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

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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 normalisation 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 ($p_{trend} > 0.05$). 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 ($p_{trend} = 0.063$; $OR_{allelic} = 1.87$, 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 canaliculi 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/m^2) 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**.

Statistical analysis

Each SNP was tested for deviation from the Hardy-Weinberg equilibrium (HWE) in both the patients and controls using the chi-square (χ^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 Haploview 4.2 software package (www.broadinstitute.org/haploview/haploview). 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.

Table 1. Characteristics of the *ABCB11* and *ABCB4* nucleotide variants

Gene	rs no.	Location (bp)[a]	Consequence type	Alleles[b]	MAF[c]
ABCB11 2q31.1	**rs2287622**	chr2:168973818	missense (p.Val444Ala)	C / T	0.33
	rs3815676	chr2:169013869	intronic	A / G	0.05
	rs7577650	chr2:169034700	upstream	A / G	0.28
ABCB4 7q21.12	**rs4148826**	chr7:87445103	intronic	A / G	0.17
	rs2109505	chr7:87450090	synonymous (p.Ile237Ile)	A / T	0.17
	rs2302386	chr7:87462628	intronic	A / G	0.13

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

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_{trend} > 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	Alleles[a]	MAF			OR (95%CI); p-value[b]		
			Cases	Controls	p_{trend}-value	Allelic model[c]	Dominant model[d]	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[f]	0.17 (0.01–2.93); 0.128[f]	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[f]
	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[f]
	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[f]
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[f]	0.24 (0.01–4.19); 0.374[f]	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[f]
	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[f]
	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[f]
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[f]
	rs3815676	A / G	0.00	0.02	0.360	0.57 (0.03–9.88); 1.000[f]	0.56 (0.03–9.76); 1.000[f]	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[f]
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[f]
	rs2109505	A / T	0.21	0.13	0.063	1.87 (0.92–3.80); 0.078	1.90 (0.83–4.36); 0.125	12.24 (0.74–201.75); 0.150[f]
	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[f]

[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	–	0.977	0.904
rs2109505	0.857	–	0.875
rs2302386	0.539	0.557	–

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	–	0.115	0.422
rs3815676	0.000	–	0.485
rs7577650	0.152	0.004	–

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	Alleles[a]	MAF			OR (95%CI); p-value[b]		
			Cases	Controls	p_{trend}-value	Allelic model[c]	Dominant model[d]	Recessive model[e]
ICP (n = 67)								
ABCB11	**rs2287622**	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
	rs3815676	A / G	0.00	0.02	0.143	0.22 (0.01–3.85); 0.225[f]	0.22 (0.01–3.80); 0.222[f]	NA
	rs7577650	A / G	0.37	0.40	0.519	0.88 (0.60–1.29); 0.505	0.92 (0.54–1.58); 0.768	0.72 (0.34–1.54); 0.399
ABCB4	**rs4148826**	A / G	0.17	0.14	0.285	1.31 (0.79–2.18); 0.289	1.21 (0.68–2.17); 0.516	3.61 (0.79–16.52); 0.109[f]
	rs2109505	A / T	0.16	0.13	0.310	1.30 (0.77–2.19); 0.331	1.20 (0.67–2.17); 0.539	9.42 (0.84–105.45); 0.084[f]
	rs2302386	A / G	0.12	0.10	0.379	1.30 (0.72–2.35); 0.377	1.33 (0.70–2.53); 0.390	1.54 (0.16–15.04); 0.547[f]
Mild ICP (n = 48)								
ABCB11	**rs2287622**	C / T	0.42	0.42	0.907	0.97 (0.63–1.51); 0.904	0.97 (0.51–1.83); 0.916	0.97 (0.44–2.11); 0.931
	rs3815676	A / G	0.00	0.02	0.214	0.31 (0.02–5.36); 0.375[f]	0.30 (0.02–5.29); 0.372[f]	NA
	rs7577650	A / G	0.39	0.40	0.838	0.95 (0.61–1.48); 0.833	1.14 (0.60–2.14); 0.696	0.66 (0.27–1.64); 0.372
ABCB4	**rs4148826**	A / G	0.16	0.14	0.579	1.18 (0.65–2.15); 0.584	1.07 (0.54–2.12); 0.854	3.37 (0.60–18.92); 0.183[f]
	rs2109505	A / T	0.14	0.13	0.773	1.09 (0.58–2.05); 0.784	1.01 (0.50–2.05); 0.971	6.51 (0.40–105.94); 0.253[f]
	rs2302386	A / G	0.12	0.10	0.523	1.25 (0.63–2.48); 0.519	1.22 (0.57–2.60); 0.608	2.17 (0.22–21.36); 0.440[f]
Moderate and severe ICP (n = 19)								
ABCB11	**rs2287622**	C / T	0.36	0.42	0.479	0.77 (0.38–1.55); 0.463	0.53 (0.20–1.38); 0.186	1.20 (0.38–3.77); 0.761[f]
	rs3815676	A / G	0.00	0.02	0.437	0.79 (0.05–13.73); 1.000[f]	0.77 (0.04–13.59); 1.000[f]	NA
	rs7577650	A / G	0.32	0.40	0.343	0.70 (0.35–1.42); 0.321	0.56 (0.22–1.42); 0.216	0.87 (0.25–3.10); 1.000[f]
ABCB4	**rs4148826**	A / G	0.21	0.14	0.208	1.66 (0.74–3.74); 0.218	1.63 (0.62–4.28); 0.319	4.21 (0.45–39.64); 0.261[f]
	rs2109505	A / T	0.21	0.13	0.112	1.86 (0.82–4.21); 0.131	1.77 (0.67–4.67); 0.241	17.00 (1.02–283.21); 0.113[f]
	rs2302386	A / G	0.13	0.10	0.470	1.43 (0.54–3.81); 0.406[f]	1.61 (0.56–4.66); 0.367[f]	2.20 (0.11–44.18); 1.000[f]

[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 6. Haplotype analysis of the *ABCB11* and *ABCB4* nucleotide variants

Gene	Nucleotide variants	Haplotypes	Frequency	Case, Control Frequencies	χ^2	p-value	p_{corr}-value[a]
ABCB11	**rs2287622_rs3815676**	CA	0.576	0.596, 0.570	0.397	0.528	0.598
		TA	0.412	0.403, 0.414	0.065	0.799	0.880
	rs3815676_rs7577650	AG	0.616	0.665, 0.602	2.254	0.133	0.193
		AA	0.371	0.335, 0.381	1.268	0.260	0.342
	rs2287622_rs3815676_rs7577650	CAG	0.454	0.499, 0.442	1.803	0.179	0.453
		TAA	0.248	0.237, 0.251	0.139	0.710	1.000
		TAG	0.163	0.166, 0.162	0.013	0.910	1.000
		CAA	0.122	0.098, 0.129	1.229	0.268	0.607
ABCB4	**rs4148826_rs2109505**	AT	0.855	0.835, 0.861	0.761	0.383	0.810
		GA	0.127	0.153, 0.119	1.435	0.231	0.462
		GT	0.015	0.012, 0.016	0.195	0.659	1.000
	rs2109505_rs2302386	TA	0.860	0.846, 0.864	0.362	0.547	0.942
		AG	0.087	0.113, 0.080	1.925	0.165	0.606
		AA	0.042	0.040, 0.043	0.025	0.874	1.000
		TG	0.011	0.001, 0.014	2.128	0.145	0.510
	rs4148826_rs2109505_rs2302386	ATA	0.847	0.834, 0.851	0.288	0.591	1.000
		GAG	0.087	0.113, 0.080	1.923	0.166	0.541
		GAA	0.040	0.040, 0.040	0.002	0.969	1.000
		GTA	0.013	0.012, 0.013	0.016	0.899	1.000

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

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 genotyping 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.

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

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

Gene	rs no.	Chromosome location[a]	Alleles[b]	Primers for PCR amplification (5'–3')	PCR product length (bp)	Annealing temp. (°C)	Melt. temp. range (°C)
ABCB11 2q31.1	**rs2287622**	**chr2:168973818**	C / T	F: AGCTGTCATTTCCCCTGGT R: CACAAAGCATCTGCACCTGT	132	55	76–91
	rs3815676	**chr2:169013869**	A / G	F: GATGCCATTGCCAAGTAGA R: TCTCAGGATGGAGGCATTTC	121	55	74–89
	rs7577650	**chr2:169034700**	A / G	F: GCCAGCATGAGTCAGTTAACAC R: GAAATTGTGTCCTTCCACACAG	143	55	74–89
ABCB4 7q21.12	**rs4148826**	**chr7:87445103**	A / G	F: GTCACATTCTGGCATTCAT R: GCCTTGCAAATGTTGCTCT	120	55	70–85
	rs2109505	**chr7:87450090**	A / T	F: CTTTGTCACTAAATGCCGAGA R: TAAAGGGTTGACCAGAGTGC	97 analysis without and with spiking DNA	55	74–89
	rs2302386	**chr7:87462628**	A / G	F: TTCCTGTGTATTTCCTTCACC R: TTTGGATATCTGGTTGACTCC	139	58	72–87

[a] GRCh38 / hg38.
[b] Underline denotes the minor allele.

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