ELSEVIER

Journal of Hepatology 48 (2008) 125–132

Journal of Hepatology

www.elsevier.com/locate/jhep

Association of the multidrug-resistance-associated protein gene (*ABCC2*) variants with intrahepatic cholestasis of pregnancy☆

Silvia Sookoian[1,3,†], Gustavo Castaño[2,3,†], Adriana Burgueño[1], Tomas Fernández Gianotti[1], Carlos J. Pirola[1,*]

[1]*Instituto de Investigaciones Medicas A. Lanari, Departamento de Genetica y Biología Molecular de Enfermedades Complejas, Universidad de Buenos Aires, CONICET, Combatientes de Malvinas 3150, Buenos Aires 1427, Argentina*
[2]*Hepatología, Departamento de Medicina Interna, Hospital J.M.Penna, Almafuerte 406, Buenos Aires, Argentina*
[3]*Consejo de Investigación GCBA, Medrano 350, Buenos Aires, Argentina*

***Background/Aims*: We hypothesized that common genetic variation at *ABCC2* influences ICP susceptibility. Hence we studied the association of single nucleotide polymorphisms (SNPs) of promoter, coding and non-coding regions of *ABCC2* and intrahepatic cholestasis of pregnancy (ICP).**

***Methods*: 70 ICP patients and 112 healthy pregnant women in the third trimester of their pregnancies were included in a cross sectional study. Four tag SNPs (rs717620 A/G; rs2756105 C/T; rs2002042 C/T; rs3740066 A/G) encompassing 70 kb in chr.10 and representing 46 polymorphic sites ($r^2 > 0.8$) were genotyped. Besides, 2 additional SNPs (rs17222723 A/T and rs8187710 G/A) were included.**

***Results*: In univariate analysis, rs2002042 and rs3740066 were significantly associated with ICP ($p < 0.04$ and 0.01, respectively) but after multiple testing correction, only rs3740066 remained significantly associated with disease status ($p < 0.03$). We also observed a positive association between the rs3740066 and ALT, AST, alkaline phosphatase and total and conjugated bilirubin concentrations. Consistent with the analysis of individual markers, we observed that haplotype frequency of the *ABCC2* gene in ICP patients significantly differed from controls ($p < 0.03$).**

***Conclusions*: We found an association between the rs3740066 in exon 28 of *ABCC2* gene and ICP. The risk of disease for homozygous AA carriers is 4-fold higher (OR 4.44 CI 95% 1.83–10.78, $p < 0.001$) in comparison with GG carriers.**

***Keywords*: Bile; Liver; MRP2; Multidrug-resistance-associated protein; Gene variant; *ABCC2*; Intrahepatic cholestasis of pregnancy**

Received 26 April 2007; received in revised form 27 July 2007; accepted 13 August 2007; available online 23 October 2007
Associate Editor: M. Trauner

☆ The authors declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript except SS, AB and CJP who belong to Consejo Nacional de Investigaciones Científicas y Técnicas.

* Corresponding author. Tel.: +54 11 4514 8701x167; fax: +54 11 4523 8947.
E-mail addresses: carlospirola@ciudad.com.ar, pirola.carlos@lanari.fmed.uba.ar (C.J. Pirola).

† Both authors contributed equally to this work

Abbreviations: ICP, intrahepatic cholestasis of pregnancy; MRP2, multidrug-resistance-associated protein; SNPs, single nucleotide polymorphisms; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a cholestatic disorder of the second and third trimesters of pregnancy that complicates otherwise normal pregnancies, persisting until delivery and disappearing spontaneously after parturition.

While ICP poses little maternal risk, there is an increased risk to the fetus in the occurrence of fetal distress, increased risk for prematurity and intrauterine death [1,2].

ICP is a complex condition whose etiology is likely to involve several environmental, hormonal and

0168-8278/$32.00
doi:10.1016/j.jhep.2007.08.015

genetic factors [3,4]. However, although the pathogenesis of ICP remains unknown, there is evidence of a genetic predisposition for the disease. For instance, familial clustering [5] and uneven geographical distribution [4] support the genetic origin of the disease. Lastly, ICP patients usually describe itching in mother and sisters [6], and sisters of affected patients have a 12-fold increased risk of developing ICP [7].

Several studies have provided data about the association of gene variants of different hepatobiliary ATP-binding cassette (ABC) transporters and increased risk of cholestasis of pregnancy. For instance, gene variants of the genes encoding the *ABCB4* and *ABCB11* were associated with severe forms of ICP [8–11]. In addition, three novel non-synonymous mutations in exon 14 of *MDR3* were found in ICP patients [12] as well as a new splicing site mutation in this gene may be associated with stillbirths and gallstone disease in a recently published family study [13]. Sequence variation in the *ATP8B1* gene was also evaluated [10,14]. Surprisingly, there are no data about the *ABCC2* gene variants and ICP.

Nevertheless, gene mapping for complex disorders is further complicated if there are multiple variants which are presumed to work in concert to produce the altered phenotype. For that reason, we chose the candidate gene approach selecting a gene based on function.

The fact that the canalicular membrane hepatocyte transporters represent the rate-limiting step in bile formation [15] explains that the majority of the candidate genes studied for ICP comprise those codifying for the transport systems relevant for bile formation.

The active transport of solutes across the canalicular membrane of hepatocytes is forced by an array of export pumps that belong to the ABC family. As the multidrug-resistance-associated protein (MRP2) mediates the excretion of a wide range of amphipathic anionic substrates, including bilirubin diglucuronide, estradiol 17-b-glucuronide and sulfate conjugates [15], we hypothesized that common genetic variation at ABC-transporter encoding gene *MRP2* (*ABCC2*) influences ICP susceptibility. Hence we studied the association of single nucleotide polymorphisms (SNPs) of promoter, coding and non-coding regions of *ABCC2* as well as the haplotype frequencies with ICP.

2. Patients and methods

2.1. Settings and study design

Between June 1, 2003 and June 1, 2006 we performed a cross sectional study in normal pregnant women and patients with ICP in a county hospital of the city of Buenos Aires. ICP prevalence was calculated to be 1.04% among 6731 pregnancies.

2.2. Patients

During the study period, 70 ICP patients attending at the Department of Obstetrics and Gynecology and referred to the Liver Unit and 112 unrelated healthy pregnant women in their third trimester of pregnancy and coming to the hospital during the same study period were included.

The same proportion of women in both cases and controls of three neighboring countries of Argentina (Peru, Bolivia and Paraguay) were included, and to ensure homogeneity of the genetic background control population was matched on ethnicity, area of residence and time of recruitment with affected individuals.

Diagnosis of ICP was based on the following criteria: 1-presence of pruritus occurring during the second half of an otherwise uneventful pregnancy, 2-the presence of abnormalities in liver function test suggestive of ICP: serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) greater than 40 or 35 IU/L, respectively, 3-elevated levels of fasting total bile salts (SBA) $>$ 12 μmol/L SBA, 4-no skin lesions caused by systemic diseases that could cause pruritus and 5-spontaneous resolution of clinical symptoms and laboratory findings after delivery. All the patients were referred during pregnancy.

Additional inclusion criteria were the absence of infection by hepatitis viruses (HAV, HBV and HCV, AxSYM, all Abbott Park, USA) autoimmune diseases, moderate to severe alcohol intake, HIV infection (Enzyme-Linked Immunoabsorbent Assay, Organon Teknika), biliary obstruction and the use of drugs or alternative medicine therapy known to precipitate cholestasis.

Patients with a verified family history of ICP were classified as familial ICP while those without a family history (or whose family history could not be verified) were termed sporadic cases.

Complete medical, obstetrical and perinatal data were recorded. Apgar score [16] and gestational age assessed by the Capurro method were registered in all newborns [17].

In addition, all relevant clinical variables such as gestational age, birth weight, mode of delivery, and perinatal morbidity were registered for each pregnancy.

2.3. Methods

2.3.1. Liver function tests

Serum samples were obtained after a fasting period of 12-h, and aliquots were frozen at −20 °C until bile acid determinations were performed. ALT, AST, alkaline phosphatase and total and conjugated bilirubin concentrations were carried out by routine automated techniques.

Serum total bile acids were measured by capillary electrophoresis as previously reported (normal value $<$12 μmol/L) [18].

This study was performed according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board and the Bioethical Committee of our Institution. Written consent was obtained in every case.

2.3.2. Genotype and haplotype analysis

The genetic analyses were done on genomic DNA extracted from white blood cells by a standard method as previously described [19].

To assess the contribution of *ABCC2* gene variants to ICP, we selected tag SNPs (tSNPs) by using phase II genotyping data from the HapMap project for Caucasians from the CEU dataset (http://www.hapmap.org). Settings: minor allele frequency (MAF) $\geqslant$ 0.10, that predict the remaining SNPs with a minimum R^2 of 0.8 using the tagger tool (http://www.broad.mit.edu/mpg/tagger/) [20]. We identified tSNPs using an aggressive tagging approach [20].

Besides, we included 2 additional SNPs (rs17222723 A/T and rs8187710 G/A) because in a recent report studying the extent of inter-individual variability in the expression of the canalicular ABC-transporters, individual susceptibility to develop acquired forms of cholestatic liver diseases was shown (please note that rs17222723 is the actual nomenclature for the rs8187694, and then both SNPs are identical) [21].

Genotyping was performed by a high-throughput genotyping method involving PCR amplification of genomic DNA with two tailed allele-specific primers that introduce priming sites for universal energy-transfer-labeled primers as previously described [22].

Haplotype frequencies and linkage disequilibrium (LD) measures were estimated using the Haploview package.

PLINK software was used for assessing association between SNPs and affection status and quantitative traits (http://pngu.mgh.harvard.edu/purcell/plink/) and for testing Hardy–Weinberg equilibrium. Controlling for multiple testing was done by permutation test (100.000 permutations) to obtain an empirical p value. Permutation procedures provide a computationally intensive approach to generating significance levels empirically [23].

Testing SNP haplotype analysis was performed by both WHAP software (a method for testing SNP haplotype associations with qualitative and quantitative traits in samples of individuals with or without parental genotype data) [24] (http://pngu.mgh.harvard.edu/purcell//whap/) and Haploview (Haploview is a software package that provides computation of linkage disequilibrium statistics and population haplotype patterns from primary genotype data in a visually appealing and interactive interface) (http://www.broad.mit.edu/mpg/haploview/) [25].

To test for significant differences in SNPs and haplotype distributions between cases and controls, permutation tests were performed.

2.4. Statistical analysis

Quantitative data were expressed as median and range, unless otherwise indicated. We assessed differences between groups by means of the Mann-Whitney U test. Genotype frequencies were analyzed by means of the χ^2 test. We used the CSS/Statistica program package, StatSoft V 6.0 (Tulsa, USA), to perform these analyses.

3. Results

Clinical, obstetrical and perinatal characteristics of the patients with intrahepatic cholestatis of pregnancy and healthy pregnant controls are shown in Table 1. Family history of ICP was significantly different in cases and controls ($p < 0.0002$). Between ICP patients, 10 of them mentioned family history of the disease while 40 cases were sporadic. The remaining 20 patients did not know about family history of the disease. In the control group, no woman showed a family history of ICP. In both cases and controls, women had singleton deliveries.

Gestational age at delivery, neonate birth weight and Apgar score in ICP patients were significantly lower in ICP when compared with controls (Table 1). Type of delivery was significantly different when ICP patients were compared with controls: vaginal delivery was observed in 65.8% of the controls and 32.5% of ICP patients, cesarean was indicated in 28.8% of the controls and 44.22% of ICP patients and induced labor occurred in 5.4% of the controls and 23.28% of ICP patients ($p < 0.02$).

As expected, liver function tests in patients with ICP differed significantly from healthy pregnant controls (Table 1).

3.1. ABCC2 gene variants

To minimize the number of markers selected for genotyping for the candidate gene *ABCC2,* we selected four haplotype-tagging SNPs showing a minor allele frequency >10% (rs717620 A/G; rs2756105 C/T; rs2002042 C/T; rs3740066 A/G) encompassing 70 kb in chr.10 (101.532.567–101.601.283) and representing 46 polymorphic sites ($r^2 > 0.8$) considering the HapMap project data (http://www.hapmap.org). Table 2 illustrates the tSNPs characteristics.

A power estimation in the utilized sample of 70 cases and 112 controls was performed for single-point allelic effects for an odds ratio of 2.0 at a nominal significance level of 0.05 [26] for HapMap-predicted MAF of 0.23 (rs717620) to 0.36 (rs3740066) of a potential susceptibility marker and a prevalence of the disease of 1%. This analysis gave us an estimated power of 80% or greater under the additive model (additive genetic variance is the variance that results from the additive effects of alleles at each contributing locus) except for the additional rs172222723 and rs8187710 for which the statistical power is much less than 80% (27 and 34%, respectively) owing to the lower MAF of these variants (rs172222723: 3% and rs8187741: 3.8%, respectively).

Table 1
Clinical, biochemical, obstetrical and perinatal characteristics of the patients with intrahepatic cholestatis of pregnancy (ICP) and healthy pregnant controls

Characteristics	ICP patients (Median, range)	Healthy pregnant controls (Median, range)	p level
Age (years)	27, 30	26, 31	NS
Number of pregnancies	2, 7	2, 13	NS
Gestational age at delivery (weeks)	36, 22	38, 17	0.02
Neonate birth weight (g)	2950, 1990	3400, 2850	<0.001
Neonate Apgar score	9, 3	10, 4	<0.001
Total bilirubin (μmol/L)	13, 35	6, 27	<0.001
Direct bilirubin (μmol/L)	4, 23	2, 6	<0.001
Asparte aminotransferase (IU/L) AST	53, 394	17, 34	<0.001
Alanine amino transferase (IU/L) ALT	71, 546	14, 49	<0.001
Alkaline phophatase (IU/L)	655, 1377	232, 636	<0.001

Results are expressed as median, range.

Table 2
Tag single nucleotide polymorphisms of the *ABCC2* gene genotyped in the study

Location in the *ABCC2* gene	dsSNP rs # Cluster ID	Hetero-zygosity	Function	ds SNP allele	Protein residue	Aminoacid position	HapMap MAF
Exon 1	rs717620	0.260	Untranslated	A/G	–	–	0.23 (A)
Intron 2	rs2756105	0.397	Intron	C/T	–	–	0.36 (T)
Intron 19	rs2002042	0.42	Intron	C/T	–	–	0.30 (T)
Exon 25	rs17222723	0.320	Nonsynonymous	A/T	Glu [E] Val [V]	1188	0.06 (A)
Exon 28	rs3740066	0.417	Synonymous	A/G	Ile [I]	1324	0.34 (A)
Exon 32	rs8187710	0.115	Nonsynonymous	A/G	Tyr [Y] Cys [C]	1515	0.06 (A)

MAF: minor allele frequency.

No marker showed departure from Hardy–Weinberg equilibrium, indicating robust genotyping performance in this study (data not shown).

In univariate analysis, the comparison of genotype frequencies of the four tSNPs in cases and controls showed significant differences only for the rs2002042 and rs3740066 (p value <0.04 and 0.01, respectively). However, after the multiple comparison correction by 100.000 permutations, only the association between the rs3740066 and ICP remained positive ($p < 0.03$). Genotype distributions of 6 SNPs in cases and controls and cumulative OR using proportional odds model (Liu–Agresti method [27]) are shown in Table 3. In addition, the calculated risk for homozygous AA carriers of the rs3740066 is OR 4.44 (CI 95% 1.83–10.78, $p < 0.001$) and for heterozygous AG carriers is OR 1.65 (CI 95% 0.76–3.64, $p = 0.12$) in comparison with GG carriers. The Cochran–Armitage test for trend showed a p value = 0.02. Fisher's exact Test for allele frequency differences for the rs3740066 showed a significant difference between patients and controls (75 A alleles in ICP patients and 81 in controls; 65 G alleles in ICP patients and 143 controls, OR: 2.04, CI% 1.30–3.20, $p = 0.002$).

We observed that the A allele of the rs3740066 was over-represented in patients with a family history of ICP (AA 50% and AG + GG: 50%) in comparison to the others (AA group 13%, AG + GG: 87%) (Fisher exact test $p = 0.05$).

Using the WHAP software, we further evaluated the LD pattern for the mentioned SNPs. The estimation of haplotypes from genotype data showed 8 possible combinations covering >95% of the common haplotypes with a frequency higher than 0.01 (Table 4).

Table 3
Genotype distribution of 6 SNPs in cases and controls

dsSNP rs # Cluster ID	Genotype	ICP patients *n* (%)	Controls *n* (%)	Cumulative OR (95% CI)	*p* Value
rs717620	AA	6 (8.3)	4 (3.3)	0.707 (0.368–1.359	0.30
	AG	17 (25)	28 (25)		
	GG	47 (66.7)	80 (71.7)		
rs2756105	CC	17 (24.2)	41 (36.3)	1.684 (0.934–2.910)	0.09
	CT	36 (51.5)	51 (45.5)		
	TT	17 (24.2)	20 (18.2)		
rs2002042	CC	43 (61.1)	46 (41.2)	0.397 (0.218–0.723)	0.003
	CT	21 (30.6)	41 (36.7)		
	TT	6 (8.3)	25 (22.1)		
rs17222723	AA	0	0	4.08 (0.885–18.1)	0.09
	AT	2 (2.6)	12 (10.8)		
	TT	68 (97.4)	100 (89.2)		
rs8187710	AA	0	0	4.46 (0.976–20.42)	0.06
	AG	2 (2.8)	13 (11.8)		
	GG	68 (97.2)	99 (88.2)		
rs3740066	GG	16 (22.5)	44 (39.1)	2.58 (1.452–4.592)	0.001
	AG	33 (47.5)	55 (49.3)		
	AA	21 (30)	13 (11.6)		

Odds ratios (OR) and 95% confidence intervals (95%CI). Cumulative OR using proportional odds model (Liu–Agresti method [27]) is shown. Cumulative OR stands for the cumulative effect of the two genotypes (heterozygous and homozygous in comparison with homozygous for the other allele in the first row taking as the reference group). p value stands for nominal p.

Table 4
Estimated haplotype frequency distributions of *ABCC2* gene SNPs in ICP and control chromosomes

Haplotype Number	dsSNP rs # Cluster ID						Haplotype Frequency	
	rs1	r2	rs3	rs4	rs5	rs6	Intrahepatic cholestasis of pregnancy (*n*: 140)	Healthy pregnant controls (*n*: 224)
H1	G	C	T	G	T	G	0.310	0.327
H2	G	T	C	A	T	G	0.222	0.180
H3	G	C	C	G	T	G	0.208	0.172
H4	A	T	C	A	T	G	0.189	0.151
H5	G	C	T	A	T	G	0.029	0.052
H6	G	T	C	G	T	G	0.016	0.052
H7	G	T	C	G	A	A	0.011	0.038
H8	A	C	T	G	T	G	0.014	0.027

Haplotypes are composed by variants of rs1: rs717620, rs2: rs2756105, rs3: rs2002042, rs4: rs3740066, rs5: rs17222723 and rs6: rs8187710. Number of chromosomes examined. Global significance: $p < 0.02$; Haplotype 6 compared with all others: $p < 0.004$ and Haplotype 6 and 7 vs all others: $p < 002$ for the difference between subject groups. p values stand for empirical P after permutation test.

Consistent with the analysis of individual markers, we observed that the global *ABCC2* gene variant haplotype frequencies in ICP patients significantly differed from that in controls ($p < 0.03$). Trying to dissect the association signal, we performed the analysis using the WHAP software removing individual markers one by one, and we observed that no other SNPs but rs2756105 contributed an additional gene effect than that of rs3740066. Furthermore, among all the haplotypes, H6 alone explains much of the global effect ($p < 0.005$). However, grouping H6 and H7 vs all others a more significant effect was reached ($p < 0.002$).

We also assessed the association between the rs3740066 and quantitative traits by PLINK software. Thus, we observed a positive association between the rs3740066 and ALT, AST, alkaline alkaline phosphatase and total and conjugated bilirubin concentrations. No significant association was observed with any obstetrical and perinatal characteristics (Table 5). Serum total bile acids were significantly correlated (Spearman R: 0.30, $p < 0.01$) with A allele (mean ± SD: GG 5.10 ± 10.8, GA 14.2 ± 23.9 and AA 20.6 ± 26.0).

Table 5
Biochemical, obstetrical and perinatal characteristics in the whole population according to *ABCC2* rs3740066 genotypes

	AA *n*: 33 (Median, range)	GG + GA *n*: 149 (Median, range)	*p* level
Age, years	26, 23	25, 31	0.59
Gestational age at delivery (weeks)	36, 21	37, 21	0.07
Neonate birth weight (g)	2965, 2100	3100, 2990	0.09
Apgar score	9.5, 1	10, 4	0.09
Total bilirubin (μmol/L)	9, 33	8, 37	0.01
Direct bilirubin (μmol/L)	3, 18	2, 24	0.01
AST (IU/L)	34, 339	19, 283	0.001
ALT (IU/L)	37.5, 530	17, 391	<0.01
Alkaline phophatase (IU/L)	467, 1322	331, 1132	<0.01

Results are expressed as median, range.

4. Discussion

In an attempt to identify genetic variants in the *ABCC2* (*MRP2*) gene underlying risk or individual susceptibility of ICP, we genotyped 6 SNPs and evaluated the corresponding haplotype combinations in a case-control association study. We observed a significant association between the rs3740066 A/G in the coding region (exon 28) of *ABCC2* gene and intrahepatic cholestasis of pregnancy, and the estimated risk of disease for homozygous AA carriers was 4-fold higher in comparison with GG carriers. As far as we know, this is the first paper to provide data about the contribution of the *ABCC2* gene variants to the risk of the ICP.

Consistent with this result, the association with the haplotype 6 (GTCGTG) and 7 (GTCGAA) provided additional evidence for an association between the variant and the ICP phenotype ($p < 0.002$). These haplotypes shared the characteristics of containing a sub-haplotype for rs717620, rs2756105 and rs3740066 (GTG). On removing SNP rs3740066 from the haplotypes we found no significant differences between cases and controls.

Studies in patients with Dubin–Johnson Syndrome (DJS) have contributed valuable information about *ABCC2* genomic organization and the structure and function of ABCC2 protein [28] despite the fact that the role of genetic variants in the *ABCC2* gene was reported in cholestatic diseases such as DJS [29] and primary biliary cirrhosis [30], no studies reported the role of the *ABCC2* gene polymorphisms in ICP. Considering that some *ABCC2* mutations found in DJS patients are associated with the loss of transport activity and/or the ability to traffic to the apical membrane by the single amino acid alteration [31], it is possible that the *ABCC2*

SNPs may also be associated with alterations in protein function, transcriptional gene activity and/or splicing and translation of the mRNA. Consequently, *ABCC2* variants may be responsible for the interindividual differences in the susceptibility to ICP.

Although association does not necessarily mean a causal-relationship, in this case several lines of evidence support the association between the *ABCC2* rs3740066 and ICP. First, the biological plausibility of this relation as *ABCC2* has been implicated in drug and estrogen induced cholestasis [32]. In accordance with this finding, although the major physiological role of ABCC2 is to transport conjugated metabolites into the bile canaliculus, previous data demonstrated that a major metabolite of human estrogen metabolism, estradiol-17-β-D-glucuronide ($E_2$17βG), has been shown to be transported by both MRP2 and MRP3 [33].

Besides, this metabolite, with a significantly increased level during pregnancy and considered to be related to the pathogenesis of intrahepatic cholestasis of pregnancy, is secreted into the bile mainly by ABCC2 [34].

Second, as has previously been mentioned, canalicular bile secretion represents the rate-limiting step in bile formation and the conjugate export pump ABCC2 plays an important role in the ATP-dependent transport system [15]. One may thus speculate that a gene variant leading to an altered function of the canalicular transport system involved in bile secretion may also participate in the etiology of the ICP. In fact, we observed that A allele of the rs3740066 was significantly and positively correlated with the plasma total bile acid concentration. In addition, it has been shown that the MRP2 substrate $E_2$17βG trans-inhibits the bile salt export pump (which may induce cholestasis) [35]. In this context, the possibility that the rs3740066 may influence bile salt levels in ICP by increasing MRP2-activity may be considered.

The rs3740066 in the exon 28 is a synonymous SNP. However, it might still be detrimental to gene function if it affects, for instance, alternative splicing regulation by disrupting exonic splicing enhancer [36] or silencer-binding elements.

Although it deserves further investigation, it is tempting to speculate that the exon 28 reported variant may affect the splicing machinery altering disease severity and with a potential role as a genetic modifier. Moreover, Kimchi–Sarfaty et al. [37] provide evidence that naturally occurring silent SNPs can affect *in vivo* protein folding and, consequently function. The study shows that substrate specificity of P-glycoprotein, the product of the multidrug resistance 1 (*MDR1*) gene, is altered by SNPs presumed to be synonymous and silent.

As a final point, a number of novel SNPs in the *ABCC2* gene have recently been analyzed in healthy Japanese subjects [38] and also in cell lines from human tumors [39], suggesting that the C-24T in the 5′flanking region linked with C3972T in the exon 28 may be of importance in the regulation of the *ABCC2* gene, the coding variant (the rs3740066) being associated with the promoter activity of the gene. When we applied the pairwise r^2 method for the analysis of LD of the studied variants, we observed from the HapMap data that the rs717629 at the promoter region had a moderate squared correlation coefficient value (r^2: 0.441) at which the alleles can be captured by rs3740066. Despite the fact that in our population, both variants are in a low to moderate LD (Spearman R 0.34, $p < 0.001$), we did not observe any significant association between rs717620 and ICP. Since the allele G of rs717620 seems to participate in ICP-associated haplotypes, this matter deserves further investigation.

Lastly, a power estimation with our sample size gave us a statistically power of 80% or greater under the additive model. In addition, it is worth mentioning that previous reports about candidate gene association studies in ICP patients used similar sample size [8,10,40,41] probably denoting difficulties in recruiting well-characterized patients owing to the low prevalence of the disease.

In conclusion, the pathogenesis of the ICP remains unknown but seems to be related to the effects of hormonal factors on the liver of genetically predisposed women. Thus it is reasonable to speculate that mild malfunction of canalicular transporters (particularly ABCC2 protein), which causes no disease outside pregnancy, may lead to clinical symptoms of cholestasis when the transporter capability to secrete substances, for instance estrogens, is exceeded as occurs in pregnancy.

Regarding ICP, it seems that no single gene is sufficient for disease. Consequently, other candidate genes have been successfully evaluated in the risk of the disease, for example, genes encoding the hepatobiliary ABC transporters for phospholipids (*ABCB4*) and the bile salt export pump (*ABCB11*) [8,9,12,11], all of them conveying biologically meaningful associations.

We hope our study can serve as a primer since further research is needed to confirm and extend the current findings improving the power of the study by increasing the number of subjects and reveal the intimate mechanism by which the *ABCC2* variant at exon 28 or linked variants not explored in our study may lead to protein dysfunction. Extending the knowledge of the molecular basis underling disease variability in patients with ICP may aid the development of novel therapeutic approaches to diminish fetal morbimortality associated to the disease.

Acknowledgements

This study was partially supported by the Consejo de Investigación del Gobierno de la Ciudad de Buenos

Aires and by Grants B119 (Universidad de Buenos Aires), PICT 25920 (Agencia Nacional de Promoción Científica y Tecnológica) and PIP 5195 (Consejo Nacional de Investigaciones Científicas y Técnicas). S.S., A.B. and C.J.P. belong to Consejo Nacional de Investigaciones Científicas y Técnicas.

References

[1] Rioseco AJ, Ivankovic MB, Manzur A, Hamed F, Kato SR, Parer JT, et al. Intrahepatic cholestasis of pregnancy: a retrospective case-control study of perinatal outcome. Am J Obstet Gynecol 1994;170:890–895.

[2] Laatikainen T, Tulenheimo A. Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. Int J Gynaecol Obstet 1984;22:91–94.

[3] Reyes H, Baez ME, Gonzalez MC, Hernandez I, Palma J, Ribalta J, et al. Selenium, zinc and copper plasma levels in intrahepatic cholestasis of pregnancy, in normal pregnancies and in healthy individuals, in Chile. J Hepatol 2000;32:542–549.

[4] Reyes H. Review: intrahepatic cholestasis. A puzzling disorder of pregnancy. J Gastroenterol Hepatol 1997;12:211–216.

[5] Hirvioja ML, Kivinen S. Inheritance of intrahepatic cholestasis of pregnancy in one kindred. Clin Genet 1993;43:315–317.

[6] Dalen E, Westerholm B. Occurrence of hepatic impairment in women jaundiced by oral contraceptives and in their mothers and sisters. Acta Med Scand 1974;195:459–463.

[7] Eloranta ML, Heinonen S, Mononen T, Saarikoski S. Risk of obstetric cholestasis in sisters of index patients. Clin Genet 2001;60:42–45.

[8] Wasmuth HE, Glantz A, Keppeler H, Simon E, Bartz C, Rath W, et al. Intrahepatic cholestasis of pregnancy: the severe form is associated with common variants of the hepatobiliary phospholipid transporter gene ABCB4. Gut 2007;56:265–270.

[9] Pauli-Magnus C, Lang T, Meier Y, Zodan-Marin T, Jung D, Breymann C, et al. Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy. Pharmacogenetics 2004;14:91–102.

[10] Savander M, Ropponen A, Avela K, Weerasekera N, Cormand B, Hirvioja ML, et al. Genetic evidence of heterogeneity in intrahepatic cholestasis of pregnancy. Gut 2003;52:1025–1029.

[11] Mullenbach R, Linton KJ, Wiltshire S, Weerasekera N, Chambers J, Elias E, et al. ABCB4 gene sequence variation in women with intrahepatic cholestasis of pregnancy. J Med Genet 2003;40:e70.

[12] Floreani A, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, et al. Intrahepatic cholestasis of pregnancy: three novel MDR3 gene mutations. Aliment Pharmacol Ther 2006;23:1649–1653.

[13] Schneider G, Paus TC, Kullak-Ublick GA, Meier PJ, Wienker TF, Lang T, et al. Linkage between a new splicing site mutation in the MDR3 alias ABCB4 gene and intrahepatic cholestasis of pregnancy. Hepatology 2007;45:150–158.

[14] Painter JN, Savander M, Ropponen A, Nupponen N, Riikonen S, Ylikorkala O, et al. Sequence variation in the ATP8B1 gene and intrahepatic cholestasis of pregnancy. Eur J Hum Genet 2005;13:435–439.

[15] Trauner M, Meier PJ, Boyer JL. Molecular pathogenesis of cholestasis. N Engl J Med 1998;339:1217–1227.

[16] Apgar V. The newborn (Apgar) scoring system. Reflections and advice. Pediatr Clin North Am 1966;13:645–650.

[17] Capurro H, Konichezky S, Fonseca D, Caldeyro-Barcia R. A simplified method for diagnosis of gestational age in the newborn infant. J Pediatr 1978;93:120–122.

[18] Castano G, Lucangioli S, Sookoian S, Mesquida M, Lemberg A, Di SM, et al. Bile acid profiles by capillary electrophoresis in intrahepatic cholestasis of pregnancy. Clin Sci (Lond) 2006;110:459–465.

[19] Kawasaki ES. Sample preparation from blood, cells, and other fluids. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols. A guide to Methods and Applications. San diego: Academic Press, Inc.; 1990. p. 146–152.

[20] de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. Nat Genet 2005;37:1217–1223.

[21] Meier Y, Pauli-Magnus C, Zanger UM, Klein K, Schaeffeler E, Nussler AK, et al. Interindividual variability of canalicular ATP-binding-cassette (ABC)-transporter expression in human liver. Hepatology 2006;44:62–74.

[22] Myakishev MV, Khripin Y, Hu S, Hamer DH. High-throughput SNP genotyping by allele-specific PCR with universal energy-transfer-labeled primers. Genome Res 2001;11:163–169.

[23] Zhao JH, Curtis D, Sham PC. Model-free analysis and permutation tests for allelic associations. Hum Hered 2000;50:133–139.

[24] Purcell S, Daly MJ, Sham PC. WHAP: haplotype-based association analysis. Bioinformatics 2007;23:255–256.

[25] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263–265.

[26] Skol AD, Scott LJ, Abecasis GR, Boehnke M. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat Genet 2006;38:209–213.

[27] Liu IM, Agresti A. Mantel–Haenszel-type inference for cumulative odds ratios with a stratified ordinal response. Biometrics 1996;52:1223–1234.

[28] Toh S, Wada M, Uchiumi T, Inokuchi A, Makino Y, Horie Y, et al. Genomic structure of the canalicular multispecific organic anion-transporter gene (MRP2/cMOAT) and mutations in the ATP-binding-cassette region in Dubin–Johnson syndrome. Am J Hum Genet 1999;64:739–746.

[29] Hashimoto K, Uchiumi T, Konno T, Ebihara T, Nakamura T, Wada M, et al. Trafficking and functional defects by mutations of the ATP-binding domains in MRP2 in patients with Dubin–Johnson syndrome. Hepatology 2002;36:1236–1245.

[30] Kullak-Ublick GA, Baretton GB, Oswald M, Renner EL, Paumgartner G, Beuers U. Expression of the hepatocyte canalicular multidrug resistance protein (MRP2) in primary biliary cirrhosis. Hepatol Res 2002;23:78–82.

[31] Keitel V, Kartenbeck J, Nies AT, Spring H, Brom M, Keppler D. Impaired protein maturation of the conjugate export pump multidrug resistance protein 2 as a consequence of a deletion mutation in Dubin–Johnson syndrome. Hepatology 2000;32:1317–1328.

[32] Gerk PM, Vore M. Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. J Pharmacol Exp Ther 2002;302:407–415.

[33] Ito K, Oleschuk CJ, Westlake C, Vasa MZ, Deeley RG, Cole SP. Mutation of Trp1254 in the multispecific organic anion transporter, multidrug resistance protein 2 (MRP2) (ABCC2), alters substrate specificity and results in loss of methotrexate transport activity. J Biol Chem 2001;276:38108–38114.

[34] Takikawa H, Yamazaki R, Sano N, Yamanaka M. Biliary excretion of estradiol-17 beta-glucuronide in the rat. Hepatology 1996;23:607–613.

[35] Stieger B, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. Gastroenterology 2000;118:422–430.

[36] Cartegni L, Krainer AR. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. Nat Genet 2002;30:377–384.
[37] Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, et al. A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 2007;315:525–528.
[38] Suzuki H, Sugiyama Y. Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. Adv Drug Deliv Rev 2002;54:1311–1331.
[39] Itoda M, Saito Y, Soyama A, Saeki M, Murayama N, Ishida S, et al. Polymorphisms in the ABCC2 (cMOAT/MRP2) gene found in 72 established cell lines derived from Japanese individuals: an association between single nucleotide polymorphisms in the 5′-untranslated region and exon 28. Drug Metab Dispos 2002;30:363–364.
[40] Eloranta ML, Heiskanen JT, Hiltunen MJ, Mannermaa AJ, Punnonen KR, Heinonen ST. Multidrug resistance 3 gene mutation 1712delT and estrogen receptor alpha gene polymorphisms in Finnish women with obstetric cholestasis. Eur J Obstet Gynecol Reprod Biol 2002;105:132–135.
[41] Eloranta ML, Hakli T, Hiltunen M, Helisalmi S, Punnonen K, Heinonen S. Association of single nucleotide polymorphisms of the bile salt export pump gene with intrahepatic cholestasis of pregnancy. Scand J Gastroenterol 2003;38:648–652.