Functional polymorphisms in the *CYP3A4, CYP3A5,* and *CYP21A2* genes in the risk for hypertension in pregnancy.

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Abstract.

An intronic single nucleotide polymorphism (SNP) in the *CYP3A5* gene (*CYP3A5*3;* SNP rs776746) affects RNA splicing and enzymatic activity. The *CYP3A5*3* frequency increased with distance from the equator and natural selection has been proposed to explain the worldwide distribution of this allele. CYP3A activity has been related with the risk for hypertension in pregnancy, a major cause of morbidity and mortality among women, and *CYP3A5*3* could reduce the risk for this disease in populations from regions with high sodium and water availability. The *CYP3A*5 genotype was related with blood pressure in the general population, but the effect on the risk for hypertension in pregnancy has not been evaluated.

We compared the allele and genotype frequencies of three functional SNPs in the *CYP3A5* (rs776746), *CYP3A4* (rs2740574), and *CYP21A2* (rs6471) genes between pregnant women who developed hypertension (n=250) or who remained normotensive (control group, n=250). In addition, we sequenced the full *CYP3A5* coding sequence in 40 women from the two groups to determine whether some gene variants could explain the risk for hypertensive pregnancies in our population.

Allele and genotype frequencies did not differ between hypertensive and normotensive women for the three *CYP* variants. We did not find *CYP3A5* nucleotide changes that could explain a higher risk for hypertension in pregnancy. Our data suggests that the variation in *CYP3A5*, *CYP3A4*, and *CYP21A2* did not contribute to the risk for hypertension in pregnancy in our population.

Key words: P450 enzymes; CYP3A5; hypertension in pregnancy; natural selection.

Introduction.

Cytochrome P450 (CYPs) enzymes metabolize a variety of diverse compounds, either endogenous (e.g. hormones) and exogenous (e.g. drugs). Members of the CYP3A subfamily participate in the metabolism of bile acids and steroids (such as aldosterone, testosterone, and estrogens), but also in the biotransformation of *xenobiotics* such as immunosuppressive drugs [1-3]. There is a wide variability in the clearance of CYP3A substrates, and DNA polymorphisms in the genes encoding these CYPs could explain the difference in dose requirements and secondary effects of many drugs [4-7].

In humans, there are four *CYP3A* genes (*CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*) clustered in a region of about 220 kb in chromosome 7q21 [8]. CYP3A4 is expressed at high levels in adult liver and small intestine, and CYP3A5 is also expressed in kidney [4]. While CYP3A4 represents most of the CYP3A protein in humans, the CYP3A5 content shows an extreme interindividual variability [9,10]. This heterogeneous expression is mainly due to a single nucleotide polymorphism (SNP) in intron 3 of *CYP3A5* (6986 G/A; SNP rs776746) which results in the incorrect splicing of the pre-mRNA to give an aberrant mRNA that is degraded [10-12]. The 6986 A is also known as *CYP3A5*3* (being *CYP3A*1* the 6986 G allele; see the *Cytochrome P450 Homepage* for a description of the variation at the P450 genes, http://drnelson.utmem.edu/CytochromeP450.html), and individuals who are *CYP3A5*3* homozygotes have a reduced CYP3A5 activity (non expressers) compared to carriers of at least one *CYP3A5*1* allele [13]. This expression variability explained the differences in dose requirements in patients who are treated with drugs that are cleared by CYP3A5. For instance, *CYP3A5*3* homozygotes would require a lower dose of the immunosuppressive drug tacrolimus to reach the blood concentration target, compared to *CYP3A5*1* homozygotes [7,14,15].

The frequency of *CYP3A5*3* varies between populations, with the lowest (<25%) in African Sub-Sahara populations and the highest (>95%) in some European regions [10, 12]. In the United States the *CYP3A5*3* frequency is significantly lower among blacks, and this could partly explain the higher mean blood pressure in individuals of African ascent through a mechanism that involves the conversion of cortisol to 6b-hydroxycortisol in the kidney followed by increased sodium and water retention [16-19]. Some authors have proposed a “sodium retention hypothesis” to explain the correlation between *CYP3A5* allele frequencies and distance from equator [20, 21]. According to this hypothesis, the first human populations would have adapted to the hot African climate through

the selection of gene variants that increased the retention of sodium and water [22]. Natural selection could have favored the *CYP3A5*3* allele by conferring an advantage in populations living in warmer regions and under high sodium availability [21, 23].

In addition to the diverse *CYP3A5*3* frequency across human populations, there is a remarkable interpopulation difference with regard to the frequency of other *CYP3A5* variants and haplotype structure, with an excess of rare variants [21]. Overall, these findings strongly argued in favor of a selective pressure on this gene, with the intensity of selection varying with latitude. Hypertension in pregnancy has been proposed as a putative disadvantage associated with *CYP3A5* variants. Approximately 5% of pregnant women develop hypertension, and this represents one of the main causes of morbidity and mortality associated with pregnancy [24-27]. In favor of the hypothesis that pregnancy hypertension could be associated with CYP3A activity was the increased urinary levels of 6b-hydroxycortisol found in women with preeclampsia, a condition characterized by hypertension and proteinuria in pregnancy [28, 29]. The association between *CYP3A5*1* and hypertension also supported a role for this variant in hypertension in pregnancy [18, 30, 31].

To our knowledge no study about the association between *CYP3A5* variants and hypertension in pregnancy has been reported. To address this issue, we genotyped the common *CYP3A5*3* allele and searched for new *CYP3A5* variants in a group of Spanish women who developed hypertension during pregnancy or who remained normotensives. In addition, we also genotyped these women for two variants in the *CYP3A4* and *CYP21A2* genes.

Methods.

Patients and controls.

Our study included a total of 250 women who developed hypertension after the 20th week of pregnancy. Of these, 125 (50%) had proteinuria and were diagnosed with pre-eclampsia. The control group was composed by a total of 250 women who did not have hypertensive pregnancies. The main characteristics of these patients and controls have been reported and are summarized in **table 1** [25, 32]. All the patients and controls were Caucasians from the region of Asturias (Northern Spain, total population one million) and were recruited by Nephrologists of Hospital Universitario Central de Asturias (HUCA). All the patients and controls gave their informed consent to participate in the study, and this was approved by the Ethical Committee of HUCA.

***CYP3A4/CYP3A5* genotyping.**

Genomic DNA was obtained from blood leukocytes and patients and controls were genotyped for the *CYP3A4* -392 A/G (SNP id rs2740574; www.ensembl.org) and *CYP3A5* 6986 A/G (SNP id rs776746) polymorphisms. The *CYP3A5* was genotyped through real time PCR with a Taqman assay, following manufacturer´s instructions (Applied Biosystems; assay id. C_25201809_30; www.appliedbiosystems.com). To genotype the *CYP3A4* rs2740574 the DNA was polymerase chain reaction (PCR) amplified with specific primers: GGACAGCCAT AGA(G/A)ACAAGGGCT (forward) and AGGTTTCCATGGCCAAGTCT (reverse). This PCR renders a fragment of 448 bp, that after digestion with the restriction enzyme *BfaI* gave fragments of 278 + 170 bp (-392 G) or 257 + 170 + 21 bp (-392 A). To determine each patient/control genotype, these restriction enzyme digestions were electrophoresed on 3% agarose gels followed by ethidium bromide staining and visualization in an ultraviolet transiluminator. To confirm the accuracy of these genotyping methods, we sequenced several individuals representative of each genotype.

***CYP21A2* genotyping.**

Patients and controls were genotyped for the *CYP21A2* 1683G>T variant (SNP id rs6471, also known as *CYP21A2*15*). This was a missense change at codon 281 of CYP21A2 (V281L), and the protein containing 281L has a reduced enzymatic activity compared to the wild type (281 V) variant [33, 34]. Briefly, a fragment of 438 bp was amplified with primers that matched exons 6 (forward: GAAGAGGGATCACATCGTGGAGAT) and 8 (reverse: CCTTTTGCTTGTCCGCAG)

of *CYP21A2*. PCR fragments were digested with the restriction enzyme *Eco72I*, and after electrophoresis on 3% agarose gels the two alleles were visualized as bands of 438 bp (1683 T; 281 L) or 317 + 121 bp (1683 G; 281 V) (**supplementary figure 1**). To confirm the accuracy of this genotyping method, we sequenced individuals representative of each genotype.

***CYP3A5* sequencing.**

The full *CYP3A5* coding region (13 exons, including the 5´ and 3´ untranslated sequences) was sequenced in 20 hypertensive patients (10 with a *CYP3A5*1/*3* and 10 *CYP3A5*3/*3*) and 20 normotensive controls (10 with a *CYP3A5*1/**3 and 10 *CYP3A5*3/*3*). Fragments were PCR-amplified with primers that matched the intronic flanking regions (**supplementary table 2)**. These fragments were purified and both strands sequenced with BigDye chemistry in an ABI3130 automated system (Applied Biosystems, Foster City, CA, USA). Sequences were compared with the *CYP3A5* reference sequence (ENSG00000106258 for the genomic; ENST00000222982 for the transcript; www.ensembl.org).

Statistical analysis.

Allele and genotype frequencies were compared between patients and controls through a χ^2 test. The χ^2 was also used to analyse the deviation from the Hardy–Weinberg equilibrium of the genotype frequencies. The SPSS package (v. 11.0) was used for all the statistical analysis. The frequencies for all the possible haplotypes defined by the *CYP3A4* and *CYP3A5* loci were calculated with the Cubic exact solutions for the estimation of pairwise haplotype frequencies (www.oege.org/software/cubex). This programm estimates the haplotype frequencies, the normalised linkage disequilibrium (LD) parameter (D´), and the LD correlation coefficient between two loci [35].

Results.

Allele and genotype frequencies in patients and controls.

Allele and genotype frequencies did not differ between hypertensive and normotensive pregnant women for the *CYP3A4*, *CYP3A5*, and *CYP21A2* variants (**Table 2**). This suggested that they did not contribute to the risk of developing hypertension during pregnancy in our population. The observed genotype frequencies in the two groups did not differ from those expected under the Hardy-Weinberg equilibrium. All the 15 patients with fetal death were homozygous for the common variants at the three loci, and this suggested that the rare alleles were not associated with an increased risk for fetal death. We found a significant linkage disequilibrium between the two *CYP3A4* and *CYP3A5* SNPs in patients (D´=0.54, p<0.001) and controls (D´=0.53, p<0.001). We did not find significant differences for the estimated frequencies of the four haplotypes between patients and controls (**supplementary table 2**).

***CYP3A5* sequencing.**

The main hypothesis of our study was that functional variants in the *CYP3A5* gene could be associated with a reduced risk for hypertension in pregnancy. In addition to genotype and compare the *CYP3A5*3* frequencies between patients and controls, we also sequenced the *CYP3A5* coding sequence in 40 patients and 40 controls. All the **1/*3* patients and controls were also heterozygotes for a previously reported polymorphism in the 3´ untranslated region (T/C SNP; rs15524). All the *CYP3A5*3* homozygotes were also TT, and the only control woman **1/*1* was C/C. In addition to the common rs15524 SNP, we also found a rare nucleotide change (intron 4, SNP rs28365094) in two patients and one control.

Duscusion.

Hypertension is one of the main complications of pregnancy, and represents a major cause of morbidity and mortality among pregnant women [24]. Genes associated with hypertension in pregnancy might be under natural selection, and those variants that reduced the risk could be favored. This hypothesis has been proposed to explain the gradient of *CYP3A5*1/*3* frequencies [21]. Other genes linked to hypertension have functional alleles that showed a worldwide variation consistent with different selection pressures during the out-of-Africa expansion [36, 37]. The expression of CYP3A5 in human kidney is largely determined by *3A5*3* and this could affect mineralocorticoid synthesis and sodium and water retention [5]. The *CYP3A5* genotype predicted renal CYP3A activity and blood pressure in healthy African-Americans [18]. CYP3A5 activity could affect the risk of developing hypertension in pregnancy, a major cause of mortality in pregnant women and fetal death. This could have been a natural selection mechanism for *CYP3A5* variation [21]. To determine whether *CYP3A5* variants were linked to the risk of developing hypertension during pregnancy, we compared the allele and genotype frequencies between hypertensive and normotensive (the control group) pregnancies.

The *CYP3A5*3* was almost fixed in our population, as reported for other European populations [12]. In our population, *3A5*1* (the "expresser" allele) was not a risk factor for hypertension in pregnancy. Patients and controls were also genotyped for two variants in the *CYP3A4* and *CP21A2* genes. The *CYP3A4 -92 G* (*CYP3A4*1B*) allele has been linked to higher gene expression, and could thus be associated to traits related to higher CYP3A4 enzymatic activities [5, 38]. The *CYP21A2 281L* variant was linked to a reduced enzymatic activity and associated with "non-severe" congenital adrenal hyperplasia (CAH) [33]. In our population, homozygosity for this variant is the most common genotype among patients with CAH (Coto et al., unpublished work). We did not find significant differences for the two *CYP3A4* and *CYP21A2* variants in our patients/controls, indicating that they did not contribute to the risk for hypertension in pregnancy in our population. A total of 15 patients (6%) lost their fetuses, and all these women were homozygous for the most frequent allele at the three genes. Hypertension in pregnancy was associated with a low rate of maternal death in our population (<0,5%) [25, 32]. None of the women in our study died as a consequence of the disease, and thus we cannot exclude that these genes variants were associated with an increased risk for mortality among pregnant women.

The high *CYP3A5*3* frequency in populations distant from the equator, together with the very low rate of nucleotide variation and the skew toward rare variants, prompted some authors to propose a natural selection mechanism that shaped the current distribution of allele frequencies. A genetic signature consistent with selection against the *CYP3A4*1B* allele in non-African populations has also been reported [21]. Our ancestral African environment was characterized by a hot climate that favored body heat dissipation through sweating and the subsequent loss of salt and water [20, 22, 39, 40]. In cooler Northern regions the thermodynamic requirement shifted to heat conservation, favoring those mechanisms that reduced water and sodium conservation. In this context, a reduction in the activity of enzymes that participate in the synthesis of aldosterone could be beneficial. Aldosterone increased the reabsorption of sodium and water, increasing blood volumen and, therefore, blood pressure [41]. Aldosterone is a mineralocorticoid synthesised from cholesterol in the adrenal cortex and P450 enzymes play a major role in this process. For instance, CYP21 (21-hydroxilase) catalyses the synthesis of deoxycorticosterone, a precursor of aldosterone and cortisol, and CYP3A enzymes are involved in the hydroxilation of glucocorticoids and mineralocorticoids. To determine whether some *CYP3A5* variants (other that *CYP3A5*1/*3*) were associated with hypertension in pregnancy, we sequenced the full coding and the intronic flanking regions in a group of patients and controls.

We found a common variant in the 3´ UTR region (SNP rs15524) in complete linkage disequilibrium with the *CYP3A5*1/*3*, but no *CYP3A5* variants were associated with hypertensive pregnancy in our population. Although we did not see an effect of the *CYP3A5* variants on the risk for hypertension in pregnancy, we cannot exclude an association in earlier times in response to some environmental or dietetic factor. A natural selection force in the ancient environment different to hypertension in pregnancy and increased fetal and/or maternal death could have modeled the allele distribution nowadays. For instance, many P450 enzymes evolved to detoxify natural foreign chemicals and the exposure to *xenobiotics* in some populations could have had an effect on the selection of CYP variants, shaping the allele distribution found in modern populations [1, 42].

In conclusion, we did not find significant associations between hypertension in pregnancy and three DNA polymorphisms at the *CYP3A4*, *CYP3A5* and *CYP21A2*. The sequencing of *CYP3A5* in patients and controls did not support a role for DNA variants at this gene in hypertension in pregnancy. We did not find signals of natural selection on these genes variants through a higher risk for hypertension in pregnancy.

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Table 1. Main characteristics of the hypertensive and normotensive pregnancies.

	Hypertensives (n=250)	Normotensives (n=250)
Mean age (years)	30.4 ± 6.5	29.2 ± 5.5
Systolic BP (mmHg)	157 ± 18	117 ± 11
Dyastolic BP (mmHg)	99 ± 11	69 ± 9
Duration of pregnancy (mean weeks at birth)	37.5 ± 3.5	39 ± 1.5
Newborn weigth (mean g)	2,890 ± 790	3,260 ± 435
Fetal death	15 (6%)	1 (1%)
Maternal death	0	0

Table 2. Allele and genotype frequencies for the *CYP3A4*, *CYP3A*5, and *CYP21A2* polymorphisms in hypertensive and normotensive pregnancies.

	Hypertensives N=250	Normotensives N=250
CYP3A5*3/*3	218 (87%)	220 (88%)
*1/*3	32 (13%)	29 (12%)
*1/*1	0	1 (<1%)
*CYP3A5*3*	0.94	0.94
*CYP3A5*1*	0.06	0.06
CYP3A4*1/*1	240 (96%)	238 (95%)
*1/*1B	10 (4%)	12 (5%)
*1B*1B	0	0
*CYP3A4*1*	0.98	0.97
*CYP3A4*1B*	0.02	0.03
CYP21 V/V	230 (92%)	232 (93%)
V/L	20 (8%)	18 (7%)
L/L	0	0
CYP21 V	0.96	0.96
CYP21 L	0.04	0.04

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