Physiol. Res. 67 (Suppl. 3): S499-S510, 2018

May Circulating Steroids Reveal a Predisposition to Intrahepatic Cholestasis of Pregnancy in Non-Pregnant Women?

P. ŠIMJÁK[1], M. HILL[2], A. PAŘÍZEK[1], L. VÍTEK[3], M. VELÍKOVÁ[2], M. DUŠKOVÁ[2], R. KANCHEVA[2], J. BULANT[2,4], M. KOUCKÝ[1], Z. KOKRDOVÁ[1], K. ADAMCOVÁ[1], A. ČERNÝ[1], Z. HÁJEK[1], L. STÁRKA[2]

[1]Department of Gynecology and Obstetrics, General University Hospital and First Faculty of Medicine, Charles University in Prague, Czech Republic, [2]Institute of Endocrinology, Prague, Czech Republic, [3]Institute of Medical Biochemistry and Laboratory Diagnostics and Fourth Department of Internal Medicine, General University Hospital and First Faculty of Medicine of Charles University in Prague, Czech Republic, [4]Department of Pediatrics and Adolescent Medicine, General University Hospital and First Faculty of Medicine of Charles University in Prague, Czech Republic

Received August 28, 2018
Accepted September 14, 2018

Summary

Intrahepatic cholestasis of pregnancy (ICP) is a frequent liver disorder, mostly occurring in the third trimester. ICP is not harmful to the mothers but threatens the fetus. The authors evaluated steroid alterations in maternal and mixed umbilical blood to elucidate their role in the ICP development. Ten women with ICP were included in the study. Steroids in the maternal blood were measured by Gas Chromatography-Mass Spectrometry (GC-MS) (n=58) and RIA (n=5) at the diagnosis of ICP, labor, day 5 postpartum, week 3 postpartum and week 6 postpartum. The results were evaluated by ANOVA consisting of the subject factor, between subject factors ICP, gestational age at the diagnosis of ICP and gestational age at labor, within-subject factor Stage and ICP × Stage interaction. The 17 controls were firstly examined in the week 36 of gestation. ICP patients showed reduced CYP17A1 activity in the C17,20 lyase step thus shifting the balance between the toxic conjugated pregnanediols and harmless sulfated 5α/β-reduced-17-oxo C19 steroids. Hence, more toxic metabolites originating in maternal liver from the placental pregnanes may penetrate backward to the fetal circulation. As these alterations persist in puerperium, the circulating steroids could be potentially used for predicting the predisposition to ICP even before next pregnancy.

Key words

Intrahepatic cholestasis of pregnancy • Liver enzymes • Steroid metabolism • Gas chromatography-mass spectrometry • Prediction

Corresponding author

M. Hill, Department of Steroids and Proteohormones, Institute of Endocrinology, Národní 8, 116 94, Prague, 116 94, Czech Republic. E-mail: mhill@endo.cz

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is the most frequent liver disorder in pregnancy. The disease is associated with an altered biliary secretion of bile acids (BA) and other biliary lipids and usually induces placental oxidative stress and apoptosis. Although ICP is associated with a discomfort in pregnant women, it does not seriously harm them. Instead, ICP represents a serious risk and difficult-to-predict complications in the fetus including preterm birth, meconium flux into the amniotic fluid, respiratory distress syndrome, and sudden intrauterine fetal death (Simjak *et al.* 2014). The risk of ICP rises with escalating levels of pregnancy steroids during the third trimester of pregnancy. Furthermore, the risk of ICP is higher in multiple pregnancies with multiple sources of fetal steroid precursors such as sulfated C21 and C19 Δ^5 steroids (Parizek *et al.* 2015). These steroid sulfates enter the placenta, which desulfates them and converts the unconjugated steroids to progestogens and estrogens. Placental progestogens may be converted to their 5α-reduced metabolites and, after

entering the maternal circulation, these substances may be subsequently sulfated and/or glucuronized in the maternal liver. As we have previously reported, the conjugation of 5α/β-reduced-20-oxo-pregnanes in the maternal compartment increases with the approaching term (Hill *et al.* 2010a). During enterohepatic circulation, both progesterone and estrogen metabolites may be transformed into cholestatic compounds (Reyes 2008). Although the maternal liver plays a key role in the catabolism of maternal progesterone, the formation and catabolism of female sex hormones is not limited to the maternal liver, ovaries, and adrenals. There is a bidirectional flux to the fetal-placental unit and the fetal adrenal and placenta synthesize a substantial part of the substrates for the production of active hormones and consequently their noxious catabolites. About half of the progesterone metabolism occurs in extrahepatic sites (Hill *et al.* 2010a, Reyes 2016). In ICP patients, the sulfated progesterone catabolites are increased 4-10 times when compared with healthy pregnant women, while glucuronidated metabolites remain unaffected by either pregnancy or ICP. The patients also show an increased ratio of 3α- to 3β-hydroxysteroid sulfates in serum (Reyes 2016). ICP symptoms withdraw shortly after birth as the placental conversion of fetal steroids disappears, and the activity of the fetal adrenal zone declines (Glantz *et al.* 2008, Lammert *et al.* 2000).

ICP is probably associated with an overproduction of sulfated progesterone metabolites such as pregnanolone isomers and pregnanediols and the pathophysiology of ICP may be influenced by enzymes interconverting the 3β-hydroxy, 3-oxo-, and 3α-hydroxy-pregnanes and by selective defect in steroid sulfate conjugation affecting the biliary secretion of steroid metabolites (Glantz *et al.* 2008, Meng *et al.* 1997). The key role of progesterone catabolism in the pathophysiology of ICP is also shown by reduced progesterone levels and elevated concentrations of its metabolites in women with asymptomatic hypercholanemia, which is accompanied by moderately elevated total bile acids (TBA) (>11 μM) but normal aminotransferases (Pascual *et al.* 2002). The increased levels of sulfated pregnanediols may also predict the ICP onset (Abu-Hayyeh *et al.* 2016, Parizek *et al.* 2016) and the overproduction of conjugated progesterone catabolite isopregnanolone (3β,5α-THP) sulfate inhibits the *de novo* synthesis of hepatic farnesoid X receptor (FXR), which may result in a cholestatic phenotype (Abu-Hayyeh *et al.* 2013, Rizzo *et al.* 2005). Data from animal models reveal that estradiol and reduced pregnane steroids inhibit the bile salt export pump (BSEP), which is responsible for bile acids (BAs) secretion while the progesterone itself is inactive (Byrne *et al.* 2002, Vallejo *et al.* 2006).

In the mild ICP, some reports indicate that the fetuses of mothers with ICP have elevated circulating cortisol and dehydroepiandrosterone sulfate (DHEAS) probably due to increased activity of both the maternal *zona fasciculata* and *zona reticularis*. However, in severe ICP, there is a suppressed stress response system (Wang *et al.* 2011) and other authors record lower levels of DHEAS and estrogens in women with ICP (Leslie *et al.* 2000).

Although the aforementioned data indicate an important role for steroids in the pathophysiology of ICP, there is still a number of questions to be addressed. In our previous study, we evaluated the differences between maternal circulating steroids in women with ICP before the beginning of the therapy by ursodeoxycholic acid (UDCA) to assess potential importance of maternal circulating steroids for the diagnosis of ICP (Parizek *et al.* 2016). Here we followed the time profiles of maternal circulating steroids from late pregnancy to week 6 postpartum to explain the associations of individual steps of steroid metabolism (during pregnancy, at labor and in postpartum period) with the etiopathogenesis of ICP to examine whether the steroidogenesis in patients remains altered in the postpartum period. This may be helpful for the discovery of potential markers of ICP predisposition in non-pregnant women.

Methods

Subjects

Ten pregnant women with ICP were included in the study. The mean gestational age at the onset of ICP was 35.8±1.7 weeks (range 29-38). All women with ICP had increased TBA levels (≥8 μmol/l), 90 % of them exhibited elevated aminotransferases and half of them suffered from pruritus. Other causes of cholestasis were excluded by detailed clinical and laboratory examinations. Four cases of severe ICP (TBA>40 μmol/l) were recorded. All pregnancies were singleton. Gestational diabetes mellitus was an exclusion criterion for both controls and patients. The control group consisted of 17 women with uncomplicated singleton pregnancies, who underwent laboratory examination in the week 36 of pregnancy. No case of maternal pruritus was recorded and serum aminotransferase activities and

TBA were normal in all controls. At delivery, the mean gestational age of patients with ICP and controls was 37.5±0.7 and 39.4±1.1 weeks, respectively. Pregnancy ended prematurely in one case of ICP (10 %), whereas in the control group no case of delivery before 37 weeks of pregnancy occurred. From the total of ICP-complicated pregnancies, 40 % were terminated by cesarean section, in contrast to 35 % in the control group. The mean APGAR score at the first minute was 9.3±0.6 and 9.2±0.8, and at the fifth minute was 10.0±0.0 and 9.7±0.5 for the ICP and control groups, respectively. From the ICP patients, 7 were treated with 750-1000 mg UDCA/day for 3-14 days after the first blood collection (stage "Dg") and 3 patients did not use UDCA. The Ethics Committee of the General University Hospital and 1st Faculty of Medicine of Charles University in Prague approved this study. Written informed consent was obtained from all study participants before any study related procedures had been carried on.

Sample collection

In the ICP patients, the blood samples were firstly collected in the 3rd trimester after diagnosis of ICP and before starting the treatment with UDCA while the controls were firstly examined in the week 36 of gestation (stage (Dg)). Both groups were further monitored at labor (Lab), day 5 postpartum (d5), week 3 postpartum (wk3) and week 6 postpartum (wk6). The treatment with UDCA in ICP patients was ceased immediately after labor. Serum was obtained after centrifugation for 5 min at 2,000×g at 0 °C and stored at -20 °C until analyzed.

Liver function tests

Standard serum biochemical markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP) and bilirubin were determined on an automatic analyzer (Modular analyzer, Roche Diagnostics GmbH, Mannheim, Germany), using routine laboratory assays. The TBA were quantified using an EIA kit from Trinity Biotech USA Inc. (Jamestown, NY, USA).

Steroid analysis

The levels of 38 unconjugated steroids and 25 steroid conjugates were measured in the maternal serum and serum from mixed umbilical blood. The steroid metabolome included C21 Δ^5 steroids, C19 Δ^5 steroids, C21 Δ^4 steroids, C19 Δ^4 steroids, estrogens, C21 5α/β-reduced steroids and C19 5α/β-reduced steroids. Most of the steroids were measured by GC-MS using our previously published method (for details see Hill *et al.* 2010c). However, instead of the system GCMS-QP2010 Plus we used the GCMS-TQ8040 in this study (both systems were from Shimadzu (Kyoto, Japan)). The analysis was conducted in Q3-SIM mode. 17-hydroxypregnenolone and 17-hydroxypregnenolone sulfate were measured using our previously published RIA methods (Hill *et al.* 1999, Vcelakova *et al.* 2007) while 17-hydroxyprogesterone, cortisol, and testosterone were quantified by RIA kits from Immunotech (Marseille, France).

Statistical analysis

The differences in levels of steroids and liver function test during late gestation, at labor, and during three periods postpartum (day 5, week 3, and week 6 postpartum) were evaluated using a mixed-design analysis of variance (ANOVA) model. In this model, the fixed effects factors were between-subjects variables and the stage effect was a within-subjects variable (all subjects were measured in all periods). The between-subject factors were ICP (ICP positive vs. controls), gestational age at the diagnosis of ICP (GA, Dg: <week 36 vs. ≥ week 36 of gestation, samples from controls were collected in week 36 of gestation) and gestational age at labor (GA, labor: <week 38 vs. ≥week 38 of gestation). The factors GA, Dg and GA, labor were incorporated into the model to control for changing steroid levels in the fetomaternal circulation during gestation (Hill *et al.* 2010a, Hill *et al.* 2014). The within-subject factor was Stage (diagnosis of ICP, labor, day 5 postpartum, week 3 postpartum, week 6 postpartum) and the model also included the ICP × Stage interaction testing the significance of divergence between time profiles of controls and patients. The mixed-design ANOVA model was followed by least significant difference multiple comparisons. Respecting the skewed data distribution and non-constant variance in most dependent variables, these were transformed by power transformations to achieve data symmetry and homoscedasticity prior to further data processing (Meloun *et al.* 2000). The homogeneity and distribution of the transformed data were checked by residual analysis as described elsewhere (Meloun *et al.* 2002). The statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA) was used for the ANOVA testing.

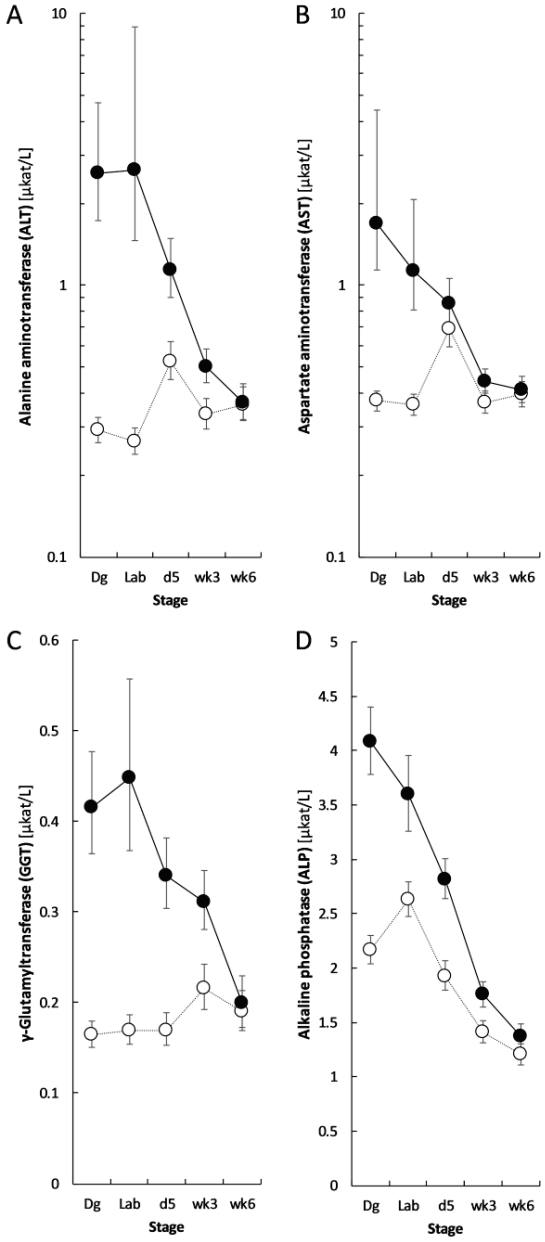

Fig. 1. Profiles of liver function tests in late pregnancy, at labor and postpartum in women with ICP and controls. Full and empty circles with error bars represent the re-transformed means with their 95 % confidence intervals for ICP patients and controls, respectively. The differences in levels of steroids and liver function test during late gestation, at labor, and during three periods postpartum (day 5, month 3, and month 6 postpartum) were evaluated using ANOVA model consisting of between-subject factors ICP (ICP positive vs. controls), gestational age at the diagnosis of ICP (GA, Dg: <week 36 vs. ≥week 36 of gestation, samples from controls were collected in week 36 of gestation) and gestational age at labor (GA, labor: <week 38 vs. ≥week 38 of gestation), within-subject factor Stage and ICP×Stage interaction. Respecting the skewed data distribution and non-constant variance in most dependent variables, these were transformed by power transformations to achieve data symmetry and homoscedasticity prior to further data processing. Statistical significance of the factors and interaction was as follows: **A**) ICP: $F=115.5$, $p<0.001$; Stage: $F=15.2$, $p<0.001$; ICP×Stage: $F=22.3$, $p<0.001$; **B**) ICP: $F=51.4$, $p<0.001$; ICP×Stage: $F=14.4$, $p<0.001$; **C**) ICP: $F=90.5$, $p<0.001$; ICP×Stage: $F=9.8$, $p<0.001$; **D**) ICP: $F=77.4$, $p<0.001$; ICP×Stage: $F=8.7$, $p<0.001$.

Results

The profiles of circulating C21 steroids in women with intrahepatic cholestasis of pregnancy (ICP) and controls (C) at the diagnosis of ICP in the 3rd trimester (Dg), at labor (Lab), day 5 postpartum (d5), week 3 postpartum (wk3) and week 6 postpartum (wk6) are shown in Table 1 as well as the corresponding data for C19 steroids and estrogens. Liver function tests show pronounced between-group differences in pregnancy, but these differences diminish d5 and wk3 and disappear in wk6. As expected, all profiles of liver function tests exhibit significant ICP × Stage interaction demonstrating significantly different courses of the corresponding profiles for controls and patients (Fig. 1). Alternatively, the steroid profiles, as well as ratios of C21 to C19 steroids, keep the between-group differences from stage Dg up to stage wk6 postpartum (Table 1, Figs 2 and 3). Only the profile of conjugated 5α-pregnane-3α,20α-diol shows a significant interaction ICP × Stage indicating different courses of time profiles for ICP group and controls (steeper fall within stages Lab and wk6 in the patients, Table 1). While the levels of C21 steroids tend to higher levels in the patients, the concentrations of C19 steroids are mostly lower (Table 1, Fig. 2) in this group. Moreover, several profiles of C21 to C19 steroid ratios are also regularly shifted to higher values in the patients when compared to controls (Fig. 3). Estrogen profiles mostly do not significantly differ except the levels of estrone sulfate showing a significant shift to lower values and estriol sulfate levels showing the opposite trend (Table 1). In the mixed umbilical blood of ICP patients, we found higher levels of pregnenolone, conjugated and unconjugated 5α-pregnane-3α,20α-diol and cortisol but lower levels of androgen metabolites epiandrosterone, etiocholanolone sulfate and estrogens estrone and estradiol sulfate (Table 2).

Discussion

In contrast to the liver function tests showing divergent time profiles in patients and controls due to fading concentrations of noxious substances in the former group (Fig. 1), the profiles of steroid levels (Table 1, Fig. 2) and steroid ratios (Fig. 3) were less divergent or parallel particularly in sulfated C19 steroids. This may indicate permanently impaired steroidogenesis in women with ICP irrespectively of the pregnancy status and a possible use of the circulating steroids for estimation of predisposition to ICP even in nonpregnant women.

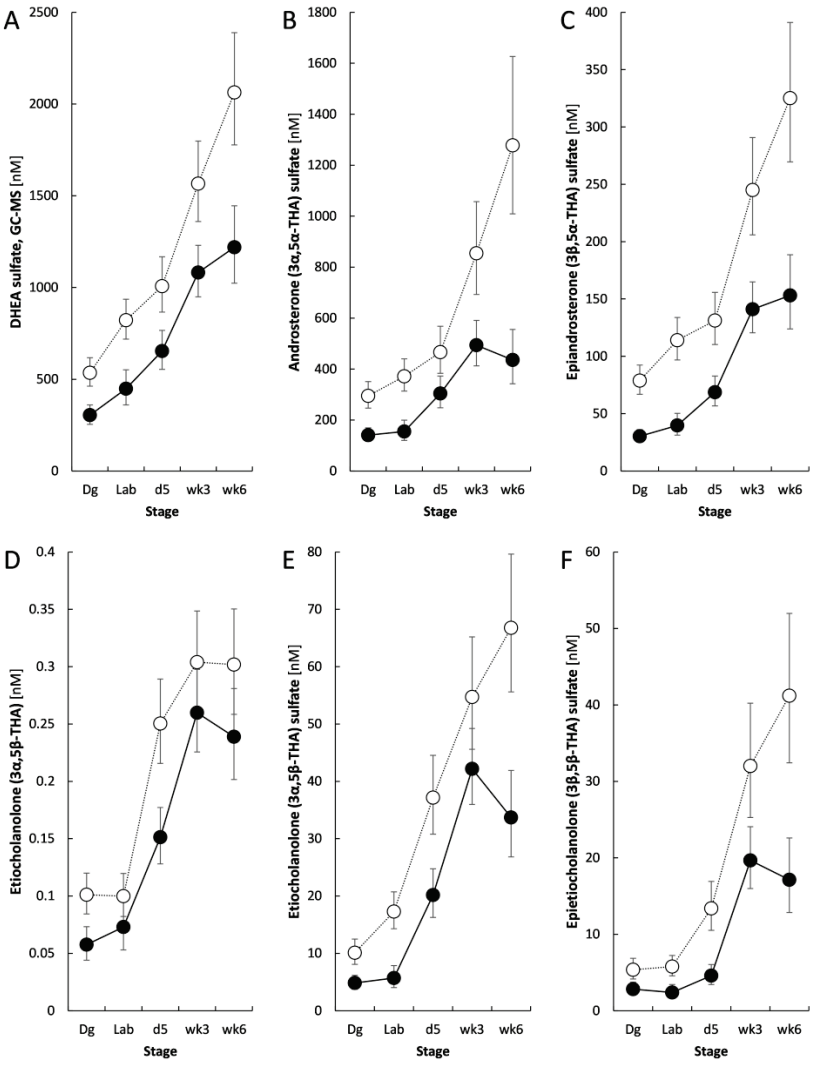

Fig. 2. Profiles of 17-oxo-C19 steroids showing significant differences between women with ICP and controls in late pregnancy, at labor and postpartum. The statistical method, symbols, and drawings are the same as for Figure 1. Statistical significance of the factors and interaction was as follows: **A**) ICP: F=37.8, p<0.001; ICP×Stage: F=0.3, p=0.876; **B**) ICP: F=47.2, p<0.001; ICP×Stage: F=1.6, p=0.197; **C**) ICP: F=69.5, p<0.001; ICP×Stage: F=1.1, p=0.346; **D**) ICP: F=14.1, p<0.001; ICP×Stage: F=0.9, p=0.485; **E**) ICP: F=37.2, p<0.001; ICP×Stage: F=1.5, p=0.216; **F**) ICP: F=34.9, p<0.001; ICP×Stage: F=0.9, p=0.463.

UDCA treatment in most of our ICP-patients after diagnosis of ICP is an important confounding factor for the steroid metabolism between stages Dg and Lab. Nevertheless, the first stage Dg was not influenced by UDCA as the blood withdrawal was performed before the beginning of medication and a similar situation was in postpartum periods when the excessive formation of progesterone was terminated at labor and the UDCA treatment was finished. Concerning the situation after the beginning of UDCA medication to labor, some authors did not found a significant influence of UDCA on steroid metabolism (Estiu *et al.* 2015) and others reported a suppressed synthesis of 5α/β-reduced progesterone metabolites (Abu-Hayyeh *et al.* 2016). In the present study, the levels of 5α/β-reduced progesterone metabolites at labor remain increased despite the UDCA treatment. The small number of ICP patients untreated by UDCA (n=3) does not allow an exact comparison whether the androgen levels significantly differ between UDCA-treated and UDCA-untreated ICP patients (n=7) and no data is available in the literature concerning the UDCA effect on conjugated C19 steroids. However, the circulating levels of sulfated androgens in untreated ICP patients at labor are apparently unaltered when compared with the UDCA-treated patients. When considering data from others, the pregnane levels in UDCA-untreated ICP patients would be either unaltered or even higher than in the treated ICP patients (Abu-Hayyeh *et al.* 2016, Estiu *et al.* 2015). Therefore, the steroid alterations in the UDCA-untreated ICP patients at labor against the control group would be either comparable or even more explicit than in the case of UDCA-treated women. Thus, our most important finding of permanent alterations in maternal steroidogenesis in ICP patients is not derived from the UDCA treatment but from the predisposition of a part of the female population to ICP due to an attenuated activity of *zona reticularis*.

Table 1. Steroids (nM) quantified in the circulation of women with intrahepatic cholestasis of pregnancy (ICP) and controls (C) at the diagnosis of ICP in the 3rd trimester, at labor, day 5 postpartum, week 3 postpartum and week 6 postpartum. Only the steroids with a significant effect of ICP are shown.

Steroid		Stage					ANOVA
		Diagnosis of ICP	Labor	Day 5	Week 3	Week 6	
17-hydroxypregnenolone sulfate	C	8.7 (6.7, 11)	9.9 (7.7, 13)	4.9 (3.4, 6.9)	5.8 (4.1, 8)	8 (5.5, 11)	ICP: F=6.5, p=0.013; Stage: F=4.1, p=0.005; ICP×Stage: F=0.1, p=0.969
	ICP	14 (11, 17)	15 (11, 20)	7.9 (5.8, 10)	8.7 (6.6, 11)	9.6 (6.9, 13)	
Progesterone	C	260 (210, 330)	130 (110, 160)	3.3 (2.7, 4)	0.85 (0.67, 1.1)	0.56 (0.42, 0.74)	ICP: F=13.6, p<0.001; Stage: F=774.3, p<0.001; ICP×Stage: F=1.5, p=0.21
	ICP	290 (230, 360)	280 (210, 390)	5.5 (4.5, 6.6)	1.1 (0.92, 1.4)	0.97 (0.74, 1.2)	
20α-dihydroprogesterone	C	55 (47, 65)	59 (50, 70)	3.5 (2.8, 4.3)	0.56 (0.38, 0.77)	0.55 (0.36, 0.79)	ICP: F=65.8, p<0.001; Stage: F=489.6, p<0.001; ICP×Stage: F=0.9, p=0.477
	ICP	110 (91, 130)	140 (110, 170)	10 (8.2, 12)	2.1 (1.7, 2.6)	1.4 (0.99, 1.8)	
16α-hydroxyprogesterone	C	20 (15, 26)	15 (12, 20)	0.79 (0.61, 1)	0.52 (0.39, 0.67)	0.56 (0.42, 0.74)	ICP: F=6.9, p=0.011; Stage: F=213.5, p<0.001; ICP×Stage: F=0.4, p=0.838
	ICP	27 (20, 36)	30 (20, 48)	1.2 (0.95, 1.5)	0.63 (0.48, 0.82)	0.79 (0.59, 1)	
5β-dihydroprogesterone	C	0.81 (0.68, 0.99)	0.51 (0.43, 0.61)	0.16 (0.14, 0.19)	0.15 (0.12, 0.17)	0.13 (0.11, 0.16)	ICP: F=11.7, p=0.001; Stage: F=75.6, p<0.001; ICP×Stage: F=1.4, p=0.23
	ICP	0.87 (0.71, 1.1)	0.68 (0.51, 0.92)	0.21 (0.17, 0.24)	0.23 (0.19, 0.27)	0.23 (0.19, 0.28)	
5α-pregnane-3α,20α-diol (3α,5α-PD)	C	13 (11, 15)	12 (11, 14)	4.5 (3.6, 5.6)	2 (1.5, 2.5)	1.3 (0.93, 1.8)	ICP: F=10.3, p=0.002; Stage: F=95, p<0.001; ICP×Stage: F=0.5, p=0.757
	ICP	16 (14, 19)	17 (14, 21)	5.1 (4, 6.3)	3.1 (2.5, 3.9)	2.5 (1.8, 3.3)	
Conjugated 5α-pregnane-3α,20α-diol	C	4900 (4100, 5900)	5600 (4800, 6700)	410 (330, 500)	30 (22, 41)	24 (16, 34)	ICP: F=31.5, p<0.001; Stage: F=720.2, p<0.001; ICP×Stage: F=5.8, p<0.001
	ICP	12000 (10000, 15000)	16000 (12000, 20000)	900 (730, 1100)	44 (34, 57)	21 (13, 31)	
Conjugated 5α-pregnane-3β,20α-diol	C	10000 (8200, 13000)	9800 (7900, 12000)	1700 (1300, 2300)	83 (47, 130)	60 (29, 100)	ICP: F=12.7, p<0.001; Stage: F=316, p<0.001; ICP×Stage: F=1.2, p=0.319
	ICP	19000 (15000, 23000)	23000 (17000, 30000)	2200 (1600, 2900)	150 (100, 210)	110 (59, 180)	
Conjugated 5β-pregnane-3β,20α-diol (3β,5β-PD)	C	820 (680, 980)	930 (780, 1100)	290 (230, 380)	21 (13, 32)	19 (12, 30)	ICP: F=7.5, p=0.008; Stage: F=187.9, p<0.001; ICP×Stage: F=1.2, p=0.313
	ICP	1100 (920, 1300)	1300 (980, 1600)	280 (210, 360)	40 (29, 55)	57 (38, 84)	
DHEA sulfate	C	540 (460, 620)	820 (720, 940)	1000 (870, 1200)	1600 (1400, 1800)	2100 (1800, 2400)	ICP: F=37.8, p<0.001; Stage: F=59.4, p<0.001; ICP×Stage: F=0.3, p=0.876
	ICP	300 (250, 360)	450 (360, 550)	650 (550, 770)	1100 (950, 1200)	1200 (1000, 1400)	
16α-hydroxy-DHEA	C	0.81 (0.58, 1.1)	0.56 (0.41, 0.78)	0.15 (0.11, 0.22)	0.13 (0.088, 0.18)	0.14 (0.092, 0.2)	ICP: F=13.7, p<0.001; Stage: F=25, p<0.001; ICP×Stage: F=0.3, p=0.907
	ICP	0.32 (0.23, 0.44)	0.35 (0.22, 0.56)	0.077 (0.054, 0.11)	0.068 (0.049, 0.095)	0.062 (0.041, 0.094)	
16α-hydroxy-DHEA sulfate	C	8.1 (6.2, 11)	15 (11, 19)	4.6 (3.4, 6.2)	6 (4.4, 8.3)	3.4 (2.5, 4.8)	ICP: F=10.2, p=0.002; Stage: F=16, p<0.001; ICP×Stage: F=1.7, p=0.17
	ICP	5.6 (4.3, 7.4)	11 (7.8, 17)	3.2 (2.4, 4.4)	1.9 (1.4, 2.5)	2.1 (1.5, 3.1)	

Table 1. (continued)

Steroid		Diagnosis of ICP	Stage: Labor	Stage: Day 5	Week 3	Week 6	ANOVA
Androstenediol	C	0.48 (0.42, 0.56)	0.87 (0.75, 1)	0.91 (0.76, 1.1)	0.87 (0.73, 1.1)	0.91 (0.74, 1.1)	ICP: F=5.9, p=0.018; Stage: F=9.7, p<0.001; ICP×Stage: F=1, p=0.424
	ICP	0.46 (0.39, 0.54)	0.59 (0.48, 0.73)	0.77 (0.65, 0.93)	0.79 (0.67, 0.94)	0.61 (0.5, 0.74)	
5-androstene-3β,7β,17β-triol	C	0.069 (0.058, 0.083)	0.087 (0.073, 0.1)	0.063 (0.052, 0.078)	0.092 (0.075, 0.11)	0.11 (0.091, 0.15)	ICP: F=10.4, p=0.002; Stage: F=3.3, p=0.016; ICP×Stage: F=0.8, p=0.537
	ICP	0.059 (0.049, 0.071)	0.071 (0.054, 0.093)	0.047 (0.039, 0.057)	0.055 (0.046, 0.067)	0.065 (0.052, 0.082)	
Androsterone (3α,5α-THA)	C	0.33 (0.29, 0.38)	0.36 (0.32, 0.42)	0.34 (0.29, 0.4)	0.21 (0.19, 0.24)	0.3 (0.25, 0.35)	ICP: F=11, p=0.001; Stage: F=10, p<0.001; ICP×Stage: F=0.4, p=0.804
	ICP	0.25 (0.22, 0.29)	0.26 (0.22, 0.32)	0.3 (0.26, 0.35)	0.18 (0.16, 0.2)	0.21 (0.18, 0.25)	
Androsterone sulfate	C	290 (250, 350)	370 (310, 440)	470 (380, 570)	850 (690, 1100)	1300 (1000, 1600)	ICP: F=47.2, p<0.001; Stage: F=36.3, p<0.001; ICP×Stage: F=1.6, p=0.197
	ICP	140 (120, 170)	160 (120, 200)	300 (250, 370)	490 (410, 590)	440 (340, 560)	
Epiandrosterone sulfate	C	79 (67, 92)	110 (97, 130)	130 (110, 160)	240 (210, 290)	330 (270, 390)	ICP: F=69.5, p<0.001; Stage: F=58.1, p<0.001; ICP×Stage: F=1.1, p=0.346
	ICP	30 (25, 36)	40 (31, 50)	69 (57, 83)	140 (120, 160)	150 (120, 190)	
Etiocholanolone (3α,5β-THA)	C	0.1 (0.084, 0.12)	0.1 (0.082, 0.12)	0.25 (0.22, 0.29)	0.3 (0.26, 0.35)	0.3 (0.26, 0.35)	ICP: F=14.1, p<0.001; Stage: F=53.2, p<0.001; ICP×Stage: F=0.9, p=0.485
	ICP	0.058 (0.044, 0.073)	0.073 (0.053, 0.097)	0.15 (0.13, 0.18)	0.26 (0.23, 0.3)	0.24 (0.2, 0.28)	
Etiocholanolone sulfate	C	10 (8.1, 12)	17 (14, 21)	37 (31, 45)	55 (46, 65)	67 (56, 80)	ICP: F=37.2, p<0.001; Stage: F=82.4, p<0.001; ICP×Stage: F=1.5, p=0.216
	ICP	4.8 (3.7, 6.2)	5.7 (4, 7.9)	20 (16, 25)	42 (36, 49)	34 (27, 42)	
Epietiocholanolone (3β,5β-THA) sulfate	C	5.4 (4.2, 6.9)	5.8 (4.6, 7.2)	13 (11, 17)	32 (25, 40)	41 (32, 52)	ICP: F=34.9, p<0.001; Stage: F=61.3, p<0.001; ICP×Stage: F=0.9, p=0.463
	ICP	2.8 (2.1, 3.7)	2.4 (1.6, 3.4)	4.6 (3.4, 6.1)	20 (16, 24)	17 (13, 23)	
Estrone sulfate	C	260 (190, 340)	310 (240, 410)	4 (2.9, 5.4)	0.71 (0.5, 0.99)	0.94 (0.66, 1.3)	ICP: F=18.2, p<0.001; Stage: F=377.3, p<0.001; ICP×Stage: F=1.4, p=0.26
	ICP	130 (94, 170)	170 (110, 250)	1.1 (0.77, 1.5)	0.33 (0.22, 0.48)	0.61 (0.4, 0.9)	
Estriol sulfate	C	43 (31, 61)	58 (41, 81)	1.7 (1.2, 2.4)	0.34 (0.22, 0.52)	0.25 (0.15, 0.39)	ICP: F=4.6, p=0.036; Stage: F=227.3, p<0.001; ICP×Stage: F=0.3, p=0.871
	ICP	79 (56, 110)	120 (73, 190)	2.5 (1.7, 3.6)	0.49 (0.34, 0.69)	0.29 (0.17, 0.47)	

ICP and C – ICP patients and controls, respectively, F – F-statistic, p – statistical significance of factors in ANOVA model.

Table 2. Steroids (nM) in mixed umbilical blood from patients with intrahepatic cholestasis of pregnancy (ICP) and controls (C) at labor.

Variable	Group		ANOVA	
	C	ICP	F	p
Pregnenolone	48 (38, 63)	31 (27, 37)	6.7	0.018
Cortisol	280 (190, 420)	130 (100, 180)	5.9	0.025
Estrone	130 (100, 180)	54 (40, 71)	11	0.004
Estradiol sulfate	7 (5.6, 8.5)	3.9 (3, 4.8)	7.9	0.011
5α-pregnane-3α,20α-diol	4.27 (2.96, 5.94)	7.6 (5.98, 9.55)	4.8	0.041
Conjugated 5α-pregnane-3α,20α-diol	859 (613, 1180)	3900 (3020, 5080)	31.2	<0.001
Epiandrosterone	0.209 (0.157, 0.289)	0.132 (0.107, 0.163)	4.6	0.046
Etiocholanolone sulfate	1.93 (1.45, 2.53)	0.921 (0.709, 1.18)	9.2	0.007

ICP and C – ICP patients and controls, respectively, F – F-statistic, p – statistical significance of factors in ANOVA model.

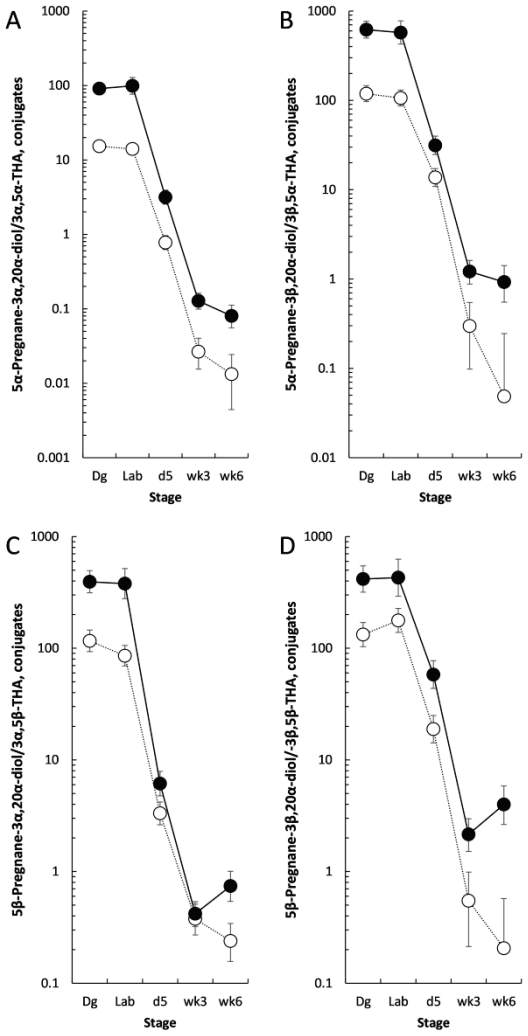

Fig. 3. Profiles of ratios of conjugated pregnanediols to corresponding conjugated 17-oxo-5α/β-reduced androstanes in late pregnancy, at labor and postpartum in women with ICP and controls. The statistical method, symbols, and drawings are the same as for Figure 1. Statistical significance of the factors and interaction was as follows: **A**) ICP: F=155.1, p<0.001; ICP×Stage: F=4.6, p=0.002; **B**) ICP: F=84.4, p<0.001; ICP×Stage: F=4.4, p=0.003; **C**) ICP: F=43.4, p<0.001; ICP×Stage: F=5.4, p<0.001; **D**) ICP: F=51.6, p<0.001; ICP×Stage: F=1.1, p=0.346.

Based on the present results and data from others (Biason-Lauber *et al.* 2013, Kamrath *et al.* 2013), we completed a diagram illustrating the formation and transplacental transport of conjugated pregnanediols in pregnancy (Fig. 4). We hypothesize that pregnenolone sulfate of fetal origin is converted to progesterone in placenta and subsequently also to 20α-dihydroprogesterone during the transplacental passage. These steroids together with a part of placental progesterone synthesized from the maternal LDL cholesterol (Hill *et al.* 2010a, Hill *et al.* 2010b) penetrate into the maternal compartment. Maternal liver and further tissues gradually convert the progestogens to conjugates of pregnanolone isomers and pregnanediols with a shift to the latter substances in ICP patients due to augmented activity of liver AKR1C1 converting steroid 20-oxo- to 20α-hydroxy-group. This shift is indicated by more pronounced differences between patients and controls for 20α-dihydroprogesterone then for progesterone (Table 1). Pregnanolone isomers and possibly also the pregnanediols are converted to the corresponding 5α/β-reduced C19 steroids by CYP17A1 *via* the alternative "backdoor" pathway. Whereas our data indicate reduced CYP17A1 activity for the ICP patients in the C17,20 lyase step, the balance between the toxic sulfated pregnanediols and harmless 5α/β-reduced-17-oxo C19 steroids is shifted to the former substances in these patients. Therefore, more quantities of toxic steroid metabolites in ICP patients may penetrate backwards into the fetus. The differences in the mixed umbilical blood are less pronounced than in the maternal blood. However, the main noxa, conjugated 5α-pregnane-3α,20α-diol and its unconjugated counterpart are elevated in the umbilical blood as well. Hence, the altered catabolism of placental steroids in the maternal but not in

the fetal compartment is critical for the development of ICP. Reduced levels of estrone, estradiol sulfate in the mixed umbilical blood of patients may partly reflect the lower activity of maternal *zona reticularis* and perhaps also the fetal zone of the fetal adrenal (FZFA) due to hereditary predisposition to lower activities of the aforementioned tissues as estrogens originate from androgens.

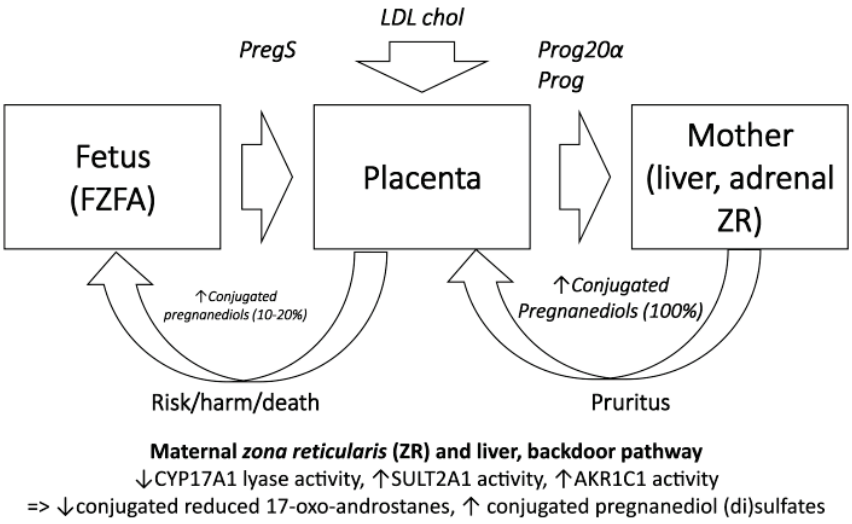

Fig. 4. Simplified scheme of formation and transport of conjugated pregnanediols in pregnancy. FZFA – fetal zone of the fetal adrenal, PregS – pregnenolone sulfate, LDL chol – maternal LDL cholesterol, Prog – progesterone, Prog20α – 20α-dihydroprogesterone, ZR – zona reticularis in maternal adrenal cortex, CYP17A1 – steroid 17-hydroxylase-17,20-lyase, SULT2A1 – steroid sulfotransferase, AKR1C1 – subfamily1C type 1 aldoketoreductase (20-oxo → 20α-hydroxy).

ICP commonly manifests in the third trimester, and the risk of ICP for the fetus increases with increasing gestational age. Moreover, the levels of conjugated pregnanediols also exhibit an increasing trend in this period (Abu-Hayyeh *et al.* 2016) and then rapidly decrease after labor (Reyes 2016). In humans and great apes, the activity of the FZFA is controlled by the placental corticoliberin (CRH), the expression of which exponentially rises with approaching term (Hill *et al.* 2010a, Petraglia *et al.* 2010, Sirianni *et al.* 2005, Smith *et al.* 2009). The attenuated activity of CYP17A1 C17,20-lyase step in ICP patients might be associated with a reduced expression of the placental CRH in these subjects as suggested by Zhou *et al.* (2013). The authors demonstrate lower CRH expression in the placenta from ICP patients with an unchanged placental expression of mRNA for both CRH and the type 1 CRH receptors (CRH-R1). They also report a considerably slower increase of circulating CRH levels with advancing gestation in the ICP group against controls. Millona *et al.* (2012) report that BAs strongly inhibit the CYP17A1 expression in mouse liver, which is FXR-dependent. In addition, Anakk *et al.* (2011) demonstrate that FXR and small heterodimer partner (SHP) knockout mice exhibit strong upregulation of CYP17A1 mRNA, CYP17A1 protein. At the same time, the knockout mice show increased circulating 17-hydroxyprogesterone at suppressed DHEA levels.

The adult *zona reticularis* is a successor of FZFA and reinstates its functioning from adrenarche reaching maximum activity in the second decade and gradually goes out till senescence (Hill *et al.* 2010a, Hill *et al.* 2010b, Rege *et al.* 2016, Sulcova *et al.* 1997). We hypothesize, that there is a predisposition to permanently attenuated CYP17A1 activity for the lyase step in women with the history of ICP from adrenarche to senescence irrespectively of the pregnancy status. The reduced activity of CYP17A1 C17,20-lyase step in ICP patients might also be associated with an impaired expression of CYB5 enzyme (Rege *et al.* 2016) in the maternal *zona reticularis*. We have also found attenuated activity of the CYP17A1 C17,20 lyase step in further pathologies such as IgA nephropathy (Sterzl *et al.* 2017), mood and anxiety disorders (Duskova *et al.* 2015) and Alzheimer's disease (Vankova *et al.* 2016). This insufficiency was sometimes accompanied by reduced sulfotransferase activity (Vankova *et al.* 2015).

In conclusion, our results indicate persistent changes in steroid metabolism in women with ICP. Although we do not directly evaluate enzyme activities, but used metabolomic data for their qualitative estimation, the results indicate slightly increased C17-hydroxylation in the steroid Δ^5 pathway at pronouncedly weakened C17,20 lyase step even considering that both steps are catalyzed by the same CYP17A1 enzyme (but on different active sites).

Whereas the steroid alterations in ICP patients persist in the puerperium, the circulating steroids could be potentially utilized in predicting the predisposition to ICP even in non-pregnant women of fertile age. However, further studies are needed to confirm this hypothesis.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Grants 17-30528A from AZV CR (Czech Health Research Council), OPPK CZ.2.16/3.1.00/21518 Equipment for metabolomics research and the project MZ CR for conceptual development of research organization 00023761 (Institute of Endocrinology, Prague, Czech Republic) supported this study.

References

ABU-HAYYEH S, PAPACLEOVOULOU G, LOVGREN-SANDBLOM A, TAHIR M, ODUWOLE O, JAMALUDIN NA, RAVAT S, NIKOLOVA V, CHAMBERS J, SELDEN C, REES M, MARSCHALL HU, PARKER MG, WILLIAMSON C: Intrahepatic cholestasis of pregnancy levels of sulfated progesterone metabolites inhibit farnesoid X receptor resulting in a cholestatic phenotype. *Hepatology* **57**: 716-726, 2013.

ABU-HAYYEH S, OVADIA C, LIEU T, JENSEN DD, CHAMBERS J, DIXON PH, LOVGREN-SANDBLOM A, BOLIER R, TOLENAARS D, KREMER AE, SYNGELAKI A, NOORI M, WILLIAMS D, MARIN JJ, MONTE MJ, NICOLAIDES KH, BEUERS U, OUDE-ELFERINK R, SEED PT, CHAPPELL L, MARSCHALL HU, BUNNETT NW, WILLIAMSON C: Prognostic and mechanistic potential of progesterone sulfates in intrahepatic cholestasis of pregnancy and pruritus gravidarum. *Hepatology* **63**: 1287-1298, 2016.

ANAKK S, WATANABE M, OCHSNER SA, McKENNA NJ, FINEGOLD MJ, MOORE DD: Combined deletion of Fxr and Shp in mice induces Cyp17a1 and results in juvenile onset cholestasis. *J Clin Invest* **121**: 86-95, 2011.

BIASON-LAUBER A, MILLER WL, PANDEY AV, FLUCK CE: Of marsupials and men: "Backdoor" dihydrotestosterone synthesis in male sexual differentiation. *Mol Cell Endocrinol* **371**: 124-132, 2013.

BYRNE JA, STRAUTNIEKS SS, MIELI-VERGANI G, HIGGINS CF, LINTON KJ, THOMPSON RJ: The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* **123**: 1649-1658, 2002.

DUSKOVA M, HILL M, BICIKOVA M, SRAMKOVA M, RIPOVA D, MOHR P, STARKA L: The steroid metabolome in men with mood and anxiety disorders. *Physiol Res* **64** (Suppl 2): S275-S282, 2015.

ESTIU MC, MONTE MJ, RIVAS L, MOIRON M, GOMEZ-RODRIGUEZ L, RODRIGUEZ-BRAVO T, MARIN JJ, MACIAS RI: Effect of ursodeoxycholic acid treatment on the altered progesterone and bile acid homeostasis in the mother-placenta-foetus trio during cholestasis of pregnancy. *Br J Clin Pharmacol* **79**: 316-329, 2015.

GLANTZ A, REILLY SJ, BENTHIN L, LAMMERT F, MATTSSON LA, MARSCHALL HU: Intrahepatic cholestasis of pregnancy: amelioration of pruritus by UDCA is associated with decreased progesterone disulphates in urine. *Hepatology* **47**: 544-551, 2008.

HILL M, HAMPL R, LUKAC D, LAPCIK O, POUZAR V, SULCOVA J: Elimination of cross-reactivity by addition of an excess of cross-reactant for radioimmunoassay of 17alpha-hydroxypregnenolone. *Steroids* **64**: 341-355, 1999.

HILL M, PARIZEK A, CIBULA D, KANCHEVA R, JIRASEK JE, JIRKOVSKA M, VELIKOVA M, KUBATOVA J, KLIMKOVA M, PASKOVA A, ZIZKA Z, KANCHEVA L, KAZIHNITKOVA H, ZAMRAZILOVA L, STARKA L: Steroid metabolome in fetal and maternal body fluids in human late pregnancy. *J Steroid Biochem Mol Biol* **122**: 114-132, 2010a.

HILL M, PARIZEK A, JIRASEK JE, JIRKOVSKA M, VELIKOVA M, DUSKOVA M, KLIMKOVA M, PASKOVA A, ZIZKA Z, GERMANOVA A, KOUCKY M, KALOUSOVA M, STARKA L: Is maternal progesterone actually independent of the fetal steroids? *Physiol Res* **59**: 211-224, 2010b.

HILL M, PARIZEK A, KANCHEVA R, DUSKOVA M, VELIKOVA M, KRIZ L, KLIMKOVA M, PASKOVA A, ZIZKA Z, MATUCHA P, MELOUN M, STARKA L: Steroid metabolome in plasma from the umbilical artery, umbilical vein, maternal cubital vein and in amniotic fluid in normal and preterm labor. *J Steroid Biochem Mol Biol* **121**: 594-610, 2010c.

HILL M, PASKOVA A, KANCEVA R, VELIKOVA M, KUBATOVA J, KANCHEVA L, ADAMCOVA K, MIKESOVA M, ZIZKA Z, KOUCKY M, SARAPATKOVA H, KACER V, MATUCHA P, MELOUN M, PARIZEK A: Steroid profiling in pregnancy: a focus on the human fetus. *J Steroid Biochem Mol Biol* **139**: 201-222, 2014.

KAMRATH C, HARTMANN MF, WUDY SA: Androgen synthesis in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Horm Metab Res* **45**: 86-91, 2013.

LAMMERT F, MARSCHALL HU, GLANTZ A, MATERN S: Intrahepatic cholestasis of pregnancy: molecular pathogenesis, diagnosis and management. *J Hepatol* **33**: 1012-1021, 2000.

LESLIE KK, REZNIKOV L, SIMON FR, FENNESSEY PV, REYES H, RIBALTA J: Estrogens in intrahepatic cholestasis of pregnancy. *Obstet Gynecol* **95**: 372-376, 2000.

MELOUN M, HILL M, MILITKY J, KUPKA K: Transformation in the PC-aided biochemical data analysis. *Clin Chem Lab Med* **38**: 553-559, 2000.

MELOUN M, MILITKY J, HILL M, BRERETON RG: Crucial problems in regression modelling and their solutions. *Analyst* **127**: 433-450, 2002.

MENG LJ, REYES H, AXELSON M, PALMA J, HERNANDEZ I, RIBALTA J, SJOVALL J: Progesterone metabolites and bile acids in serum of patients with intrahepatic cholestasis of pregnancy: effect of ursodeoxycholic acid therapy. *Hepatology* **26**: 1573-1579, 1997.

MILONA A, OWEN OM, WILLEMSEN EC, WILLIAMSON C, VAN MIL SW: Exploring a role for Cyp17a1 in the pathogenesis of cholestasis. In: *XXII International Bile Acid Meeting – Hepatic and Extrahepatic Targets of Bile Acid Signaling.* HÄUSSINGER D, BEUERS U, TRAUNER M (eds), Proceedings of Falk Symposium 184, Vienna, 2012, S. Karger AG, Basel, Switzerland, 2013, pp. 65.

PARIZEK A, DUSKOVA M, VITEK L, SRAMKOVA M, HILL M, ADAMCOVA K, SIMJAK P, CERNY A, KORDOVA Z, VRABLIKOVA H, BOUDOVA B, KOUCKY M, MALICKOVA K, STARKA L: The role of steroid hormones in the development of intrahepatic cholestasis of pregnancy. *Physiol Res* **64** (Suppl 2): S203-S209, 2015.

PARIZEK A, HILL M, DUSKOVA M, VITEK L, VELIKOVA M, KANCHEVA R, SIMJAK P, KOUCKY M, KOKRDOVA Z, ADAMCOVA K, CERNY A, HAJEK Z, STARKA L: A comprehensive evaluation of steroid metabolism in women with intrahepatic cholestasis of pregnancy. *PLoS One* **11**: e0159203, 2016.

PASCUAL MJ, SERRANO MA, EL-MIR MY, MACIAS RI, JIMENEZ F, MARIN JJ: Relationship between asymptomatic hypercholanaemia of pregnancy and progesterone metabolism. *Clin Sci (Lond)* **102**: 587-593, 2002.

PETRAGLIA F, IMPERATORE A, CHALLIS JR: Neuroendocrine mechanisms in pregnancy and parturition. *Endocr Rev* **31**: 783-816, 2010.

REGE J, KARASHIMA S, LERARIO AM, SMITH JM, AUCHUS RJ, KASA-VUBU JZ, SASANO H, NAKAMURA Y, WHITE PC, RAINEY WE: Age-dependent increases in adrenal cytochrome b5 and serum 5-androstenediol-3-sulfate. *J Clin Endocrinol Metab* **101**: 4585-4593, 2016.

REYES H: Sex hormones and bile acids in intrahepatic cholestasis of pregnancy. *Hepatology* **47**: 376-379, 2008.

REYES H: Sulfated progesterone metabolites in the pathogenesis of intrahepatic cholestasis of pregnancy: another loop in the ascending spiral of medical knowledge. *Hepatology* **63**: 1080-1082, 2016.

RIZZO G, RENGA B, MENCARELLI A, PELLICCIARI R, FIORUCCI S: Role of FXR in regulating bile acid homeostasis and relevance for human diseases. *Curr Drug Targets Immune Endocr Metabol Disord* **5**: 289-303, 2005.

SIMJAK P, PARIZEK A, VITEK L, CERNY A, ADAMCOVA K, KOUCKY M, HILL M, DUSKOVA M, STARKA L: Fetal complications due to intrahepatic cholestasis of pregnancy. *J Perinat Med* **43**: 133-139, 2015.

SIRIANNI R, MAYHEW BA, CARR BR, PARKER CR JR, RAINEY WE: Corticotropin-releasing hormone (CRH) and urocortin act through type 1 CRH receptors to stimulate dehydroepiandrosterone sulfate production in human fetal adrenal cells. *J Clin Endocrinol Metab* **90**: 5393-5400, 2005.

SMITH R, SMITH JI, SHEN X, ENGEL PJ, BOWMAN ME, MCGRATH SA, BISITS AM, MCELDUFF P, GILES WB, SMITH DW: Patterns of plasma corticotropin-releasing hormone, progesterone, estradiol, and estriol change and the onset of human labor. *J Clin Endocrinol Metab* **94**: 2066-2074, 2009.

STERZL I, HILL M, STARKA L, VELIKOVA M, KANCEVA R, JEMELKOVA J, CZERNEKOVA L, KOSZTYU P, ZADRAZIL J, MATOUSOVIC K, VONDRAK K, RASKA M: Patients with IgA nephropathy have altered levels of immunomodulatory C19 steroids. Glucocorticoid therapy with addition of adrenal androgens may be the choice. *Physiol Res* **66** (Suppl 3): S433-S442, 2017.

SULCOVA J, HILL M, HAMPL R, STARKA L: Age and sex related differences in serum levels of unconjugated dehydroepiandrosterone and its sulphate in normal subjects. *J Endocrinol* **154**: 57-62, 1997.

VALLEJO M, BRIZ O, SERRANO MA, MONTE MJ, MARIN JJ: Potential role of trans-inhibition of the bile salt export pump by progesterone metabolites in the etiopathogenesis of intrahepatic cholestasis of pregnancy. *J Hepatol* **44**: 1150-1157, 2006.

VANKOVA M, HILL M, VELIKOVA M, VCELAK J, VACINOVA G, LUKASOVA P, VEJRAZKOVA D, DVORAKOVA K, RUSINA R, HOLMEROVA I, JAROLIMOVA E, VANKOVA H, BENDLOVA B: Reduced sulfotransferase SULT2A1 activity in patients with Alzheimer's disease. *Physiol Res* **64** (Suppl 2): S265-S273, 2015.

VANKOVA M, HILL M, VELIKOVA M, VCELAK J, VACINOVA G, DVORAKOVA K, LUKASOVA P, VEJRAZKOVA D, RUSINA R, HOLMEROVA I, JAROLIMOVA E, VANKOVA H, KANCHEVA R, BENDLOVA B, STARKA L: Preliminary evidence of altered steroidogenesis in women with Alzheimer's disease: have the patients "OLDER" adrenal zona reticularis? *J Steroid Biochem Mol Biol* **158**: 157-177, 2016.

VCELAKOVA H, HILL M, LAPCIK O, PARIZEK A: Determination of 17alpha-hydroxypregnenolone sulfate and its application in diagnostics. *Steroids* **72**: 323-327, 2007.

WANG C, CHEN X, ZHOU SF, LI X: Impaired fetal adrenal function in intrahepatic cholestasis of pregnancy. *Med Sci Monit* **17**: CR265-CR271, 2011.

ZHOU F, HE MM, LIU ZF, ZHANG L, GAO BX, WANG XD: Expression of corticotrophin-releasing hormone and its receptor in patients with intrahepatic cholestasis of pregnancy. *Placenta* **34**: 401-406, 2013.