STUDY OF THE RELATIONSHIP BETWEEN VARIANTS OF CYP2C9 ENZYME AND ACUTE INTERMITTENT PORPHYRIN MANIFESTATION

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Abstract: Objective: The manifestation of Acute Intermittent Porphyria (AIP) requires many factors. In situations of high demand of heme, initial enzyme of the pathway in parallel to P450 cytochrome genes (CYPs) are induced in response to drugs. It was suggested that variants in CYP2C9, would play a role in AIP manifestation, according to the theory of Thunell LS (2006). Method: It was studied genotype and allele frequency of some SNPs in a control and AIP patients groups, then was analyzed the relation of those found variants with heme precursors. We amplified by PCR a fragment covering from intron 1 to intron 3. All obtained data were analyzed by Statistical programs VcStat® and InfoStat®. Results: We detected 4 different polymorphisms: two missense variants in exon 3 g.8633C>A and g.8652G>T and two SNPs no translated g.8187G>C and g.8436T>C in introns 1 and 2, respectively. And there were not significant frequencies in all studied groups. Conclusions: The presence of genotypes G/C-C/T was in major frequency in AIP men, which also have high levels of ALA and PBG comparing with women. Worldwide, this study clearly demonstrated for the first time that AIP men with G/C-C/T variants could have a high possibility to trigger the disease due to porphyrinogenic drug consumption, as a mainly reason to development the disease in male patients.

Keywords – AIP, CYP2C9, manifestation, HMBS, SNPs

INTRODUCTION

The porphyrias are a heterogeneous group of metabolic disorders that result from the decreased activity of a specific enzyme of the heme biosynthetic pathway and are characterized by the overproduction and excretion of heme precursors in urine and/or stool and their accumulation in certain tissues. They can be classified in hepatic, erythropoietic or hepatoporphyrroepoietic depending on the main site of expression of the specific enzymatic defect. Alternatively, they can be classified in cutaneous or acute depending on their clinical manifestations. Specific patterns of accumulation of the heme precursors δ-aminolaevulinic acid (ALA), porphobilinogen (PBG) and porphyrins are associated with characteristic clinical features such as acute neurovisceral attacks, skin lesions or both [1-3].

A parcial deficiency of the activity of the third enzyme, porphobilinogen deaminase (PBGD EC 4.3.1.8) in hemo biosynthesis pathway, cause a disease Acute Intermittent Porphyria (AIP, OMIM 176000) is the most common of the acute hepatic porphyrias in Argentina. It is an autosomal dominant disorder with incomplete penetrance.

The clinical symptoms are: acute neurovisceral signs which include various peripheral and central nervous system manifestations, intermittent attacks of abdominal pain, constipation, vomiting, hypertension, tachycardia, fever and Acute attacks develop exposing to diverse porphyrinogenic agents [4-6].

In cases of high demand of heme, leads to an overstimulation of its hepatic synthesis and accumulation of porphyrinic precursors ALA and PBG in AIP carriers and result into the clinical onset of the disease. There are many factors which require a whole use of heme like: lipophilic drugs, organic solvents, components in red wine and coloured spirits including alcohols and congeners, cannabis, terpenes, biocides, steroid hormones, various kinds of stress possibly including oxidative stress, fluctuations in female sex hormone spectrum, major surgery, fasting, hard exercise, or infection or other intercurrent illness [7-14]. ALAS1 gene (ubiquitous isoform of the aminolevulinate synthase) encodes the limiting enzyme of heme synthesis and is regulated by heme negative feedback [15]. ALAS1 and P450 cytochrome genes (CYPs), in parallel, are induced in response to drugs.
which have an effect on some Nuclear Receptors (NRs) [16]. CYPs enzymes are the main liver heme proteins and are highly polymorphic. CYPs are engaged in phase 1 and phase 2 metabolisms of xenobiotics, hormones, and are also involved in the synthesis of endogenous steroid compounds, specially CYPs 2 and 3 species, which by far dominate the human hepatic hemoprotein pool [17-19].

CYP2C9 is one of these hepatic enzyme which play an important role in the oxidation of both xenobiotic and endogenous compounds. Warfarin and phenytoin and other routinely prescribed drugs such as acenocoumarol, tolbutamide, losartan, glipizide, and some nonsteroidal anti-inflammatory drugs [20]. CYP2C9 makes up about 18% of the CYPs protein in liver microsomes. CYP2C9’s gene is located on chromosome 10q24.2 (GenBanK id1559). The activity suffers a decrease of 88% and 95% with the two variants CYP2C9*2 and CYP2C9*3 respectively, compared with the wild-type (wt) enzyme CYP2C9*1. CYP2C9*2 (rs1799853) is a polymorphism that replaces to cysteine by arginine at position 144 in exon 3. In contrast, CYP2C9*3 (rs1057910) is another polymorphism at position 359 in exon 7, that replaces leucine by isoleucine[21-23]. There are other SNPs that have been described in other zones in the gene and have effect on its activity and/or expression.

It was suggested that SNPs in CYP2C9, would play an important role in AIP manifestation [24, 25]. In this work, we study the genotype and allele frequencies of CYP2C9*2 (C/T variant) and others in our population in general and in the AIP population specially, to investigate if these variants play some role in the AIP crisis, according to the theory of Thunell.S [24, 25]. To our knowledge, this is the first study done in Argentinean AIP’s patients.

**MATERIAL AND METHODS**

**Study population**

All samples were obtained from the blood DNA Bank of CIPYP - Hospital de Clínicas de Buenos Aires. Forty one healthy random controls and fifty nine AIP patients (15 men and 44 women) were selected from thirty two unrelated AIP families carrying different HMB5 gene mutations. Thirty Latent AIP (L-AIP): patients carrying the mutation without clinical and biochemical manifestations, twenty-nine Manifested AIP (M-AIP): patients carrying the mutation with clinical and biochemical manifestations. Considering that we are working with a finite population, 331 AIP total patients in our Center, our sample size was statistically appropriate. The major part of females presented as mainly triggering factors: fasting, stress, etc. Instead, in males, AIP is manifested by drugs consuming.

Informed consent was obtained from all patients following the standards of UNESCO Declarations-DD.HH Genome and Genetic Data (https://www.unesco.org/shs/ethics), Declaration of Helsinki was followed and the study was approved by the Institutional Research Ethics Committee of the Research Center on Porphyrins and Porphyrias (CIPYP) - National Scientific and Technical Research Council (CONICET), University of Buenos Aires (UBA).

**Biochemical studies**

ALA and PBG were measured in urine (collected during 24h) by ionic chromatography exchange and quantified by spectrophotometry. Reference range: (ALA ≤ 4 mg/24h – PBG ≤ 2 mg/24h), the details of methodology were as described by Batlle [26].

**DNA extraction, PCR reactions, purification and sequencing**

We amplified by PCR a fragment from +8007 bp to +8843 bp a span that covers part of intron 1 to part of intron 3. The reaction mixture contained primers forward: 5’-TGGCTTGAAACATCAGGGCCATC-3’ and primer reverse: 5’-TGCTCTCAGGCTAAACCCC-3’. The reaction mixture (50 µl) contained 0.2 mM dNTPs, 1.5mM MgSO4, 1 U of goTaq DNA polymerase (Promega®) and each oligonucleotide primer at 0.5 mM. Thermal cycling conditions were as follows: 95°C for 2 min; followed by 35 cycles of denaturation at 95°C for 30 s; annealing at 60°C for 1 min; and extension at 72°C for 1 min. A final extension step was performed at 72°C for 5 min. PCR products were purified with commercial kit Accu Prep PCR Purification Kit (BIONEER) and automatically sequenced in both forward and reverse directions by Macrogen Company (Korea).

**Statistical analysis**

Allele and genotype frequencies were calculated in the studied population. The results obtained were further evaluated for Hardy-Weinberg equilibrium. Then, vccSTAT® and InfoStatDB® programs were applied to analyze the frequencies of found SNPs and their effect on the trigger of AIP.

**RESULTS**

**Frequencies of SNPs in studied population:**

In our work, we detected four different polymorphisms in the studied zone shown in Table 1: two missense variants in exon 3 (g.8633C>T and g.8652G>A) and two SNPs no translated (g.8187G>C and g.8436T>C) in both introns 1 and 2, respectively. However, they have been already reported in the NCBI database or in the Human CYPAllele Nomenclature Committee.

The Fisher exact test (by VccSTAT®) was used to calculate differences in allele and genotype frequencies of CYP2C9*2, odds ratio and 95% confidence interval, considering the total population (100). The frequencies found for C/C (homozygous) and C/T (heteryozygous) genotypes were 0.7 and 0.2, respectively, while genotype T/T was not present in our population. On the other hand, the allele C was the most frequent (0.8). And there were no differences in all studied groups, as shown in Table 1 and Figure 1.

In the studied fragment, there were two SNPs that appeared in introns 1 and 2, present in 21% and 19%, respectively. Neither of them have an effect on the activity of the enzyme. Polymorphisms rs9332119 (intron 1) and rs1799853 (exon 3) notably appeared together in all cases.

CYP2C9*8, rs7900194 (exon 3), was only found in one control sample as shown in Table 1. This variant is present with major frequency in African–American population [27, 28].

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As we mentioned above, the patients were selected from 32 unrelated families and with varied HMBS mutations. The variant p:G111R was more common, due to its major frequency in AIP patients in Argentina [3]. ALA and PBG were measured in urine of all studied patients, when biochemical diagnostic was done, Table 2 showed the ranges of AIP precursors in different groups. We decided to analyze statistically the relation between these parameters and CYP2C9 SNPs.

InfoStat/P© (version 2018) was used. When we compared the values of ALA and PBG versus the presence of the corresponding genotype T/T and T/C (variants found in intron 2), no significant value was found, Figure 2.

**Table (1):** Frequency distribution of CYP2C9 polymorphisms in 100 Argentinean samples.

<table>
<thead>
<tr>
<th>rs</th>
<th>Region</th>
<th>Position</th>
<th>Change</th>
<th>Amino-acid effect</th>
<th>Frequency (n)</th>
<th>Name</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9332119</td>
<td>Intron 1</td>
<td>+8187</td>
<td>G&gt;C</td>
<td>No translated**</td>
<td>21/100</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>rs9332120</td>
<td>Intron 2</td>
<td>+8436</td>
<td>T&gt;C</td>
<td>No translated**</td>
<td>19/100</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>rs1799853</td>
<td>Exon 3</td>
<td>+8633</td>
<td>C&gt;T</td>
<td>R144C missense</td>
<td>21/100</td>
<td>CYP2C9*2</td>
<td>↓88%</td>
</tr>
<tr>
<td>rs7900194</td>
<td>Exon 3</td>
<td>+8652</td>
<td>G&gt;A</td>
<td>R150H missense</td>
<td>1/100</td>
<td>CYP2C9*8</td>
<td>↑Activity</td>
</tr>
</tbody>
</table>

**Figure (1):** genotype and allele frequencies of CYP2C9*2 in Argentinean population.

(a)- Genotype frequency and (b)- Allele frequency in all studied groups.

**Relationship between SNPs and AIP manifestation:**

As we mentioned above, the patients were selected from 32 unrelated families and with varied HMBS mutations. The variant p:G111R was more common, due to its major frequency in AIP patients in Argentina [3]. ALA and PBG were measured in urine of all studied patients, when biochemical diagnostic was done, Table 2 showed the ranges of AIP precursors in different groups. We decided to analyze statistically the relation between these parameters and CYP2C9 SNPs.

InfoStat/P© (version 2018) was used. When we compared the values of ALA and PBG versus the presence of the corresponding genotype T/T and T/C (variants found in intron 2), no significant value was found, Figure 2.

**Figure (2):** Analyze the role of variants of intron 2 in AIP manifestation by InfoStat/P©, it was not found difference in both groups.
We considered both variants of intron 1 and exon 3 of CYP2C9 (genotypes G/C-C/T): who have both variants G/C in intron 1 and C/T in exon 3, and patients with wt variants: who have G/G-C/C variants in both regions. When we compared the levels of ALA and PBG in urine of studied patients, it was found a tendency of elevated levels of ALA and PBG in the urine of the total patients with variants G/C-C/T comparing with others with wt variants as demonstrated in Figure 3.

<table>
<thead>
<tr>
<th>AIP markers</th>
<th>Ranges in M-AIP</th>
<th>Ranges in L-AIP</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>2.4-22.4 mg/24h</td>
<td>0.5-2 mg/24h</td>
<td>ALA &lt; 4 mg/24h</td>
</tr>
<tr>
<td>PBG</td>
<td>6.7-73.9 mg/24h</td>
<td>0.7-2 mg/24h</td>
<td>PBG &lt; 2 mg/24h</td>
</tr>
</tbody>
</table>

Table (2): ALA and PBG values obtained from biochemical analysis measured in latent and manifested patients

Figure (3): InfoStat/P© was used to analyze the role of variants of intron 1 and exon 3 in AIP manifestation. Elevated values of ALA and PBG were observed in patients who had G/C-C/T.

The previous results leaded us to divide the patients by gender, taking into account the presence of G/C-C/T variants were in major frequency in men than in women 0.63, 0.37, respectively, (Fisher test: p-value was 0.03). We observed high levels of ALA and PBG in the urine of men with G/C-C/T variants comparing with men with wt variants. On the other side, women group’s wt and G/C-C/T showed the same level of ALA and PBG, Figure 4.

Figure (4): Comparison of ALA and PBG in male and female groups. High levels of ALA and PBG were noticed in males groups with G/C-C/T (* p < 0.05).

InfoStat/P© (Two independent groups analysis) was applied to the groups of men with wt variants and with G/C-C/T variants, significant p values were found, 0.01, 0.02 for ALA and PBG, respectively. However, there were no significant differences in comparison with female group’s wt and G/C-C/T genotypes, Figure 5.

Figure (5): Comparison of ALA and PBG values in male and female groups by InfoStat/P©. (* P < 0.05).
DISCUSSION
As it is well known, porphyria manifestation depends on various factors such as fasting, stress, drugs, and others. AIP is manifested principally in women rather than men, about 80% of our patients are women. Many groups in different countries are researching about potential precipitating drugs with the aim of helping patients to have a more secure and personalized diagnosis to avoid acute attacks caused by special porphyrinogenic drugs which are metabolized by CYP2C9.

In our Center, many projects are carried out about this subject. In this work, we have focused our attention on CYP2C9 enzyme, taking into account that this protein is very polymorphic, and that it was suggested that it would play an important role in the manifestation of AIP [24]. So we analyzed (for the first time in Argentinian people and specially in AIP patients), the frequency of the allele and genotype variants of the +8007 bp to +8843 bp region of CYP2C9’s gene by sequencing, including variant CYP2C9*2 which has been reported in another work in our country [29].

In our results, we found that the genotype frequency for general population and also for AIP samples of CYP2C9*2 was similar to those described for other populations of Caucasian origin [30, 31]. Moreover there was no difference comparing L-AIP and M-AIP. We also observed that the presence of CYP2C9*2 was always accompanied with the variant of intron 1. As indicated in this site [https://www.ncbi.nlm.nih.gov/snp/rs9332119#frequency_tab which showed similar frequency results in an European population , close to our population. We detected another SNP in intron 2 that had no clinical effect, and with major frequency comparing with an unique work published [32].

In another part of this study, we analyzed the effect of these variants on AIP manifestation, measured as the values of ALA and PBG in urine. We did not find any relationship between the variants of intron 2 (T/T and T/C) and the levels of ALA and PBG. Instead, there was a tendency of elevated levels of ALA and PBG in the urine of the total AIP patients with G/C-C/T comparing with wt variants. When we divided patients by gender, we observed higher levels of ALA and PBG in the urine of male patients who have G/C-C/T compared with males with wt variants. But in the female’s group, it was found the same level of ALA and PBG in both wt variants and G/C-C/T patients. InfoStat/P confirmed a significant p-value in the comparison between male’s groups.

CONCLUSION
As it was considered before, AIP is principally manifested in women, because they are more exposed to porphyrinogenic factors such as dieting, stress, and contraceptives. For this reason, the number of males in our AIP population was very low, about 20%. In spite of this, our results clearly demonstrated that the men with G/C-C/T variants would have a high possibility to trigger the disease, which would be justified due to drug consumption in all of them, according to their clinical histories.

LIST OF ABBREVIATIONS
A  Adenine
C  Cytosine
bp  Base pairs
CYP2C9  Cytochrome P450 family 2 subfamily C member 9
CYP2C9*2  Cytochrome P450 family 2 subfamily C member 9, variante 2
CYP2C9*3  Cytochrome P450 family 2 subfamily C member 9, variante 3
DNA  Desoxirribonucleic acid
G  Guanine
HMBS  Hydroxymethylbilane synthase
kb  Kilobases
wt  Wild-tipe

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest concerning this article. No non-financial conflicts of interest exist for any of the authors.

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