background: Hyperhomocysteinemia, a thrombotic risk factor, may have several causes. Among the genetic causes of hyperhomocysteinemia, there are polymorphisms in the enzymes methylenetetrahydrofolate reductase (C677T) and cystathionine β-synthase (C699T, C1080T, and 844ins68). Although the frequency of hyperhomocysteinemia in our country is high, there is no evidence about the frequencies of these polymorphisms.

Methods: We analyzed 80 healthy individuals from several regions in our country. We evaluated the fasting and post-oral methionine load plasma Hcy and the genotypes in order to obtain the allele frequencies of the polymorphisms C677T of methylenetetrahydrofolate reductase and C699T, C1080T, and 844ins68 of the cystathionine β-synthase.

Results: No individual had deficiency of folic acid, vitamins B12, or B6, but 80% had post-oral methionine load hyperhomocysteinemia. We found a significant increase in the Hcy plasma concentration associated with age and gender. Only the polymorphism C1080T was significantly associated with hyperhomocysteinemia.

Conclusion: There is an association between fasting and post-oral methionine load plasma Hcy concentrations with the allelic frequencies of the polymorphisms C669T, 844ins68, and C1080T of the cystathionine β-synthase and C667T of the methylenetetrahydrofolate reductase in healthy Mexican individuals. As compared with individuals with normal fasting or post-oral methionine load Hcy plasma levels, only C1080T was significantly associated with hyperhomocysteinemia.

Keywords

Homocysteine, hyperhomocysteine, oral methionine load, cystathionine beta-synthase, methylenetetrahydrofolate reductase

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Introduction

Hyperhomocysteinemia (HHC) is a pathological entity characterized by a high concentration of plasma homocysteine (Hcy) (>15μmol/L).[1] HHC has been considered as an important risk factor for neural tube defects and for diseases such as atherosclerosis and coronary artery disease, cerebrovascular events, neuropsychiatric disorders such as schizophrenia, and adverse effects during the pregnancy.[2] HHC induces endothelial dysfunction[3,4] because of several mechanisms including increased oxidative stress and elevated production of reactive oxygen species (ROS) which up-regulate the expression of angiotensin-converting enzymes. ROS may bind with nitric oxide (NO) to generate $ONOO^-$ which may further deteriorate the endothelial dysfunction.[5] HHC also alters the release of endothelin-1, NO, prostacyclin, angiotensin II, and thromboxane A_2. All these mediators are important as vasodilators and regulators of the vascular function,[6] having a critical impact on other endothelial functions such as anti-oxidative and anti-inflammatory effects.[7] Other undesirable effects of HHC, such as deregulation of the hydrogen sulfide signaling pathway, disturbances in lipoprotein metabolism, protein *N*-homocysteinylation, cellular hypomethylation, apoptosis of endothelial, and smooth muscle cells,[8] and neurodegeneration,[9] have been described.

Hcy, a non-essential amino acid generated during the methionine metabolism, is metabolized through two pathways: remethylation and transsulfuration. In remethylation, Hcy forms methionine by the addition of a methyl group from 5-methyltetrahydrofolate or betaine. 5-methyltetrahydrofolate results in the conversion of dietary folic acid to 5,10-methyltetrahydofolate and finally to 5-methyltetrahydrofolate by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). The reaction of remethylation with 5-methyltetrahydrofolate occurs in all tissues and vitamin B12 participates as a co-factor. The reaction with betaine is limited to the liver, and it is independent of vitamin B12. In the transsulfuration pathway, Hcy is converted to cystathionine by cystathionine β-synthase (CBS) and finally to cysteine using vitamin B6 as a co-factor.[10] HHC is due to several factors such as age and gender, genetic disorders linked to enzymatic deficiencies, ethnicity, life-style factors, poor vitamin intake, liver, renal or thyroid disease, comorbidities, and drug use,[11,12] and differences in the definitions of HHC.[13,14] Although establishing the true frequency of HHC is an almost impossible task because of the number of variables described in association with this phenomenon, more information about this metabolic abnormality and its frequency is given in the literature. On the contrary, although the frequency of some mutations, namely, MTHFR C677T, is evenly described world-wide (around 32%),[13] the frequencies for other mutations are also widely variable among different regions of the world.

In an important percentage of individuals with fasting normal concentrations, Hcy may significantly rise when the patient is challenged with an oral methionine load (OML). This procedure induces high concentrations of plasmatic Hcy and discovers genetic or acquired abnormalities, mainly in the transsulfuration pathway.[15]

Because atherothrombotic disease is a main cause of death and a public health problem in the world as in Mexico, it is important to identify the likely impact of each atherothrombotic risk factor to identify more people at risk of developing these complications. Based on the quite specific genetic background of the Mexican population, we hypothesized that the frequencies of the mutations associated with HHC could be different from those described in other countries. Therefore, our objective was to determine the fasting and post-oral methionine load (POML) plasma concentrations of Hcy in healthy Mexican individuals and to identify the allelic frequencies of polymorphisms 844ins68 in exon 8, C699T in exon 6, C1080T in exon 10 of the CBS, as well as the C667T mutation of the MTHFR in individuals with POML HHC.

Methods

Study design and period

A hospital-based, observational, descriptive, cross-sectional, non-randomized study was conducted from 1 August 2017 to 30 August 2019.

Population

Apparently healthy male or female individuals, older than 18 years, from different regions of our country were included. Individual exclusion criteria were liver or renal disease, hypothyroidism, known thrombotic abnormalities, and recent use of vitamin supplements or medications known to interfere with the metabolism of folic acid (methotrexate, phenytoin, among others). Eligible patients were informed about the characteristics of the study protocol, and a signed informed consent was obtained before blood was drawn. A non-validated questionnaire was applied to all individuals to obtain information about their personal and family history as well as data about their life style.

Study protocol

At the first day, a fasting 5 mL blood sample was drawn in a vacuum glass tube containing ethylenediaminetetraacetic acid (EDTA), and another 5 mL sample was collected in a tube without an anticoagulant (Beckton Dickinson, Franklin Lakes, NJ, USA). On the second day, the same samples of blood were collected 8 h after intake of an OML. OML was performed as previously described using high performance liquid chromatography (HPLC) grade L-methionine (Sigma-Aldrich Chemicals, St. Louis, MO) (100 mg/kg of body weight) diluted in orange juice.[16] Patients were asked to drink

the methionine during a 20 min period, and 8 h later, a blood sample was obtained. Both fasting and AOML blood samples were maintained on ice until they were centrifuged at 5000×*g* for 10 min to obtain platelet-poor plasma and serum. Three aliquots from each sample were frozen at −70°C until processing. Plasma was used for quantification of Hcy, while serum was used to measure levels of vitamin B12 (reference ranges: 150 to 900 pg/mL) (Access Immunoassay System, Beckman Coulter, Fullerton, CA).[17] Intra-erythrocyte folic acid was evaluated using microbiological assay (reference ranges: >6 ng/mL).[18]

Participants were classified as controls when they had both normal fasting and POML Hcy plasma concentrations and normal plasma concentrations of vitamin B12 and folic acid. Those individuals with Hcy plasma concentrations either fasting or POML above 15 μmol/L[19] and with normal plasma levels of vitamin B12 and folic acid were considered as the study subjects.

Hcy measurement

Total plasma Hcy concentrations were evaluated using HPLC with fluorescence detection.[20] The in-house HPLC assay is a modification of the method reported by Carducci et al.[21] Briefly 100 μL of the sample was reduced to free Hcy with dithioethreitol. After protein precipitation with 6% perchloric acid, Hcy was carboxymethylated with iodoacetate before derivatization with o-phthaldialdehyde. HPLC was carried out on an Alliance 2690 and a 474 scanning fluorescence detector (338 nm excitation, 425 nm emission; both from Waters Technology Corp, Milford, MA, USA). Separation was performed on a Kingsorb C18 analytical column, 150 × 4.6 mm^2, 3 μm (Phenomenex, Torrance, CA), using a 10 μL injection volume. The analysis was carried out in a linear gradient from 78% of solvent A to 70% A from 7 min and 100% B from 13 min. Mobile phase A was 0.02M sodium phosphate buffer pH 7.0, tetrahydrofuran 96:4; mobile phase B was 0.02M sodium phosphate buffer pH 7, acetonitrile 45:55.

Polymorphism analysis

DNA was extracted using the QIAamp DNA Blood Mini Kit DNA (Qiagen Inc., Valencia, CA, USA), from lymphocytes obtained from blood anticoagulated with EDTA. Once the DNA was extracted, its integrity was confirmed in a 1% agarose gel and its purity and concentration were assayed by spectrophotometry.

Polymorphism C667T. Detection was performed using the polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) technique. The following primers were used for amplification: 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and 5′-AGG ACG GTG CGG TGA GAG TG-3′ by PCR using 30 ng of genomic DNA, 1.5 mM MgCl2, primers of 0.2 pmol/μL each, dNTP of 0.1 mM each, and 0.05 U of Taq DNA polymerase (Promega) in the buffer recommended by the supplier. The polymorphism was checked using PCR parameters, which were as follows: 3 min initial denaturation at 92°C, followed by 30 cycles of 92°C/60 s, 58°C/60 s, and 72°C/30 s, and a final extension of 7 min at 72 C°. A 198 bp product was obtained which was digested with *HinfI*. The presence of the polymorphism generates a restriction site for the enzyme and two fragments are obtained, 177 and 21 bp, which were then separated in a 3% agarose gel.

Polymorphism C699T. This polymorphism does not create a restriction enzyme recognition site and was therefore analyzed by Primer Introduced Restriction Analysis. A mutagenic sense oligonucleotide (5'-CAGCAACCCCCTGGCTCAGT-3') introduces a RsaI site in the 699C allele and together with an antisense oligonucleotide (5'-TTATCGTTTGTGTCCCG-TACCG-3')[22] a genomic DNA fragment of 287 bp was amplified using approximately 100 ng genomic DNA and 100 ng of both oligonucleotides in a standard PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl) containing 2 mM MgCl2 and 200 μM dNTPs. PCR parameters were as follows: 3 min initial denaturation at 92°C, followed by 30 cycles of 92°C/60 s, 64°C/60 s, 72°C/30 s, and a final extension of 7 min at 72°C. After digestion with RsaI and resolution of the fragments on a 20% polyacrylamide gel, the 699CC genotype results in fragments of 171, 92, 20 and 4 bp, whereas the 699TT genotype shows fragments of 171, 112 and 4 bp.

Polymorphism C1080T. A genomic DNA fragment of 88 bp was amplified by PCR using both 100 ng forward (5'-CTG-GCAGCACGGTGGCGG-3') and 100 ng reverse oligonucleotides (5'-CGCACTGAGTCGGGCAGAATG-3') and approximately 100 ng genomic DNA in a standard PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl) containing 2 mM MgCl2 and 200 μM dNTPs. All samples were cycled 30 times: 1 min/92°C denaturation, 1 min/55°C annealing, and 30 s/72°C extension, preceded by an initial denaturation of 3 min/92°C and followed by a final extension of 7 min/72°C. The PCR fragment was analyzed by BstUI restriction enzyme analysis followed by separation on a 2% agarose gel. The 1080TT genotype results in an uncut fragment of 88 bp and the 1080CC genotype in two fragments of 55 and 33 bp, whereas the heterozygous C/T genotype is displayed by three fragments (88, 55, and 33 bp).

Mutations I278T and G307S. These mutations in exon 8 of the CBS gene were determined using PCR-RFLP-based assays using restriction enzymes AluI (New England Biolabs, Ipswich, MA, USA) for I278T and BsrI (New England Biolabs, USA) for G307S. The following primers were used for amplification: forward: 5′-TCCAGGCGGGGCTTTTGCTG-3′ and reverse: 5′-GCACTGTGGCCGGGCTCTGGA-3′. All samples were cycled 30 times: 1 min/92°C denaturation, 1 min/64°C

Table 1. Anthropometric Values According to Gender in Participating Subjects.

	Total (n = 80)	Women (n = 50)	Men (n = 30)	P-value
	X (SD)			
Gender	–	62.5%	37.5%	–
Age (years)	43.9 ± 19.5	45.3 ± 20.6	40.4 ± 16.5	0.245
Weight (kg)	69.8 ± 7.8	67.52 ± 7.5	73.7 ± 6.9	0.0004
Height (m)	1.6 ± 0.1	1.57 ± 0.05	1.71 ± 0.05	<0.0001
BMI (kg/m^2)	26.4 ± 2.9	27.3 ± 2.9	25.0 ± 2.2	0.0001
Waist circumference (cm)	91.7 ± 7.5	90.0 ± 10.2	93.4 ± 4.8	0.521
Hip circumference (cm)	100.8 ± 4.6	100.2 ± 5.2	101.5 ± 4.1	0.033

SD: standard deviation; BMI: Body mass index.
Means contrasted with the Mann–Whitney U-test. Statistical significance was considered when $P \leqslant 0.05$.

annealing, and 30 s/72°C extension, preceded by an initial denaturation of 3 min/92°C and followed by a final extension of 7 min/72°C.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). For the analysis of fasting Hcy plasma concentrations, a Student's t test was used. The Mann–Whitney U-test was used to analyze POML plasma Hcy concentrations. Significant differences were considered when $P \leqslant 0.05$. We also analyzed the risk of HHC based on the presence of several polymorphisms by calculating the odds ratio (OR) with a 95% confidence level. All data were analyzed with the SigmaPLot 12.1 statistical program (Systat Software, Inc., San Jose, CA, USA).

Sample size

The sample size was calculated based on a difference of proportions and following the hypothesis that the allelic frequency of the C1080T polymorphism of CBS in healthy Mexican donors without HHC (basal and post-COM) should be low or absent, ranging between 0% and 5% but equal to or greater than 50% in donors with HHC, considering the findings in other populations. In accordance with the above and considering a value of $\alpha = 0.05$ and $1-\beta = 0.8$, the estimated sample size was 19 donors per group.

Ethics

The protocol was approved by the Ethics Committees of our hospital (Instituto Mexicano del Seguro Social (REC No. 3605), approved this study (Approval No. R-2017-3609-32)), according to the ethical standards of the Helsinki Declaration of 1975 (revised in 2000). All patients were informed about the aims of the study and signed informed consent prior entering the study.

Results

Blood samples were obtained from 100 apparently healthy Mexicans, but 20 were discarded because they did not meet the inclusion criteria. In the end, only samples of 80 individuals were analyzed, 50 women (62.5%) and 30 men (37.5%). In all of them, plasma concentrations of fasting and POML Hcy, vitamin B_{12}, and intra-erythrocyte folic acid were determined. Mean ± SD of plasma vitamin B12 concentration was 453.3 ± 184.9 pmol/L (reference values: >150 pmol/L), while mean + SD intra-erythrocyte folic acid concentration was 11.63 ± 6.65 ng/mL (reference values: >6 ng/mL). All had normal B_{12} and folic acid concentrations.

Demographics of participants in the study are shown in Table 1. Mean concentration of fasting plasma Hcy was 8.66 ± 3.44 μmol/L. According to the world-wide accepted diagnostic criteria, only one patient had fasting HHC in this study (accepted upper limit = 15 μmol/L). Fasting plasma concentrations of Hcy had a normal distribution between 2.5 and 16.31 μmol/L. Plasma concentrations of both fasting and POML plasma Hcy concentrations are shown in Figure 1.

After administration of the OML, those individuals with Hcy plasma concentrations >15.0 μmol/L were also considered as carriers of HHC. Only 20% of the individuals had normal POML Hcy concentrations, and they were considered as controls. POML HHC was found in 80% of the individuals, and they were classified as having mild (plasma Hcy between 15.1 and 30 μmol/L, 20%), moderate (plasma Hcy between 30.1 and 100 μmol/L, 73.3%), and severe HHC (plasma Hcy > 100 μmol/L, 6.6%). We did not find significant differences in fasting or POML Hcy concentrations between women and men (Figure 2). However, after adjusting by age (18–40 vs 41–80 years old), we observed some significant differences. Mean fasting plasma Hcy concentrations in individuals between 18–40 years old vs 41–80 years old were significantly different (P = 0.027); however, this difference disappeared after we compared POML plasma Hcy concentrations in individuals between 18–40 vs 41–80 years old (P = 0.053). Fasting Hcy and POML Hcy plasma concentrations were not significantly different in men between

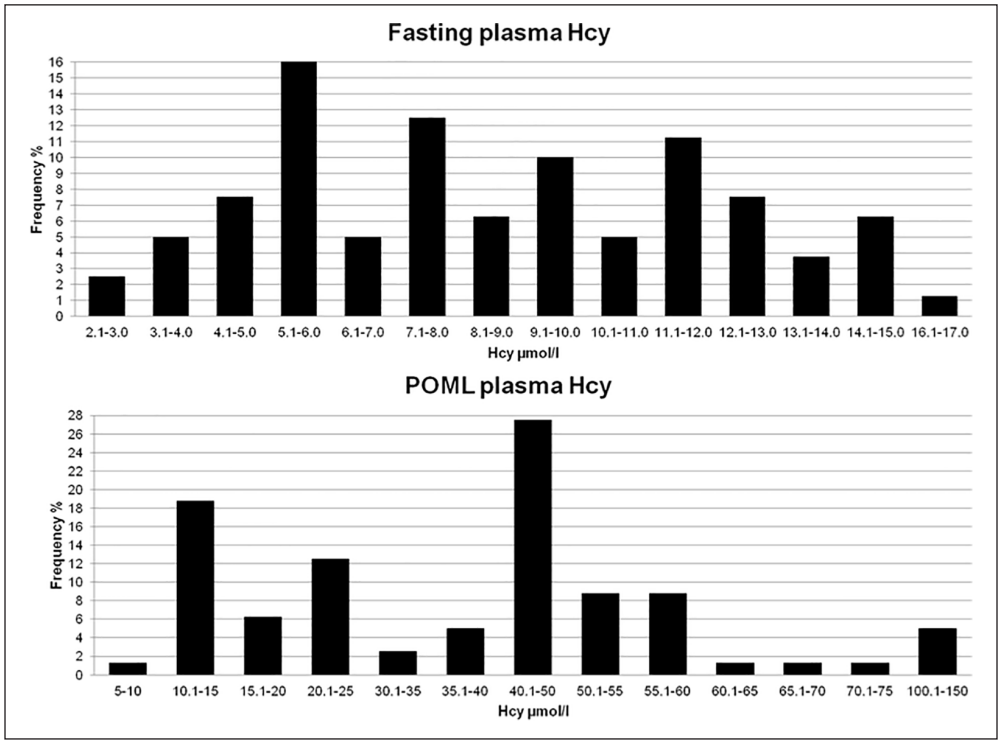

Figure 1. Distribution of fasting and post-oral methionine load (POML) homocysteine (Hcy) plasma concentrations.

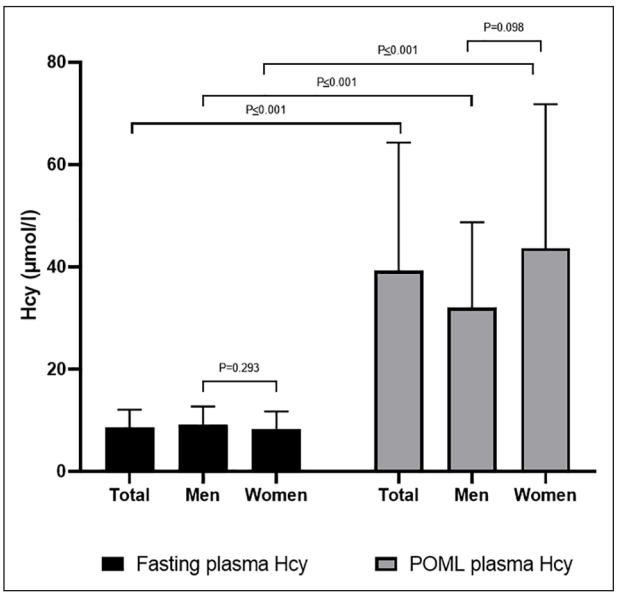

Figure 2. Fasting and post-oral methionine load (POML) plasma homocysteine (Hcy) concentrations in the study population. Significant differences were found when compared to fasting Hcy vs POML Hcy plasma concentrations in the whole group as well as between women and men. However, no significant differences were found between women and men when fasting Hcy or POML Hcy plasma concentrations were separately analyzed.
Means contrasted with the Mann–Whitney U-test*. Statistical significance was considered when $P \leq 0.05$.

18–40 vs 41–80 ($P=0.092$ and $P=0.575$, respectively). On the contrary, fasting Hcy as well as POML Hcy plasma concentrations in women between 18–40 vs 41–80 years old were significantly different ($P=0.003$ and $P=0.01$, respectively). Finally, comparison of fasting Hcy plasma concentrations between men and women in the 18–40 years old range was significantly different ($P=0.029$). This difference disappeared when we compared the fasting Hcy plasma concentrations between men and women in the 41–80 years old range ($P=0.611$) (Figure 3).

Due to the high frequency of POML HHC in our population, we considered important to determine if this phenomenon was likely due to a specific mutation (s) in the CBS gene. Therefore, we analyzed the G307S, I278T mutations and the C1080T, C699T, and 844ins68 polymorphisms of the CBS as well as the C667T polymorphism of the MTHFR. These specific genotypes in the CBS gene were chosen because they are the most frequently described in association with HHC in the worldwide literature. The C677T polymorphism was evaluated because it was previously informed with a high frequency in the Mexican population.[23] None of the individuals of this study had the G307S and I278T mutations. The mutated allele of the C667T polymorphism of the MTHFR was found with a higher frequency in those individuals with POML HHC vs controls. The mutated allele of the C1080T polymorphism was more frequently found in those individuals with POML HHC vs controls (0.65 vs 0.42,

respectively). This polymorphism was not in Hardy–Weinberg equilibrium in both study groups.

The mutated allele of the C699T polymorphism was more frequently found in individuals with POML HHC, and it was never in the homozygous status; in those individuals with POML HHC, it was not in Hardy–Weinberg equilibrium. The 844ins68 polymorphism was found with the lower frequency in the study population, and it was found with almost the same frequency in individuals with POML HHC and controls. The M/M genotype of this polymorphism was never found in our study (Table 2).

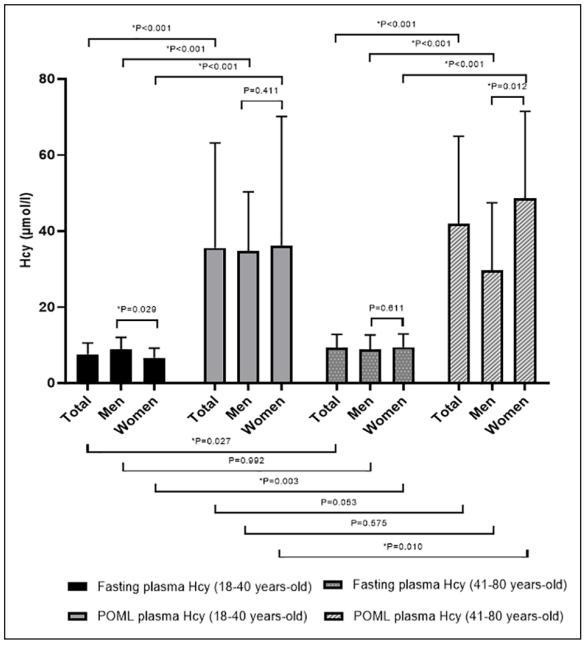

Figure 3. Fasting and post-oral methionine load (POML) plasma homocysteine (Hcy) concentration by gender and age. The whole study population was separated in two groups (18 to 40 years-old vs 41 to 80 years old).
Means were contrasted with the Mann–Whitney U-test*. Statistical significance was considered when $P \leq 0.05$.

After analyzing the likely relationship between all these polymorphisms and the presence of HHC, we found that those individuals with the C1080T polymorphism had a OR = 3.27 (P = 0.0321) (Table 3).

Discussion

In recent years, mild and moderate HHC has taken great importance in some pathological conditions because it is independently associated with an increased risk for arterial or venous thromboembolic diseases.[23] In Mexico, because almost no data about HHC have been obtained, in this study, we evaluated in healthy population the likely relationship between fasting and POML Hcy plasma concentrations and the frequency of some of the most common mutations that have been associated with HHC around the world. Fasting Hcy concentrations showed a normal distribution with a mean of 8.66 + 3.44, and these values were similar to those reported in the literature.[24,25] However, the frequency of POML HHC was quite high (80%), without association with deficiency of folic acid or vitamins B6 or B12. Moderate and severe POML HHC was present in 73.3% and 6.6% of the subjects, respectively. This is important because it has been informed that increased plasma Hcy concentrations induce endothelial dysfunction and a higher risk of thrombotic disease.[26] Although the results of our research are completely new for our population, they may not be quite surprising. Indeed, the informed frequencies of both the mutations of the enzymes of the Hcy metabolism as well as HHC are quite diverse over the world. As most of the Mexican population has a specific, well-defined genetic background, we initially hypothesized that our results would be different from those described in other populations. However, because several Latin American countries share profound genetic similarities with the Mexican population, it could be possible further suggest that our results may also be important in these countries.

Fasting plasma Hcy concentrations were significantly higher in men as compared with women but only in the group between 18 and 40 years old. This fact agrees with previous

Table 2. Genotype Frequencies on Individuals with POML HHC.

Polymorphism	W/W	%	W/M	%	M/M	%	Frequency allele M	HW (P)
Controls (n = 20)								
C667T	10	50	8	40	2	10	0.30	0.8313
C1080T	9	45	5	25	6	30	0.42	0.0289*
C699T	15	75	4	20	1	5	0.15	0.3347
844ins68	14	70	6	30	0	0	0.15	0.4299
Individuals with POML HHC (n = 60)								
C667T	24	40	22	36.7	14	23.3	0.41	0.0570
C1080T	12	20	17	28.3	31	51.7	0.65	0.0041*
C699T	32	53.3	28	46.7	0	0	0.23	0.0184*
844ins68	40	66.7	20	33.3	0	0	0.16	0.1213

W: normal allele; M: mutated allele; HW: Hardy–Weinberg equilibrium; POML: post-oral methionine load; HHC: hyperhomocysteinemia.
*$P < 0.05$ = not consistent with HW.

Table 3. Risk of HHC According to the Presence of the 667CT, 1080CT, 699CT, and 844 ins68 Polymorphisms.

Polymorphism	P-value	OR (95%CI)
C667T	0.4347	1.5 (0.5423–4.1490)
1080CT	0.0321	3.2727 (1.1064–9.6811)
699CT	0.0948	2.6250 (0.8462–8.1433)
844 ins68	0.7829	1.1667 (0.3896–3.4934)

OR: odds ratio; CI: confidence interval; HHC: hyperhomocysteinemia.

evidence showing that young women have lower plasma Hcy concentrations and that these differences disappear with age.[27] It has been proposed that, at least partially, this phenomenon could be due to the influence of sexual hormones because plasma Hcy rises after menopause.[27,28] Furthermore, it should be mentioned that renal function importantly determines the plasma Hcy concentration and, as a consequence, age-related physiological fall of the renal capacities may also help to explain the rise of plasma Hcy concentrations associated with an increasing age.[29]

Because of the high frequency of POML HHC in our population, we decided to investigate the allele frequencies of mutations I278T and G307S and the C1080T, C699T, and 844ins68 polymorphisms of the CBS gene as well as the C667T polymorphism of the MTHFR gene. The homozygous C667T trait is associated with a rise the plasma Hcy concentration up to 20%.[30,31] The frequency of the heterozygous genotype C/T of the C677T polymorphism in the Mexican population is significantly higher (almost 50%) than those described in other populations,[32] and it greatly varies among the several mestizo populations in our country. In this study, we found that the frequency of the C/T genotype is 45.8% among the control subjects and 35.9% among people with HHC. The frequency of the homozygous trait T/T in the general population is almost 30%[33] however, in this study, we found that the frequency among control and cases was 20.8% and 23.1% respectively. It is well known that the simultaneous presence of the C667T and 844ins68 polymorphisms of the CBS is significantly associated with an increased risk of occlusive arterial and venous diseases.[34] Regarding this last information, we must underline that in Mexico, the frequency of diabetes mellitus is quite high but the frequency of chronic complications associated with this metabolic disease seems also higher than those described in other countries. Due to the high frequency of POML HHC found in this study, we hypothesize that this last biochemical abnormality may have either a direct or indirect role on the high frequency of such chronic diabetic complications. Further research addressing this hypothesis is warranted.

In patients with severe HHC, it was described a specific mutation of the exon 8 of the CBS characterized by a 68 pb insertion: 53 bp are inserted in the 3′ of the intron 7 and 15 bp are inserted in the 3′ region of the exon 8.[35,36] However, recent studies showed that the inserted sequence was eliminated after the splicing of the distal region of the intron 7-exon 8, a phenomenon that results in a normal size of the mRNA of CBS in the carriers.[37] Several studies attempted to establish a relationship between this polymorphism and the presence of occlusive arterial disease, but the results were contradictory. We know that the heterozygous state of this polymorphism is not per se a risk factor for premature occlusive vascular disease, but, if combined with the C667T mutation of the MTHFR, the risk of thrombotic events rises fourfold.[38] The polymorphism 844ins68 has been described in the heterozygous presentation in almost 12% of the general population.[38] However, in our study, we found that the frequency is as high as 33.3% and 35.9% in the control group and in subjects with HHC, respectively. The homozygous state was never found in the individuals studied. In the heterozygous state, this polymorphism was considered as a risk factor for deep vein thrombosis in the Brazilian population.[34]

Regarding the C699T and C1080T polymorphisms of the CBS, some studies indicate that hetero- or homozygous carriers of the first polymorphism have lower POML plasma Hcy vs individuals with the C/C genotype. The allele 1080T is associated with POML Hcy plasma concentrations significantly lower only if the carrier has not the alleles 844ins68 and C699T.[35] In our study, we found that polymorphisms C1080T and C699T were frequently found in individuals with POML HHC. Interestingly, the polymorphism C1080T is not in the Hardy–Weinberg equilibrium, and it is likely that this lack of equilibrium may be due to a deficit of heterozygous C/T carriers with the homozygous C/C and T/T in both cases and controls. Of course, this was an involuntary sampling error after we discharged a technical mistake in the assignment of genotypes. However, this was the only polymorphism showing a significant association with POML HHC.

Genetic abnormalities leading to severe HHC (congenital homocystinuria) are homozygous or combined mutations (with other point mutations); however, it has been described that these mutations also induce mild or moderate HHC with plasma Hcy concentrations ranging between 20 and 40 μmol/L when they are present in a heterozygous state.[39] Mutations G307S and I278T in the exon 8 of the CBS are autosomal recessive traits which are considered the main cause of homocystinuria. In patients with homocystinuria, there is an increased blood concentration of methionine and Hcy as well as a high urinary excretion of Hcy. The frequency of homozygous and heterozygous homocystinuria is 1/200,000 and 1/70,000, respectively.[40] Congenital homocystinuria is the homozygous form of this disease. It is associated to fasting plasma Hcy concentrations >400 μmol/L, and its clinical manifestations include thromboembolic diseases and premature atherosclerosis. Heterozygous carriers of this mutation have lower plasma Hcy concentrations (20–40 μmol/L).[39] The I278T mutation is a pan-ethnic trait that is identified in almost 25% of all alleles of patients from different ethnical backgrounds and, characteristically, they respond

to the treatment with pyridoxine. However, in the homozygous state, this mutation is associated with a relatively mild homocystinuria phenotype. In some regions like the Netherlands, it represents <50% of the homocystinuria alleles and it is the most prevalent mutation in patients with Czech and Slovakian background.[41] On the contrary, the G307S mutation is mainly detected in the homocystinuria alleles of patients with a Celtic origin. It represents almost 70% of the homocystinuria alleles in Ireland, is associated with severe homocystinuria when it is present as a homozygous trait, and does not respond to the treatment with pyridoxine. The mutation has never been found in Italy, the Netherlands, Germany, and the Czech Republic.[41] In our study, we search for the mutations I278T and G307S, but none of the individual analyzed were carriers of these defects.

Our research has some limitations. First, we must highlight that, although the statistical analysis was significant after comparing the allele frequencies of the C1080T polymorphism between controls and donors with hepatocellular carcinoma (HCC; $p = 0.017$ (z-test + Yates correction); $P = 0.015$), the power of the test was below ($1–\beta = 0.663$ z-test + Yates correction; $1–\beta = 0.681$ chi-square + Yates correction) the expected value ($1–\beta = 0.80$). Although this represents a limitation, it does not affect the significance of our research: we found an allelic frequency of C1080T polymorphism which was significantly higher than expected. Moreover, we found a higher than expected frequency of POML HHC in our country; however, due to the design of the study and the relatively small sample size, we cannot be sure about the likely clinical impact that this finding. Second, a non-validated questionnaire was used in order to detect individuals with personal or family history of HHC or those with HHC secondary to drugs or morbid states; however, it must be stated that such an instrument has never been validated and published in the literature.

Conclusion

This research was designed to evaluate, in individuals with or without fasting or POML HHC, the allelic frequencies of the polymorphisms C669T, 844ins68, and C1080T of the CBS and the C667T mutation of the MTHFR in a sample of Mexican healthy individuals. As compared with individuals with normal fasting or POML Hcy plasma levels, only the polymorphism C1080T was significantly associated with the presence of POML HHC. Although the impact of moderate HHC on the occurrence of occlusive vascular disease is rapidly growing, the role of the genetic factors influencing the plasma levels of Hcy is not completely understood. Because POML HHC in the Mexican population is alarmingly frequent and no evidence about the genetic causes of this abnormality was previously described, we believe that our data have may have a significant impact in highlighting the causes of HHC in Mexico. Of course, further research about the problem of HHC and its impact in the general population is warranted.

Authors' note

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Ethical approval

Ethical approval for this study was obtained from the Ethics Committees of the Hospital General Regional Carlos MacGrégor Sanchez Navarro of the Instituto Mexicano del Seguro Social (REC No. 3605; Approval No. R-2017-3609-32).

Informed consent

Written informed consent was obtained from all subjects before the study.

Trial registration

This study was registered under the Trial Registry with the approval number R-2017-3609-32.

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Supplemental material

Supplemental material for this article is available online.

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