Association of $ABCB4$ and $ABCB11$ nucleotide variants with intrahepatic cholestasis of pregnancy

Milena Gruszczyńska-Losy¹, Adrianna Mostowska², Łukasz Adamczak³, Paweł P. Jagodziński¹, b, Ewa Wender-Ożegowska³, c, Małgorzata Kędzia³, d, *

¹ Gynecologic and Obstetrical University Hospital in Poznan, Poland
² Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, Poland
³ Division of Reproduction, Department of Obstetrics, Gynecology, and Gynecological Oncology, Poznan University of Medical Sciences, Poznan, Poland

* Corresponding Author: Małgorzata Kędzia, Division of Reproduction, Department of Obstetrics, Gynecology, and Gynecological Oncology Poznan University of Medical Sciences, 33 Polna Street, 60-535 Poznan, Poland, phone: +48601700022, fax: +48618419625, email: mal.gin@poczta.fm

ABSTRACT

Introduction. Intrahepatic cholestasis of pregnancy (ICP) is the most common liver disorder during gestation. The exact pathogenesis of ICP is multifactorial and still unclear. Therefore, our study aimed to check whether the selected $ABCB4$ and $ABCB11$ nucleotide variants are associated with an increased risk of ICP.

Material and Methods. ICP was diagnosed based on clinical symptoms characteristic of this disease, and confirmed by an increase in serum bile acids and transaminases, spontaneous resolution of clinical symptoms, and normalization of laboratory tests after delivery. A total of 86 pregnant women meeting the criteria were included in the study. Healthy pregnant women with uncomplicated pregnancy served as a control group (n = 310). Six common nucleotide variants in the $ABCB11$ and $ABCB4$ genes were genotyped with the use of high-resolution melting curve analysis.

Results. All tested nucleotide variants did not show significant deviation from the Hardy Weinberg equilibrium in both ICP patients and healthy women. None of the $ABCB4$ and $ABCB11$ variants were significantly correlated with the risk of ICP ($p_{\text{trend}} > 0.05$). Similar results were also obtained after the division of patients based on the TBA levels. However, in the group of patients with moderate and severe ICP, a trend toward association between the $ABCB4$ rs2109505 variant and cholestasis was observed ($p_{\text{trend}} = 0.063$; OR_allelic = 1.87, 95% CI: 0.92 – 3.80; OR_dominant = 1.90, 95% CI: 0.83 – 4.36 and OR_recessive = 12.24, 95% CI: 0.74 – 201.75).

Conclusions. Our study did not show any significant association of the analysed $ABCB4$ and $ABCB11$ nucleotide variants with an increased risk of intrahepatic cholestasis of pregnancy.

Keywords: Intrahepatic Cholestasis of Pregnancy, $ABCB4$, $ABCB11$, nucleotide variants.

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is the most common, but short-lived, liver-specific pregnancy disorder. The incidence of ICP in the Caucasian population varies between 0.5–1.5% [1]. This illness usually occurs in the second and third trimester of pregnancy and resolves shortly after partum. Although it may have a very early-onset, as early as nine weeks of gestation, it
can persist for several months after delivery [2]. ICP is very oppressive for the mother because of pruritus, which intensifies at night, but is generally a benign disease. However, from the perspective of foetal complications, there is a correlation between high serum bile acids levels, and an increased risk of an abnormal obstetric outcome connected with an elevated risk for the foetus and newborn [3]. Kawakita et al. [4], based on total bile acid (TBA) levels in maternal serum, distinguished three ranges in the course of cholestasis: mild, with TBA 10–39.9 μmol/L moderate with TBA 40–99.9 μmol/L, and severe with TBA ≥ 100 μmol/L. The authors detected a significant association between severe ICP and adverse outcomes, with increased risk of stillbirth [3, 4].

The exact pathogenesis of ICP is multifactorial and still unclear. Pregnant women with ICP have a deficiency in the excretion of bile salts to bile, which causes an increase in serum bile acids.

Intrahepatic cholestasis of pregnancy is significantly more common in the same families. The relative risk for an affected first-degree relative is 12% [5]. The risk of recurrences in the next pregnancy reaches 45% [6]. In addition, there is an increase in the frequency of ICP in geographical regions and specified ethnic groups [7, 8]. However, the genetic basis of ICP indicates familial clustering and endemic occurrences.

The genetic basis of bile transport disorders across canalicular membranes was based on rarely occurring familial syndromes, including progressive familial intrahepatic cholestasis (PFIC), and benign recurrent intrahepatic cholestasis (BRIC) [9]. These diseases result from the functional deficiency of canalicular ATP-binding cassette (ABC) transporters. In recent years, research on the contribution of genetic factors involved in bile transport disorders were also performed in pregnant women with cholestasis [10, 11].

The most extensively studied candidate gene in intrahepatic cholestasis in pregnancy is ABCB4 (OMIM *171060). The human ABCB4 gene is located on the 7q21 chromosome. This gene encodes phosphatidylcholine floppase, an ATPase also known as multidrug resistance protein 3 (MRP3). This protein belongs to the super-family of transporter proteins possessing ATP-binding cassette. A reduction of phosphatidylcholine in the bile causes an escalation of nonmicellar toxic bile acid.

The subsequent gene examined in intrahepatic cholestasis is ABCB11 (OMIM *603201). This gene is located on chromosome 2q24. The product of ABCB11 is an ABC transporter named bile salt export pump (BSEP). It actively transports conjugated bile salts into biliary canalliculi against a concentration gradient. Defective function of BSEP results in abnormal bile salt excretion to bile, leading to cholestasis [2, 11]. Additionally, biliary transporter gene mutations were also detected in severe intrahepatic cholestasis of pregnancy, which is in the main spectrum of interest due to the consequences for the foetus [13].

Therefore, the aim of our study was to check whether the selected ABCB4 and ABCB11 nucleotide variants are associated with an increased risk of ICP. In addition, we decided to examine whether their association with the risk of ICP may depend on the severity of this disease.

**Material and Methods**

**Patients and controls**

Peripheral blood samples from women with intrahepatic cholestasis in pregnancy, and healthy pregnant control subjects with uncomplicated pregnancy were collected at the Gynaecologic and Obstetrical University Hospital, Division of Reproduction at the Poznan University of Medical Sciences.

ICP was diagnosed based on clinical symptoms: pruritus in the absence of any dermatologic or other systemic medical condition causing pruritus. Confirmation of the diagnosis was made with a rise in serum bile acids (> 10 μmol/L) and transaminases (> 31 U/L), and spontaneous resolution of clinical symptoms and normalisation of laboratory tests after delivery. The exclusion criteria were: viral or autoimmune hepatobiliary disease or extrahepatic biliary obstruction. A total of 86 pregnant women meeting the criteria were included in the study. In this group, there were 67 women with single pregnancy and 19 patients with multiple pregnancies (16 twins and three triplets). The women with ICP were divided into 2 groups (n = 60 and n = 26) according to their TBA level (10–39.9 and ≥ 40.0 μmol/L, respectively). The control subjects were healthy, lean (BMI < 25 kg/m²) pregnant women with uncomplicated pregnancy (n = 310).
Written informed consent was obtained from all participating individuals. The study procedure was approved by the Local Ethical Committees of Poznan University of Medical Sciences, and was performed in accordance with the code of ethics of the Declaration of Helsinki.

SNP selection and genotyping

Single nucleotide polymorphisms (SNPs) in the ABCB4 and ABCB11 genes were identified from the relevant literature and public databases, including the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and the 1000 Genomes Browser (http://browser.1000genomes.org/index.html). SNP selection was based on their functional significance, association with the risk of ICP in previous studies, and minor allele frequencies (MAF, ≥ 5% in the Caucasian population from the 1000 Genomes Project). The characteristics of the SNPs selected for analysis (n = 6) are presented in Table 1. Genomic DNA was isolated from peripheral blood lymphocytes with the use of a DNA extraction kit (Blirt-DNA Gdansk, Gdansk, Poland). Genotyping was carried out by high-resolution melting curve analysis (HRM) on a LightCycler 96 system (Roche Diagnostics, Mannheim, Germany) with the use of 5x HOT FIREPol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia). Quality control was ensured by including 10% of the samples as duplicates. Samples that failed genotyping were removed from the statistical calculations. The primer sequences and HRM conditions are presented in Supplementary Table 1.

Table 1. Characteristics of the ABCB11 and ABCB4 nucleotide variants

| Gene     | rs no.      | Location (bp)* | Consequence type | Alleles b | MAF c |
|----------|-------------|----------------|-----------------|-----------|-------|
| ABCB11   | rs2287622   | chr2:169973818 | missense (p.Val444Ala) | C / T     | 0.33  |
| 2q31.1   | rs3815676   | chr2:169013869 | intronic         | A / G     | 0.05  |
|          | rs577650    | chr2:169034700 | upstream         | A / G     | 0.28  |
| ABCB4    | rs4148826   | chr7:87445103  | intronic         | A / G     | 0.17  |
| 7q21.12  | rs2109505   | chr7:87450090  | synonymous (p.Ile237Ile) | A / T     | 0.17  |
|          | rs2302386   | chr7:8742628   | intronic         | A / G     | 0.13  |

*GRCh38/hg38.  
b Underline denotes the minor allele.  
c MAF — minor allele frequency based on 1000 Genomes genotype data (CEU sample).

Statistical analysis

Each SNP was tested for deviation from the Hardy-Weinberg equilibrium (HWE) in both the patients and controls using the chi-square ($\chi^2$) test. The association of the ABCB4 and ABCB11 SNPs with ICP was tested with the Cochran-Armitage trend test. Odds Ratios (ORs) with 95% Confidence Intervals (95% CIs) were used to assess the strength of the association. The allelic, dominant, and recessive models were analysed. The Bonferroni correction was applied to account for multiple testing, and p-values < 0.0083 (0.05 / 6 SNPs) were considered to be statistically significant. The pair-wise linkage disequilibrium (LD) between the tested SNPs ($D'$ and $r^2$ statistics) was evaluated using the Haplovew 4.2 software package (www.broadinstitute.org/haplovew/haplovew). The same software was used to conduct a haplotype-based association analysis (sliding window approach). Statistical significance was assessed using the 1,000-fold permutation test. All statistical calculations were performed for the whole sample, and after division of the patients based on the TBA levels. In addition, separate association testing was performed after the exclusion of cases with multiple pregnancies.

Results

All tested SNPs did not show significant deviation from HWE in both ICP patients and healthy women (p > 0.05). In the controls, the MAF for the analysed variants was between 2 and 42% (Table 2). In the tested sample, the ABCB4 gene variants are moderated LD (average $r^2 = 0.65$ and $D' = 0.92$; Table 3), while the ABCB11 SNPs are in weak LD (average $r^2 = 0.05$ and $D' = 0.34$; Table 4). None of the ABCB4 and ABCB11 SNPs were significantly correlated with the risk of ICP (p_{Bonferroni} > 0.05; Table 3). Under the assumption of all analysed
Table 2. Association of the ABCB11 and ABCB4 nucleotide variants with the risk of ICP

| Gene  | SNP          | MAF   | OR (95%CI); p-valueb |
|-------|--------------|-------|----------------------|
|       | Cases Controls |       | Allelic modelc Dominant modeld Recessive model e |
|       |   | Allelesa |   | Allelic modelc Dominant modeld Recessive model e |
| ICP (n = 86) | | | | |
| ABCB11 | rs2287622 C / T | 0.42 0.42 0.899 | 0.98 (0.69–1.38); 0.897 | 1.02 (0.62–1.70); 0.929 | 0.90 (0.48–1.68); 0.734 |
| | rs3815676 A / G | 0.00 0.02 0.096 | 0.17 (0.01–2.97); 0.131 | 0.17 (0.01–2.93); 0.128 | NA |
| | rs7577650 A / G | 0.34 0.40 0.213 | 0.79 (0.56–1.13); 0.201 | 0.86 (0.53–1.41); 0.557 | 0.54 (0.26–1.15); 0.107 |
| ABCB4 | rs4148826 A / G | 0.16 0.14 0.497 | 1.18 (0.73–1.88); 0.501 | 1.10 (0.64–1.88); 0.733 | 2.77 (0.61–12.63); 0.177 |
| | rs2109505 A / T | 0.15 0.13 0.473 | 1.19 (0.73–1.93); 0.492 | 1.11 (0.64–1.91); 0.707 | 7.27 (0.65–81.38); 0.122 |
| | rs2302386 A / G | 0.11 0.10 0.695 | 1.12 (0.64–1.96); 0.693 | 1.13 (0.61–2.07); 0.699 | 1.19 (0.12–11.60); 1.000 |
| Mild ICP (n = 60) | | | | |
| ABCB11 | rs2287622 C / T | 0.43 0.42 0.972 | 1.01 (0.68–1.50); 0.971 | 1.14 (0.63–2.07); 0.657 | 0.84 (0.40–1.75); 0.636 |
| | rs3815676 A / G | 0.00 0.02 0.163 | 0.25 (0.01–4.25); 0.338 | 0.24 (0.01–4.19); 0.374 | NA |
| | rs7577650 A / G | 0.35 0.40 0.350 | 0.82 (0.54–1.23); 0.337 | 0.93 (0.53–1.64); 0.812 | 0.52 (0.21–1.26); 0.141 |
| ABCB4 | rs4148826 A / G | 0.14 0.14 0.934 | 0.98 (0.55–1.74); 0.935 | 0.87 (0.45–1.67); 0.670 | 2.66 (0.48–14.86); 0.249 |
| | rs2109505 A / T | 0.12 0.13 0.780 | 0.92 (0.50–1.69); 0.791 | 0.84 (0.43–1.64); 0.610 | 5.19 (0.32–84.14); 0.301 |
| | rs2302386 A / G | 0.09 0.10 0.934 | 0.93 (0.49–1.91); 0.933 | 0.92 (0.83–1.93); 0.826 | 1.72 (0.18–16.88); 0.511 |
| Moderate and severe ICP (n = 26) | | | | |
| ABCB11 | rs2287622 C / T | 0.40 0.42 0.757 | 0.91 (0.52–1.64); 0.749 | 0.80 (0.35–1.83); 0.589 | 1.05 (0.38–2.90); 1.000 |
| | rs3815676 A / G | 0.00 0.02 0.360 | 0.57 (0.03–9.88); 1.000 | 0.56 (0.03–9.76); 1.000 | NA |
| | rs7577650 A / G | 0.33 0.40 0.341 | 0.74 (0.40–1.35); 0.322 | 0.73 (0.32–1.62); 0.434 | 0.61 (0.18–2.09); 0.591 |
| ABCB4 | rs4148826 A / G | 0.21 0.14 0.140 | 1.67 (0.83–3.38); 0.150 | 1.74 (0.76–4.00); 0.185 | 3.03 (0.33–28.16); 0.336 |
| | rs2109505 A / T | 0.21 0.13 0.603 | 1.87 (0.92–3.80); 0.078 | 1.90 (0.83–4.36); 0.125 | 12.24 (0.74–201.75); 0.150 |
| | rs2302386 A / G | 0.13 0.10 0.365 | 1.47 (0.63–3.41); 0.367 | 1.66 (0.67–4.15); 0.272 | 1.62 (0.08–32.23); 1.000 |

a Underline denotes the minor allele.
b Chi-square analysis.
c d vs D; d is the risk allele.
d dd + Dd vs DD; d is the risk allele.
e dd vs Dd + DD; d is the risk allele.
f Fisher exact test.
MAF – minor allele frequency; OR – odds ratio; 95%CI – 95% confidence interval; NA – not applicable.

Table 3. Linkage disequilibrium values D' and r^2 for nucleotide variants tested in the ABCB4 gene

|       | rs4148826 | rs2109505 | rs2302386 |
|-------|-----------|-----------|-----------|
| rs4148826 | 1.00      | 0.977     | 0.904     |
| rs2109505 | 0.857     | 1.00      | 0.875     |
| rs2302386 | 0.539     | 0.557     | 1.00      |

Numbers denote D' and r^2 values expressed as a percentage of maximal value (1.0). D' values are presented above the diagonal, r^2 values are presented below the diagonal.

Table 4. Linkage disequilibrium values D' and r^2 for nucleotide variants tested in the ABCB11 gene

|       | rs2287622 | rs3815676 | rs7577650 |
|-------|-----------|-----------|-----------|
| rs2287622 | 1.00      | 0.115     | 0.422     |
| rs3815676 | 0.000     | 1.00      | 0.485     |
| rs7577650 | 0.152     | 0.004     | 1.00      |

Numbers denote D' and r^2 values expressed as a percentage of maximal value (1.0). D' values are presented above the diagonal, r^2 values are presented below the diagonal.
Table 5. Association of the ABCB11 and ABCB4 nucleotide variants with the risk of ICP in the group of patients after exclusion of cases with multiple pregnancies

| Gene | SNP          | Allelesa | Cases | Controls | p-valueb | MAF                   | OR (95% CI) | p-valueb | Allelic modelc | Dominant modeld | Recessive modele |
|------|--------------|----------|-------|----------|----------|-----------------------|-------------|----------|----------------|----------------|-----------------|
| ICP  |              | Allelic | Cases | Controls |          |           | C / T      | 0.40   | 0.42 | 0.658 | 0.91 (0.62–1.34); 0.647 | 0.82 (0.47–1.41); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              | model   |       |          |          |           | 0.658     | 0.647  | 0.644 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.644     | 0.464  | 0.938 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              | Dominant| Cases | Controls | p-valueb | MAF                   | OR (95% CI) | p-valueb | Allelic modelc | Dominant modeld | Recessive modele |
|      |              | model   |       |          |          |           | C / T      | 0.40   | 0.42 | 0.658 | 0.91 (0.62–1.34); 0.647 | 0.82 (0.47–1.41); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.658     | 0.647  | 0.644 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.644     | 0.464  | 0.938 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | C / T      | 0.40   | 0.42 | 0.658 | 0.91 (0.62–1.34); 0.647 | 0.82 (0.47–1.41); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.658     | 0.647  | 0.644 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.644     | 0.464  | 0.938 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              | Recessive| Cases | Controls | p-valueb | MAF                   | OR (95% CI) | p-valueb | Allelic modelc | Dominant modeld | Recessive modele |
|      |              | model   |       |          |          |           | C / T      | 0.40   | 0.42 | 0.658 | 0.91 (0.62–1.34); 0.647 | 0.82 (0.47–1.41); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.658     | 0.647  | 0.644 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.644     | 0.464  | 0.938 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | C / T      | 0.40   | 0.42 | 0.658 | 0.91 (0.62–1.34); 0.647 | 0.82 (0.47–1.41); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.658     | 0.647  | 0.644 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |
|      |              |          |       |          |          |           | 0.644     | 0.464  | 0.938 | 1.03  | 0.53 (0.28–2.01); 0.464 | 1.03 (0.53–2.01); 0.938 |

Table 6. Haplotype analysis of the ABCB11 and ABCB4 nucleotide variants

| Gene | Nucleotide variants | Haplotypes | Frequency | Case, Control Frequencies | p-value | p_corr-valuea |
|------|---------------------|------------|-----------|---------------------------|---------|----------------|
| ABCB11 | rs2287622, rs3815676 | CA         | 0.576     | 0.596, 0.570             | 0.397   | 0.528          |
|       |                     | TA         | 0.412     | 0.403, 0.414             | 0.065   | 0.799          |
|       |                     | AG         | 0.616     | 0.665, 0.602             | 2.254   | 0.133          |
|       |                     | AA         | 0.371     | 0.335, 0.381             | 1.268   | 0.260          |
|       | rs2287622, rs3815676, rs7577650 | CAG       | 0.454     | 0.499, 0.442             | 1.803   | 0.179          |
|       |                     | TAA        | 0.248     | 0.237, 0.251             | 0.139   | 0.710          |
|       |                     | TAG        | 0.163     | 0.166, 0.162             | 0.013   | 0.910          |
|       |                     | CAA        | 0.122     | 0.098, 0.129             | 1.229   | 0.268          |
|       | rs4148826, rs2109505 | AT         | 0.855     | 0.835, 0.861             | 0.761   | 0.383          |
|       |                     | GA         | 0.127     | 0.153, 0.119             | 1.435   | 0.231          |
|       |                     | GT         | 0.015     | 0.012, 0.016             | 0.195   | 0.659          |
|       | rs2109505, rs2302386 | TA         | 0.860     | 0.846, 0.864             | 0.362   | 0.540          |
|       |                     | AG         | 0.087     | 0.113, 0.080             | 1.925   | 0.165          |
|       |                     | AA         | 0.042     | 0.040, 0.043             | 0.025   | 0.874          |
|       |                     | TG         | 0.011     | 0.001, 0.014             | 2.128   | 0.145          |
|       | rs4148826, rs2109505, rs2302386 | ATA       | 0.847     | 0.834, 0.851             | 0.288   | 0.581          |
|       |                     | GAA        | 0.040     | 0.040, 0.040             | 0.000   | 0.969          |
|       |                     | GTA        | 0.013     | 0.012, 0.013             | 0.016   | 0.899          |

a p value calculated using permutation test and a total of 1,000 permutations

MAF—minor allele frequency; OR—odds ratio; 95%CI—95% confidence interval; NA—not applicable.
in inheritance models, the tested variants showed no evidence of an association with the increased risk of developing intrahepatic cholestasis during pregnancy. Similar results were also obtained after the division of patients based on the TBA levels (Table 2). Only in the group of patients with TBA levels > 40 (moderate and strong ICP), there was a trend towards association between the ABCB4 rs2109505 variant and cholestasis (p_trend = 0.063; OR_allelic = 1.87, 95% CI: 0.92–3.80; OR_dominant = 1.90, 95% CI: 0.83–4.36, and OR_recessive = 12.24, 95% CI: 0.74–201.75). Separate statistical calculations conducted in the group of patients after exclusion of cases with multiple pregnancies showed comparable results. For all tested nucleotide variants, there was no evidence for either allelic or genotypic association with the risk of ICP (Table 5). The result close to being statistically significant was also found for the ABCB4 rs2109505 variant. Under the assumption of a recessive model, this SNP was associated with 9.42-fold (95% CI: 0.84–105.45, p = 0.084) increase in the risk of ICP (all types). Haplotype analysis of ABCB4 and ABCB11 SNPs did not reveal any common haplotypes (frequency > 0.01) associated with ICP (p_corr < 0.05; Table 6). Negative results were observed for both the whole sample and after the exclusion of cases with multiple pregnancies (results not shown).

Discussion

In recent years, the association between nucleotide variants of ABCB4 and ABCB11 and liver cholestatic diseases has become increasingly apparent [14]. Research on the genetic aetiology of the development of the disease was also carried out among pregnant women with cholestasis of pregnancy [15].

In 2004, Pauli-Magnus et al. [16] performed in a group of 21 unrelated pregnant women with cholestasis and a control group of 40 healthy pregnant women, an analysis of genetic variants of the ABCB4 gene. The results showed that nearly half of the affected pregnant women have a specific ABCB4 mutation. However, the study of the genetic variants of the BSEP encoding gene (ABCB11) failed to confirm its role in the development of cholestasis of pregnancy.

Floreani et al. [17] also proved the presence of three novel non-synonymous mutations in exon 14 of the MDR3 gene (ABCB4) among 3 of 80 patients suffering from cholestasis of pregnancy (4%) and in none of the healthy women.

In pedigree studies, Schneider et al. [18], after examining 55 relatives, showed splicing mutations in the MDR3 (ABCB4) gene, which can cause cholestasis in pregnancy and may be associated with stillbirths.

In the publication by Eloranta et al. [19] a relation was shown between the existence of cholestasis and the presence of a single nucleotide polymorphism SNP (rs473351) of the ABCB11 gene in the Finnish population (57 affected and 115 healthy individuals).

However, a subsequent study by Painter et al. [20] conducted on a larger group of affected patients (n = 142), also from the Finnish population, failed to confirm these findings, suggesting that ICP is a genetically heterogeneous disease.

In 2009, Dixon et al. [21] published a study of 491 Caucasian pregnant women with ICP and 261 controls, and demonstrated that a single nucleotide polymorphism (c.1331C > T, p.Val444Ala, rs2287622) of the ABCB11 gene might affect hepatic BSEP expression and be a significant risk factor for ICP.

In our study, we analysed six common nucleotide variants of ABCB4 and ABCB11 genes but failed to show any association between them or their haplotypes and the risk of cholestasis development. The allele and genotype frequencies for all tested SNPs were similar in both patients and properly selected controls. In addition, the ABCB4 and ABCB11 variants showed no evidence of association with the severity of this disorder. However, it is worth noting that in the group of patients with moderate and severe ICP, the results for the ABCB4 rs2109505 variant were close to reaching the nominal significance threshold. Under the assumption of an allelic and dominant model, this SNP was associated with a 1.9-fold increase in the risk of ICP. For homozygous carriers of rs2109505, the risk was increased more than 12-fold. A trend towards the association between the ABCB4 rs2109505 variant and cholestasis was also demonstrated after the exclusion of all cases with multiple pregnancies from the statistical calculations. In this case, the presence of rs2109505 in a homozygous form was associated with a 17-fold greater risk for developing ICP.
Dixon et al. [22] demonstrated a connection of the polymorphic variant rs2109505 in the ABCB4 gene with the risk of cholestasis, along with two subsequent nucleotide variants in the ABCB11 gene (rs3815676 and rs7577650). The examination was carried out on a group of 563 pregnant women with cholestasis and 642 healthy pregnant women. This was the largest cohort of pregnant women with ICP examined in relation to genetics. This association was previously reported in a smaller population [23]. The rs2109505 polymorphism is a synonymous variant located at codon 237 (p.Ile237Ile) in exon 8 of the ABCB4 gene. Its contribution to disease risk via a number of different mechanisms were intensively examined. The effect of this SNP on protein function and response to inducing agents was not ascertained. It cannot be excluded that this association exists because of linkage disequilibrium between rs2109505 and a still unidentified pathogenic ABCB4 variant.

The sequencing examination of the selected genes that may be connected to cholestasis showed the presence of 12 ABCB4 mutations, 4 potential mutations of the ABCB11 gene and a donor splice site mutation (intron19) [24].

Wasmuth et al. [13] analysed the association of selected gene variants of gene encoding hepatobiliary transporters for phospholipids (ABCB4) and bile acids (ABCB11) in patients with the severe form of intrahepatic cholestasis of pregnancy in a Swedish cohort. The study, conducted among 52 patients with a TBA level > 40 μmol/L, and 52 pregnant women in the control group, revealed that specific ABCB4 gene haplotypes could represent etiological factors for the development of the severe form of ICP. The authors did not confirm this finding for genetic variants of the ABCB11 gene. Yeap et al. [2] reported nine pregnancies complicated by severe cholestasis (maximum BA level 74–370 μmol/L) in 5 women. They detected two ABCB11 mutations with significant loss of BSEP function and one homo- and four heterozygous mutations in ABCB4.

The limitation of our study is the relatively small group of patients with intrahepatic cholestasis. Identification of cholestasis based on elevated levels of bile acid applies to around 1% of pregnant women in the Caucasian population. Among those who developed cholestasis, there were patients with multiple pregnancies, for whom the mechanism of developing the ailment is most often the result of a significantly elevated level of steroid hormones (oestrogens and sulphate progesterone metabolites) in 2nd and 3rd trimesters [6], although genetic origins of the ailment may not be ruled out in that group. Hence, it is probable that the real percentage of pregnant women for whom nucleotide variants of the ABCB4 and ABCB11 genes may play a role in the ailment’s etiopathogenesis is significantly lower.

In conclusion, our study did not show any significant association of the analysed ABCB4 and ABCB11 nucleotide variants with an increased risk of intrahepatic cholestasis of pregnancy. The negative result may originate from the relatively low number of the analysed patients and controls, as well as the limited number of examined polymorphic variants. Therefore further studies are necessary to confirm the role of ABCB4 and ABCB11 variants in the etiopathology of ICP.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
The study was supported by grant no. 502-01-01110142-05618 from Poznan University of Medical Sciences.

The technical assistance of MSc Justyna Dąbrowska is gratefully acknowledged.

References

1. McIlvride S, Dixon PH, Williamson C. Bile acids and gestation. Mol Aspects Med. 2017 Aug;56:90–100.
2. Yeap SP, Harley H, Thompson R, Williamson KD, Bate J, Sethna F, et al. Biliary transporter gene mutations in severe intrahepatic cholestasis of pregnancy: Diagnostic and management implications. J Gastroenterol Hepatol. 2019 Feb;34(2):425–435.
3. Geenes V, Chappell LC, Seed PT, Steer PJ, Knight M, Williamson C. Association of severe intrahepatic cholestasis of pregnancy with adverse pregnancy outcomes: a prospective population-based case-control study. Hepatology. 2014 Apr;59(4):1482–1491.
4. Kawakita T, Parikh LI, Ramsey PS, Huang CC, Zeymo A, Fernandez M, et al. Predictors of adverse neonatal outcomes in intrahepatic cholestasis of pregnancy. Am J Obstet Gynecol. 2015 Oct;213(4):570.e1–8.
5. Eloranta ML, Heinonen S, Mononen T, Saarikoski S. Risk of obstetric cholestasis in sisters of index patients. Clin Genet. 2001 Jul;60(1):42–45.
6. Webb GJ, Elsharkawy AM, Hirschfield GM. The etiology of intrahepatic cholestasis of pregnancy: towards solving a monkey puzzle. Am J Gastroenterol. 2014 Jan;109(1):85–88.

7. Reyes H, Gonzalez MC, Ribalta J, Aburto H, Matus C, Schramm G, et al. Prevalence of intrahepatic cholestasis of pregnancy in Chile. Ann Intern Med. 1978 Apr;88(4):487–493.

8. Lee RH, Goodwin TM, Greenspoon J, Incerpi M. The prevalence of intrahepatic cholestasis of pregnancy in a primarily Latina Los Angeles population. J Perinatol. 2006 Sep;26(9):527–532.

9. van der Woerd WL, van Mil SW, Stapelbroek JM, Klomp LW, van de Graaf SF, Houwen RH. Familial cholestasis: progressive familial intrahepatic cholestasis, benign recurrent intrahepatic cholestasis and intrahepatic cholestasis of pregnancy. Best Pract Res Clin Gastroenterol. 2010 Oct;24(5):541–553.

10. Pietrek K, Kurzawinska G, Magiedowska J, Drewns K, Barlik M, Malewski Z, et al. The role of ABC transporters’ gene polymorphisms in the etiology of intrahepatic cholestasis of pregnancy. Ginekol Pol. 2018;89(7):393–397.

11. Nicolaou M, Andress EJ, Zolnerciks JK, Dixon PH, Williamsen J, Linton KJ. Canalicular ABC transporters and liver disease. J Pathol. 2012 Jan;226(2):300–315.

12. Anzivino C, Odoardi MR, Meschiari E, Baldelli E, Facchinietti F, Neri I, et al. ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population. Dig Liver Dis. 2013 Mar;45(3):226–232.

13. 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 ABCB4 gene. Gut. 2007 Feb;56(2):265–270.

14. Aamann L, Ørntoft N, Vogel I, Grønbaek H, Becher N, Vilstrup H, et al. Unexplained cholestasis in adults and adolescents: diagnostic benefit of genetic examination. Scand J Gastroenterol. 2018 Mar;53(3):305–311.

15. Reichert MC, Lammert F. ABCB4 Gene Aberrations in Human Liver Disease: An Evolving Spectrum. Semin Liver Dis. 2018 Nov;38(4):299–307.

16. 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. Farmacogenetics. 2004 Feb;14(2):91–102.

17. Floreni A, Carderi I, Paternoster D, Soardo G, Azzaro-Li F, et al. Intrahepatic cholestasis of pregnancy: three novel MDR3 gene mutations. Aliment Farmacol Ther. 2006 Jun;23(11):1649–1653.

18. Schneider G, Paus TC, Kulak-Ublick GA, Meier P, 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 Jan;45(1):150–158.

19. Eloranta ML, Häkki T, Hiltunen M, Heilaska J, 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 Jun;38(6):648–652.

20. Painter JN, Savander M, Sistonen P, Lehesjoki AE, Aittomäki K. A known polymorphism in the bile salt export pump gene is not a risk allele for intrahepatic cholestasis of pregnancy. Scand J Gastroenterol. 2004 Jul;39(7):694–695.

21. Dixon PH, van Mil SW, Chambers J, Strautnieks S, Thompson R, Lammert F, et al. Contribution of variant alleles of ABCB4 to susceptibility to intrahepatic cholestasis of pregnancy. Gut. 2009 Apr;58(4):537–544.

22. Dixon PH, Wadsworth CA, Chambers J, Donnelly J, Cooley S, Buckley R, et al. A comprehensive analy-

---

**Supplementary Table 1. Primers and HRM conditions for genotyping of the ABCB11 and ABCB4 nucleotide variants**

| Gene     | rs no.   | Chromosome location | Alleles | Primers for PCR amplification | PCR product length (bp) | Annealing temp. (°C) | Melt. temp. range (°C) |
|----------|----------|---------------------|---------|-------------------------------|-------------------------|----------------------|------------------------|
| ABCB11   | rs2287622 | chr2:168973818      | C/T     | F: AGCTGTCAATTTCCCTGTT       | 132                     | 55                   | 76–91                  |
|          | 2q31.1   |                     |         | R: CACAAAGCATCTGCACCTG       |                         |                      |                        |
|          | rs3815676 | chr2:169013869      | A/G     | F: GATGCCATTGCCAAGTGA        | 121                     | 55                   | 74–89                  |
|          |          |                     |         | R: TCTCAGGATGAGGCAATTC       |                         |                      |                        |
|          | rs7577650 | chr2:169034700      | A/G     | F: GCCAGCATGAGCTTTCAACAC     | 143                     | 55                   | 70–85                  |
|          |          |                     |         | R: GAAATGGTGCTCTCCACAC       |                         |                      |                        |
| ABCB4    | rs4148826 | chr7:87445103       | A/G     | F: GTCAATCGGCCATCTCAT        | 120                     | 55                   | 70–85                  |
|          | 7q21.12  |                     |         | R: GCCAATGCAATGCTGCTCT       |                         |                      |                        |
|          | rs2109505 | chr7:87450090       | A/T     | F: CATTGCACCCAGAGTCA         | 97                      | 55                   | 74–89                  |
|          |          |                     |         | R: RAAAGGTGTGACCAGTGC        |                         |                      |                        |
|          | rs2302386 | chr7:87462628       | A/G     | F: TTTGCTGGTATTCCCTAC        | 139                     | 58                   | 72–87                  |
|          |          |                     |         | R: TTTGGATATCTGTTGACTCC      |                         |                      |                        |

*a GRCh38/hg38.

*b Underline denotes the minor allele.
sis of common genetic variation around six candidate loci for intrahepatic cholestasis of pregnancy. Am J Gastroenterol. 2014 Jan;109(1):76–84.
23. Müllenbach R, Weber SN, Krawczyk M, Zimmer V, Sarrazin C, Lammert F, et al. A frequent variant in the human bile salt export pump gene ABCB11 is associated with hepatitis C virus infection, but not liver stiffness in a German population. BMC Gastroenterol. 2012 Jun;12:63.
24. Dixon PH, Sambrotta M, Chambers J, Taylor-Harris P, Syngelaki A, Nicolaides K, et al. An expanded role for heterozygous mutations of ABCB4, ABCB11, ATP8B1, ABCC2 and TJP2 in intrahepatic cholestasis of pregnancy. Sci Rep. 2017 Sep;7(1):11823.

25. Gonzalez MC, Reyes H, Arrese M, Figueroa D, Lorca B, Andresen M, et al. Intrahepatic cholestasis of pregnancy in twin pregnancies. J Hepatol. 1989 Jul;9(1):84–90.
26. 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. Europ J Obstet Gynecol Reprod Biol. 2002 Nov;105(2):132–135.

Acceptance for editing: 2019–05–09
Acceptance for publication: 2019–06–29