Preeclamptic disorders of pregnancy; Novel molecular insights
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Citation for published version (APA):

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CHAPTER 7

Total bile acids in the maternal and fetal compartment in relation to placental ABCG2 expression in preeclamptic pregnancy

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Submitted
ABSTRACT

Objective
To investigate total bile acid (TBA) levels in maternal and umbilical cord blood in normotensive and preeclamptic pregnancies in the context of ABCG2 placental gene expression levels, a recently reported placental bile acid transporter.

Methods
TBA levels were determined in 83 paired maternal and umbilical cord blood samples of normotensive and preeclamptic pregnancies and in 22 paired arterial and venous umbilical cord blood samples from uncomplicated term pregnancies. ABCG2 gene expression was measured in 104 human placentas by reverse transcriptase quantitative polymerase chain reaction.

Results
Both in normotensive and in preeclamptic pregnancies, TBA levels in the maternal compartment are higher than in the fetal compartment. This effect is more pronounced in preeclamptic pregnancies (p=0.007 and p<0.001 respectively). TBA levels in maternal blood are increased in preeclamptic pregnancy compared to normotensive pregnancy (p=0.016). TBA levels in arterial and venous UCB from 22 normotensive pregnancies are not statistically different.

ABCG2 expression is reduced in pregnancies where preeclampsia is further complicated by Hemolysis Elevated Liver Enzymes and Low Platelets syndrome. ABCG2 expression in human placenta is not correlated with maternal or umbilical cord TBA levels.

Discussion
Increased maternal TBA levels in preeclamptic pregnancies indicate a relation between bile acids in the maternal circulation and preeclampsia. The fact that TBA levels in the fetal compartment do not differ between normotensive and preeclamptic pregnancies combined with the fact that arterial and venous umbilical cord blood TBA levels are comparable suggests that the placenta functions as a bile acid barrier.

INTRODUCTION

The placenta serves different functions crucial for fetal growth and development. Apart from producing nutrients, energy, growth factors, cytokines and hormones, the placenta is essential for the exchange of oxygen, nutrients and waste between the maternal and fetal circulation19.

Preeclampsia (PE) is characterized by new onset hypertension after 20 weeks of gestation combined with proteinuria and is a major risk for both mother and fetus4. Although clinical symptoms manifest in the second half of pregnancy, the pathophysiological mechanism is aberrant placenta and this occurs in the first and early second trimester of pregnancy26. Subsequent aberrant release of placental produced factors such as sFLT1 into the maternal circulation cause endothelial dysfunction15.

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific disorder that usually presents in the third trimester. ICP is diagnosed by increased non-fasting or fasting serum total bile acid (TBA) levels and maternal pruritus5, PE complicates 8-25% of pregnancies that are affected by ICP3,12, which is higher than the PE incidence in the general population (1.5-6%)6,30,32 suggesting a putative relation between bile acids and the pathogenesis of PE. About 10-20% of severe preeclamptic pregnancies are further complicated by Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome3,17. We recently reported a series of four women with seven pregnancies where in 5 pregnancies ICP preceded the occurrence of severe HELLP syndrome17 suggesting that increased TBA levels relate to the development of HELLP syndrome. Further illustration of the pathophysiological relation between PE and ICP is the fact that increased numbers of syncytial knots have been described in placentas from pregnancies complicated by ICP11, a hallmark of PE4.

Cholestatic effects of reproductive hormones and their metabolites in genetically susceptible women are considered causal to the increased maternal TBA levels19.

Bile acids are present in the fetal compartment as early as 13 weeks of human gestation27. Over 90% of fetal bile acids are conjugated forms of the primary bile acids cholic acid and chenodeoxycholic acid18. Intestinal bacteria necessary to transform primary bile acids into secondary bile acids are thought to be absent in utero. The enterohepatic circulation of bile acids in utero as opposed to adult life is minimal21. In theory, the fetus is able to contribute to the maternal TBA pool as about 80% of the maternal TBA in pregnancies complicated by ICP consists of the primary bile acid cholic acid16.

The umbilical cord functions as a ‘pipeline’ between the placental and fetal compartment and contains 1 vein and 2 arteries. The umbilical vein carries oxygen- and nutrient-rich blood from placenta to fetus. The arteries transport deoxygenated blood and waste products from fetus to placenta.

Although transfer from the maternal to the fetal circulation has been documented15, it is generally believed that fetal primary bile acids are transferred across the placenta into the maternal circulation where they are eliminated by the maternal liver14. This implies increased levels in the umbilical artery compared to the umbilical cord vein as has been demonstrated.
using gas chromatography. Several bile acid transporter proteins or proteins associated with cholestatic disease are expressed in human placenta. ABCG2 and ABCB11, members of the ABC-transporter family, are able to transfer bile acids. Although ABCB11 is the main bile acid transporter in liver, its expression in human placenta is negligible. Human ABCG2 overexpressed in Chinese hamster ovary cells results in bile transport across the plasma membrane. Fetuses from homozygous Abcg2 knock-out mice show markedly higher levels of bile acids in both maternal and in fetal serum after obstructive cholestasis without difference in placental abcg2 expression. Although ABCB11 is the main bile acid transporter in liver, its expression in placenta is at negligible levels, while at least in rodents Abcg2 appears to be a prominent placental bile transporter. ABC-transporters in placenta are known to protect the fetus from xenobiotics and cellular accumulation of cytotoxic compounds.

In this study we investigate total bile acid levels in the maternal and fetal compartment in normotensive and PE pregnancies and relate these levels to the expression of ABCG2 in placenta. Because of conflicting reports, to firmly establish if net bile acid transport across the placenta does or does not occur, we additionally analysed TBA levels in paired arterial and venous umbilical cord blood (UCB) samples in normotensive term controls.

METHODS

Biosamples and patient data

Biosamples were collected through the PE And Non-PE DAtabase (PANDA); an obstetrical biosample effort, approved by the institutional review board of the Academic Medical Center, where we obtained placenta biopsies, venous umbilical cord blood and maternal blood (not necessarily fasting samples) at the time of delivery with informed consent. Placenta biopsies (59 normotensive and 45 preeclamptic) were taken from macroscopically viable (non-infarcted) cotyledons at the maternal side within 2 hours of delivery, placed in RNAlater (Ambion), processed according to the manufacturer’s instructions and stored at -80°C until use. Clinical data are listed in Supplementary Table 1. From 83 of the in total 104 included pregnancies, both UCB heparin plasma samples and maternal heparin plasma samples stored at -80°C were available. They were used for comparison of TBA levels in maternal and umbilical cord blood and to test if there is correlation between TBA levels and the expression level of ABCG2.

Additionally paired arterial and venous umbilical cord heparin plasma samples were taken immediately after birth from 22 anonymously collected placentas from normotensive term pregnancies.

PE was defined by two systolic blood pressure measurements of ≥ 140 mmHg, or diastolic measurements of ≥ 90 mmHg, at least 6 hours apart after 20 weeks’ gestation in a previously normotensive patient in combination with new-onset proteinuria (> 0.3 g /24-hour or at least ≥ 1+ on protein dipstick when urine could not be collected for 24 hours). Patients with baseline hypertension or renal disease were excluded. Hemolysis, Elevated Liver enzymes and Low Platelets (HELLP) syndrome was defined by lactate dehydrogenase ≥ 600 U/L or haptoglobin < 0.2 g/L, aspartate or alanine aminotransferase ≥ 70 U/L, and platelet count <100 *10^9/L. Apparently healthy, normotensive pregnant women were included as controls.

Measurement of TBA levels

TBA levels were quantified using the Diazyme total bile acids kit (Diazyme Laboratories, Poway, CA), according to the manufacturer’s instructions.

In Situ Hybridization

RNA in situ hybridization was performed as previously described. Probe corresponded to nucleotides 1396 to 1920 of ABCG2 (Genbank NM_001257386.1).

Tissue Preparation and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was isolated using the MagNA Pure LC RNA Isolation Kit High Performance (Roche), and reverse transcribed using AMV First Strand cDNA Synthesis Kit for reverse transcription (RT)-PCR (Roche). RT-qPCR was performed in duplicate on a LightCycler 480 system (Roche) according to the manufacturer’s protocol with 0.4 μmol/L of each primer (Invitrogen), 100 nmol/L UPL probe (Roche) and 5 μl Absolute RT-qPCR mix (ThermoScientific). Primers were designed using the Roche Universal ProbeLibrary Assay Design Center (Supplementary table 2).

Data were analysed and quantified, using the second derivative maximum for Cp determination, with the LightCycler 480 software 1.5.0 (Roche). To correct for differences in cDNA input HPRT1 was used as a reference gene for normalization.

Statistical analysis

P-values < 0.05 were considered statistically significant. Group differences were tested using a Mann-Whitney U test. Correlations were analysed by Spearman’s rank correlation. Paired Wilcoxon matched-pairs signed rank test was used to compare values of paired arterial and venous UCB samples and of paired maternal blood and UCB samples. GraphPad Prism 6 (GraphPad Software, Inc) was used as statistical package and for graphical representation.

RESULTS

Biosampling

Patient characteristics of both groups are comparable with the exception of maternal age (Table 1). Parameters relating to PE (highest maternal diastolic blood pressure and neonatal weight) are statistically different between groups. As PE is an important contributor to iatrogenic preterm delivery the primary caesarean section rate is also increased in this patient group (Table 1).
Table 1: Clinical Characteristics Summary of Studied Groups. Data are represented as median (range) or numbers (%). P-values were calculated by Mann-Whitney U test*, Chi-square† or Fisher’s exact test‡. Abbreviations: n.a. not applicable, n.d. not determined, n.s. not significant.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Normotensive n=59</th>
<th>Preeclamptic n=45</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>28 (18 - 38)</td>
<td>31 (18 - 41)</td>
<td>p&lt;0.05*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2 (17.3 – 42.5)</td>
<td>25.3 (17.5 – 36.2)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Highest diastolic BP (mmHg)</td>
<td>71 (60-94)</td>
<td>100 (90 - 120)</td>
<td>p&lt;0.001*</td>
</tr>
<tr>
<td>Urinary protein (g/24 h)</td>
<td>n.d.</td>
<td>2.1 (0.3 - 32.4)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>30 (51%)</td>
<td>26 (58%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>0 (0%)</td>
<td>17 (38%)</td>
<td>p&lt;0.001*</td>
</tr>
<tr>
<td>Primary caesarean section</td>
<td>5 (8%)</td>
<td>21 (46%)</td>
<td>p&lt;0.001*</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>34¹¹ (26¹ - 42¹¹)</td>
<td>34⁺ (28⁺ - 41⁺)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Delivery &lt;37 weeks gestation</td>
<td>32 (54%)</td>
<td>26 (58%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Delivery &lt; 34 weeks gestation</td>
<td>23 (39%)</td>
<td>21 (47%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Neonatal weight (g)</td>
<td>2375 (860 - 5300)</td>
<td>1810 (780 - 3990)</td>
<td>p&lt;0.05*</td>
</tr>
<tr>
<td>Birth percentile &lt;10</td>
<td>3 (5%)</td>
<td>7 (16%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Maternal and umbilical cord blood available</td>
<td>52 (88%)</td>
<td>31 (69%)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

TBA determinations in maternal blood and umbilical cord blood (UCB).

In both normotensive and preeclamptic pregnancies (ranging from 26+2 to 42+0 weeks of gestation) median TBA levels in maternal blood (6.7 µmol/l and 11.5 µmol/l respectively) are higher compared to median TBA levels in UCB (p=0.007 and p<0.001 respectively). Compared to normotensive pregnancies, median TBA levels in maternal blood from preeclamptic pregnancies are increased (p=0.016). However TBA levels in UCB between normotensive and preeclamptic pregnancies do not differ (5.6 µmol/liter in both normotensive UCB and PE UCB)(Figure 1A).

TBA levels measured in both maternal blood and UCB show no correlation in relation to gestational age either in normotensive controls, PE patients or PE/HELLP patients (p-values of Spearman’s correlation coefficient all not significant, data not shown).

TBA levels in arterial and venous UCB from 22 normotensive pregnancies are not statistically different with respectively 4.25 µmol/L (0.1-11.4 µmol/L) versus 4.28 µmol/L (0.1-12.9 µmol/L) (Figure 1B).
**ABCG2 expression in human placenta**

To investigate if ABCG2 (ATP-binding cassette sub-family G member 2) is involved in TBA distribution between the maternal and fetal pool or could play a role in protecting the fetal circulation from the increased maternal TBA levels in case of a preeclamptic pregnancy, we determined cellular localization and expression levels in placenta and correlated them to TBA levels in both the maternal and fetal compartment.

In situ hybridization on term normotensive placentas (n=2) using transcript-specific antisense probes for ABCG2 shows the expected localization within the (syncytiotrophoblast layer of the placenta (Figure 2).

ABCG2 is expressed in human placenta from at least 26+2 to 42 weeks of gestation (Figure 2D). In placenta, ABCG2 is an abundantly expressed transcript with a median normalized expression of 9.1 (range: 0.6 - 32.2) especially high in contrast to placental expression of the hepatic bile acid transporter ABCB11 that in our series is >1000-fold lower (median normalized expression 0.001 (with a range of zero to 0.48) (data not shown). ABCG2 mRNA expression levels are reduced in pregnancies complicated by HELLP syndrome either compared to PE pregnancies not complicated by HELLP syndrome (p=0.033) (Figure 2B) or compared to all (both normotensive and preeclamptic) pregnancies not complicated by HELLP syndrome (p=0.017) (Figure 2C). With a Spearman’s correlation coefficient of 0.21 (p=0.04) ABCG2 expression is weakly correlated to gestational age (Figure 2D).

**ABCG2 gene expression in relation to maternal or umbilical cord blood TBA levels**

There is no statistical significant relation with either maternal or UCB TBA levels with placental expression of ABCG2 (data not shown).

There is no correlation between the Δ ([TBA maternal] - [TBA UCB]) and the expression of ABCG2 (p=0.65) in placenta (data not shown).

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**Figure 2:** In situ hybridization on placental tissue. In situ hybridization using a transcript-specific probe for ABCG2 (A). Purple blue staining indicates positive hybridization signal. Comparison of RT-qPCR expression levels in placenta in normotensive and PE pregnancies and in relation to gestational age. (B-C) Box plots of normalized ABCG2 mRNA expression levels comparing preeclamptic (n=28) versus PE/HELLP (n=17) pregnancies (B) or non-HELLP (n=87) versus PE/HELLP (n=17) pregnancies (C). Data are shown as median (thick line) with interquartile range (box limits) and minimum and maximum values (whisker limits). Normalized expression levels of ABCG2 in placental samples of different gestational ages (D). Open rounds represent normotensive pregnancies (n=59), dotted rounds preeclamptic pregnancies (n=28) and closed rounds pregnancies complicated by both PE and HELLP syndrome (n=17).
DISCUSSION

Based on studies using different techniques to determine specific bile acid profiles of pregnant women with different pathologies performed between 1977 and 2001, the current view is that during pregnancy total bile acids in the fetal compartment exceed that of the maternal compartment and that fetal primary bile acids are transferred across the placenta to the maternal circulation where they are eliminated by the maternal liver. A recent small sample-sized study (n=15) confirmed this view. In our cohort of 83 paired maternal/umbilical cord blood samples investigated from pregnancies with gestational ages ranging from 26 to 42 weeks of gestation we observe the opposite. Median TBA levels in maternal blood are increased compared to UCB and this increase is more substantial in case of a preeclamptic pregnancy (Figure 1A). This discrepancy may be due to the limited sample size of previously reported series. In contrast to the other studies that report that all acquired samples were fasting, our samples were not necessarily fasting samples since they were taken just prior to delivery. In our series, no sample reached the 40 µM cut-off with a concomitant risk for fetal complications as reported in ICP pregnancies and relatively a high proportion of the PE patients (that have increased TBA levels compared to normotensive controls) delivered by caesarean section and the majority of them will have been fasting.

We observe no evidence for net bile acid transport across the placenta, as TBA levels in the umbilical cord arteries (that transport nutrients and oxygen-rich blood from the placenta to the fetus) and the umbilical cord vein (carrying waste products and deoxygenated blood from the fetus to the placenta) are similar (Figure 1B).

We analysed the expression level of ABCG2 in placenta as a function of gestational age, in relation to PE, HELLP syndrome and with respect to maternal and umbilical cord blood TBA levels. In contrast to previous papers, we observe a weak positive correlation of ABCG2 expression with gestational age.

When comparing placentas from pregnancies complicated by HELLP syndrome to those not afflicted by this complication, the expression of ABCG2 in HELLP placenta is decreased (Figure 3) in line with a previous report on a 1.8-fold down regulation of ABCG2 in HELLP. It has previously been shown that ABCG2 expression is lower in placenta from pregnancies with intra uterine growth restriction not complicated by PE. Of our 59 normotensive pregnancies only 3 neonates had a neonatal weight below the 10th percentile, thus no conclusion can be drawn from our data regarding this issue.

In Abcg2−/− mice absolute TBA levels in both the maternal and fetal compartment are increased compared to wild type mice, it could be speculated that the lower ABCG2 placental expression in HELLP syndrome placenta would relate to higher TBA levels in UCB and lower levels in the maternal circulation. This shift could not be demonstrated in the 11 HELLP paired UCB/maternal blood samples versus the 72 non-HELLP sets.

The decreased ABCG2 expression only in placenta of HELLP pregnancies was unexpected. ABCG2 promoter contains a HIF1-alpha binding site and as HIF1-alpha is known to be induced under hypoxic conditions such as PE, this theoretically would imply altered ABCG2 expression in (hypoxic) PE placenta. In PE higher placental levels of HIF1-alpha are present and this theoretically should result in increased expression of ABCG2. We do not observe increased ABCG2 expression in PE placenta. We do not observe this in the placenta tissues studied suggesting that in placenta HIF1-alpha is not the mediator of ABCG2 expression.

In conclusion: Based on our data we see no relation of ABCG2 expression in human placenta with TBA levels in either the maternal or the fetal compartment. Based on the absence of diverging TBA levels between the umbilical cord artery and vein combined with the unaltered TBA levels in cord blood in preeclamptic pregnancies (where TBA levels in the maternal circulation are increased) we conclude that the human placenta forms a barrier protecting the fetal circulation from maternal bile acids.
Chapter 7

REFERENCE LIST


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