Molecular mechanisms of pruritus in cholestasis
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Autotaxin Activity has a high Accuracy to Diagnose Intrahepatic Cholestasis of Pregnancy


ABSTRACT

**Background & Aims:** Intrahepatic cholestasis of pregnancy (ICP) is defined by pruritus, elevated total fasting serum bile salts (TBS) and transaminases, and an increased risk of adverse fetal outcome. An accurate diagnostic marker is needed. Increased serum autotaxin correlates with cholestasis-associated pruritus. We aimed to unravel the diagnostic accuracy of autotaxin in ICP.

**Methods:** Serum samples and placental tissue were collected from 44 women with uncomplicated pregnancies and 105 with pruritus and/or elevated serum transaminases. Autotaxin serum levels were quantified enzymatically and by western blotting, autotaxin gene expression by quantitative PCR.

**Results:** Serum autotaxin was increased in ICP (mean±SD: 43.5±18.2 nmol mL⁻¹ min⁻¹, n=55, p<0.0001) compared to other pruritic disorders of pregnancy (16.8±6.7 nmol mL⁻¹ min⁻¹, n=33), pre-eclampsia complicated by HELLP-syndrome (16.8±8.9 nmol mL⁻¹ min⁻¹, n=17), and pregnant controls (19.6±5.7 nmol mL⁻¹ min⁻¹, n=44). Longitudinal analysis during pregnancy revealed a marked rise in serum autotaxin with onset of ICP-related pruritus. Serum autotaxin was increased in women taking oral contraceptives. Increased serum autotaxin during ICP was not associated with increased autotaxin mRNA in placenta. With a cut-off value of 27.0 nmol mL⁻¹ min⁻¹, autotaxin had an excellent sensitivity and specificity in distinguishing ICP from other pruritic disorders or pre-eclampsia/HELLP-syndrome. Serum autotaxin displayed no circadian rhythm and was not influenced by food intake.

**Conclusions:** Increased serum autotaxin activity represents a highly sensitive, specific and robust diagnostic marker of ICP distinguishing ICP from other pruritic disorders of pregnancy and pregnancy-related liver diseases. Pregnancy and oral contraception increase serum autotaxin to a much lesser extent than ICP.
INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP), also known as obstetric cholestasis, is a pregnancy-specific liver disorder with onset mainly in the third trimester of pregnancy. ICP is characterized by pruritus, elevated serum fasting bile salts and transaminases and an increased risk of adverse fetal outcomes.\(^1\)\(^-\)\(^3\) This disorder typically affects 0.2—2\% of all pregnant women. The incidence of ICP, however, varies considerably with ethnicity and geographical location with the highest rates observed in Northern Europe and Southern America.\(^2\)\(^,\)\(^4\) Pruritus is the defining symptom of ICP which progressively worsens as the pregnancy advances. Pruritus may considerably reduce quality of life, lead to sleep deprivation, depressed mood, and even suicidal ideation in more severe cases. In contrast to other more commonly observed pruritic dermatoses of pregnancy,\(^5\) a concern in ICP is the increased risk of adverse fetal outcomes.\(^1\)\(^,\)\(^2\) ICP increases the risk of fetal distress, cardiotocography abnormalities, preterm labor and sudden intrauterine death particularly in those women with total serum fasting bile salt (TBS) levels exceeding 40 \(\mu\text{mol/L}\).\(^3\)\(^,\)\(^4\)\(^,\)\(^6\)\(^,\)\(^7\) Therefore a proper diagnosis is essential to enable pharmacological treatment with ursodeoxycholic acid (UDCA), close antenatal monitoring and potentially the induction of labor after 37 weeks with the aim of reducing fetal distress and intrauterine death.\(^8\)\(^,\)\(^9\)

The diagnosis of ICP is currently based on the presence of pruritus, raised fasting serum TBS levels above 10 \(\mu\text{mol/L}\), and/or elevated serum transaminases (in the absence of diseases that cause cholestasis or pruritus) as well as spontaneous relief of signs and symptoms within four to six weeks after delivery.\(^1\)\(^,\)\(^10\) However, diagnosis of ICP may be difficult when considering other pregnancy-associated dermatoses, liver diseases and their possible co-existence. The most sensitive marker for ICP is a raised fasting level of TBS while serum transaminases may be normal in up to 30\% of cases.\(^6\)\(^,\)\(^11\) However, an asymptomatic elevation of TBS levels, hypercholanaemia, is observed in approximately 10\% of pregnant women,\(^12\) and has been reported to affect up to 40\% of Argentinean pregnancies.\(^13\) In addition, serum TBS increase upon food intake, thereby increasing variation unless serum is collected upon fasting. Elevated serum transaminases during the 3\(^{rd}\) trimester of pregnancy are seen in women with HELLP-syndrome (hemolysis, elevated liver enzymes and low platelet count), pre-eclampsia, acute fatty liver of pregnancy and
other non pregnancy-related liver disorders including obesity. These women also hold an increased risk for fetal adverse outcomes, but have an etiology that differs from women with ICP. Furthermore, the management of these conditions is different from that of ICP.

Autotaxin (ATX) is a lysophospholipase D which is essential for angiogenesis and neuronal development during embryogenesis. Other physiological functions attributed to ATX include cellular motility, proliferation, and lymphocyte homing. The effects of ATX are largely mediated by the enzymatic formation of lysophosphatidic acid (LPA) which may act via one of at least six different LPA receptors. ATX levels have been reported to be increased during pregnancy and correlate positively with gestational age. To identify the pruritogens of cholestasis we recently screened sera from ICP women for activation of neuronal cells and identified LPA as a potent neuronal activator. LPA and ATX levels were significantly increased in ICP women compared to gestation-matched pregnant controls. LPA could be related to pruritus during ICP as intradermal injection of LPA in mice caused a dose-dependent scratch response.

In this study, we analyzed serum ATX levels in women with ICP, other pruritic dermatoses of pregnancy and pregnancy-related liver disorders in order to determine whether ATX may represent a diagnostic marker for ICP. Furthermore, ATX expression in placental tissue was analyzed to determine the source of increased circulating ATX levels during ICP, as placenta was suggested to be the source of enhanced serum ATX during uncomplicated pregnancy. Finally, the influence of oral female steroid hormones and food intake on serum ATX was determined.

**METHODS**

**Human subjects.** Peripheral venous whole blood samples were collected prospectively from pregnant women with pruritus and/or elevated serum transaminases as well as newborn babies and placental tissue from pregnant women after delivery who were seen at the Women’s and Children’s Clinic, Academic Medical Center, University of Amsterdam, Amsterdam from December 2005 until March 2010 and via the research team at Queen...
Charlotte’s and Chelsea Hospital, London from January 2006 to June 2010. Samples were also taken from non-pregnant women with a history of ICP and controls with a previous uncomplicated pregnancy. ICP cases and healthy volunteers were only enrolled after giving informed consent. The study was conducted according to the Declaration of Helsinki and approved by the local Medical Ethical Committees (Reference numbers: 21233.018.07, 05.17.0936, and 08/H0707/21). Blood samples were allowed to clot for an hour before they were centrifuged for 10 minutes at 1000 \( g \) and 4\(^\circ\)C. The serum supernatant was aliquoted and cryopreserved at -80\(^\circ\)C until measurements were performed. Placental tissue was snap frozen using liquid nitrogen and cryopreserved at -80\(^\circ\)C for later RNA isolation.

ICP was diagnosed in pregnant women with pruritus, but without rash, in conjunction with raised serum transaminases and/or fasting serum TBS (> 10 \( \mu \)mol/L), as described previously.\(^1\)\(^{10}\) If ICP was suspected but TBS and transaminases were normal at first presentation, measurements were repeated weekly and patients only classified as ICP if these parameters became abnormal. Most of the women diagnosed for ICP received UDCA treatment according to guidelines\(^8\) Women were excluded if they had signs of acute or chronic hepatitis infection (hepatitis A, B or C), other non-viral hepatitis etiologies or extrahepatic biliary obstruction following ultrasound examination. Pregnant and non-pregnant controls had no history of liver dysfunction or any complication in the current or previous pregnancies.

Pruritic dermatoses of pregnancy consisted of atopic eruption of pregnancy and polymorphic eruption of pregnancy without raised serum transaminases or serum TBS levels as defined recently.\(^19\)

The diagnosis of HELLP-Syndrome was defined according to the National Heart, Lung and Blood Institute Working Group criteria\(^20\) and was based on hemolysis (haptoglobin < 0.20 g/L and/or lactate dehydrogenase (LDH) > 600 IU/L), elevated aspartate amino transaminase (ASAT) and/or alanine amino transaminase (ALAT) > 70U/L and low platelet count (platelets < 100 x 10\(^9\)/L). Pre-eclampsia was defined as arterial hypertension with two blood pressure measurements \( \geq 140/90 \) mmHg more than 4 hours apart or a diastolic blood pressure of \( \geq 110 \) mmHg combined with proteinuria.
 (> 300mg/24 hours) that developed after 20 weeks of gestation in a formerly normotensive woman.

Healthy controls consisted of women and men without a significant past medical history. Women on oral contraceptives took either a combined pill containing both, estrogen and progestin, or a progestin-only pill.

All autotaxin and total serum bile salt measurements were performed by observers blinded to patient status and results were interpreted without knowledge of diagnosis.

**Materials.** Choline oxidase, horseradish peroxidase, and homovanillinic acid were purchased from Sigma-Aldrich (St. Louis, MO); myristoyl-lysophosphatidylcholine (LPC 14:0) was from Avanti Polar Lipids (Alabaster, AL).

**Autotaxin activity assay.** ATX activity was quantified as recently described.\(^{21}\) Briefly, serum samples were diluted and incubated with a buffer containing 1 mmol/L of LPC 14:0 for 60 min at 37°C. The lysophospholipase D activity of ATX was determined by the amount of liberated choline using an enzymatic fluorimetric method. Samples were added to a buffer containing choline oxidase (2 U/mL), horseradish peroxidase (1.6 U/mL), and homovanillinic acid as substrate for peroxidase. The increase in fluorescence was monitored at 37°C on a Novostar analyzer. Both, the inter-assay and the intra-assay variance of the assay was < 10%.

**RNA Isolation and Quantification of Transcript levels.** Total RNA was extracted from placental tissue using Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from total RNA with an oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). Real-time PCR measurements were performed at 60°C in a Lightcycler apparatus (Roche, Mannheim, Germany) with Lightcycler Faststart DNA Master Plus CYBR Green I (Roche). Transcript levels were normalized to the
housekeeping gene, 36b4 (acidic ribosomal phosphoprotein P0). For qPCR experiments, the following primer sequences were used: ATX forward: TGCAATAGCTCAGAGGAGGA; ATX reverse: AGAAGTTCAAGCTGGTGAGA; 36B4 forward: TCATCAAGGTACAAAACA; and 36B4 reverse: GCCTTGACCTTTTCAGCAAG; HPRT forward: AGTTCTGTGGCCATCTGCTT; HPRT reverse: GTTAAACAAATCCCGCCA; GAPDH forward: GTCAGTGGTGACCTTGACCT; GAPDH reverse: TGAGCTTGACAAATGTTGCG.

**Total serum bile salt determination.** Serum TBS levels were quantified using Diazyme total bile salts kit (Diazyme Laboratories, Poway, CA) according to the manufacturer’s instructions.

**SDS-PAGE and Western Blotting.** ATX was extracted from 20 μL of serum samples by incubation with immunoprecipitating ATX-antibody 5E5 (kindly provided by J. Aoki) bound to sepharose for 4 hours at 4°C. After washing, sepharose beads were incubated for 10 min at 37°C with SDS-loading buffer containing β-mercapto-ethanol and spun down. Equal amounts of supernatant were separated by SDS-PAGE and incubated with anti-ATX (1:1500, Cayman) and appropriate secondary detection reagents. Immunoreactive bands were visualized by enhanced chemiluminescence (Roche, Amersham, Buckinghamshire, UK).

**Statistical analysis.** Statistical differences were evaluated for two groups by Student’s t-test and for three or more groups by one-way ANOVA with Bonferroni correction using SPSS (version 18.0). A multivariable test score was constructed from a logistic regression model with disease status as the dependent and ATX as independent variable. Test performance was then assessed by calculating c-statistic (area under the receiver operating characteristic, ROC) and a cut-off value of 27 nmol mL⁻¹ min⁻¹ was identified as optimizing both the sensitivity and specificity of the assay. In male and female healthy controls the 95-
percentile of ATX activity had a value of 8.5 nmol mL\(^{-1}\)min\(^{-1}\).\(^{18}\) Intuitively, the c-statistic describes the probability that a randomly chosen affected patient has a higher test score than a randomly chosen unaffected control. Hence, a test with null discriminatory value has a c-statistic of 0.5, a perfect test a c-statistic of 1. For analyses comparing areas under the curves (AUC) of ROC (AUROCs) the library pROC on the statistical platform R version 3.0.2 was used. Statistical differences between the AUROCs were calculated using the Delong test. The ATX plus TBS-scores were derived from multivariable logistic regression models with the assessed diagnosis as dependent variable and ATX and TBS as independent variables. All data are expressed as means ± standard deviations (SD).

**RESULTS**

**Increased ATX activity is specific for ICP**

Serum ATX activity (mean ± SD) was markedly higher in women with ICP (43.5±18.2 nmol mL\(^{-1}\) min\(^{-1}\), n=55, p<0.0001) than in women with other pruritic dermatoses of pregnancy (16.8±6.7 nmol mL\(^{-1}\)min\(^{-1}\), n=33), HELLP-syndrome and pre-eclampsia (16.8±8.9 nmol mL\(^{-1}\)min\(^{-1}\), n=17), and pregnant controls (19.6±5.7 nmol mL\(^{-1}\)min\(^{-1}\), n=44) (Figure 1A) of comparable gestational age (for patient biochemistry, see Table 1). The number of previous pregnancies had no influence on ATX activities (Supplementary Figure 1). The enhanced ATX activity correlated with increased ATX protein content in sera from ICP women (Figure 1B).

A cut-off value for serum ATX activity of 27 nmol mL\(^{-1}\)min\(^{-1}\) was determined to maximize the sensitivity and specificity of the test results with this study population using nonparametric receiver operating characteristics curve (Figure 1C&D). Serum ATX activity above the cut-off of 27 nmol mL\(^{-1}\)min\(^{-1}\) were observed in 6% of women with pruritic dermatoses of pregnancy, 19% of women with HELLP-syndrome or pre-eclampsia and 7% of women with unaffected pregnancies. This cut-off resulted in sensitivities of 82%, 80%, and 80%, specificities of 90%, 90%, and 81%, and positive predictive values of 96%, 93%, and 93% to diagnose ICP from other pruritic dermatoses of pregnancy, pregnancy-related
liver disorders, and uncomplicated pregnancies, respectively. 100% specificity and 100% positive predictive value for the diagnosis of ICP for both groups could be reached using a higher cut-off value of 31 nmol mL$^{-1}$min$^{-1}$ which still had a remarkable sensitivity of 72%. Taken together, ATX represents a robust diagnostic marker for ICP in pregnant women.

<table>
<thead>
<tr>
<th></th>
<th>Pregnant controls (n=44)</th>
<th>Pre-eclampsia / HELLP-syndrome (n=17)</th>
<th>Pruritic disorders of pregnancy (n=33)</th>
<th>Intrahepatic cholestasis of pregnancy (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW (wks)</td>
<td>34.0 ± 4.4</td>
<td>33.2 ± 3.7</td>
<td>30.0 ± 6.5</td>
<td>35.3 ± 9.9</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>11.3 ± 4.4</td>
<td>327.5 ± 272.9</td>
<td>14.3 ± 7.0</td>
<td>104.4 ± 110.1</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>27.9 ± 5.8</td>
<td>278.6 ± 295.5</td>
<td>20.4 ± 5.2</td>
<td>64.6 ± 51.0</td>
</tr>
<tr>
<td>TBS (µmol/L)</td>
<td>2.9 ± 1.1</td>
<td>6.3 ± 5.1</td>
<td>4.1 ± 2.5</td>
<td>37.9 ± 40.7</td>
</tr>
<tr>
<td>ATX (nmol mL$^{-1}$ min$^{-1}$)</td>
<td>19.6 ± 5.4</td>
<td>16.8 ± 8.9</td>
<td>16.8 ± 6.7</td>
<td>43.5 ± 18.2</td>
</tr>
</tbody>
</table>

**Table 1:** Clinical features and serum chemistry of women with uncomplicated pregnancy, HELLP-syndrome, pruritic disorders of pregnancy and ICP. All values are expressed as mean ± SD. Abbreviations: GW = gestational week, ALT = alanine aminotransferase, AST = aspartate aminotransferase, TBS = total serum bile salts.

Similarly, the diagnostic value of total fasting serum bile salt levels and alanine aminotransferase was analysed (Supplementary Figure 2A&D). A cut of value of 10 µmol/L for TBS resulted in sensitivities of 75%, 74%, and 75%, specificities of 97%, 88%, and 100%, and positive predictive values of 98%, 93%, and 100% to diagnose ICP from other pruritic dermatoses of pregnancy, pregnancy-related liver disorders, and uncomplicated pregnancies, respectively (Supplementary Figure 2B+C).
Figure 1: Increased serum ATX activities and protein levels are specific for women with intrahepatic cholestasis of pregnancy. (A) ATX activities and (B) ATX protein levels were specifically increased in women with ICP but not pregnant women with HELLP-syndrome and/or pre-eclampsia, other pruritic disorders and women with uncomplicated pregnancy. (C&D): Non-parametric receiver operating characteristic curves for ATX activity resulted in high areas under the curve, distinguishing between women with intrahepatic cholestasis of pregnancy and HELLP-syndrome and pre-eclampsia, respectively (p<0.001) as well as between ICP and pruritic disorders of pregnancy (p<0.001).
For ALT, a cut of value of 35 U/L was associated with sensitivities of 70%, 71%, and 70%, specificities of 97%, 0%, and 100%, and positive predictive values of 98%, 69%, and 100% to diagnose ICP from other pruritic dermatoses of pregnancy, pregnancy-related liver disorders, and uncomplicated pregnancies, respectively (Supplementary Figure 2D+E). The areas under the curves of ROC (AUROCs) for ATX activity for the diagnosis of ICP vs. HELLP and vs. pruritic dermatoses of pregnancy were higher compared to the AUROCs of TBS (Supplementary Table 1), but did not reach significance when compared to TBS. However, combining ATX activity and TBS by multiple regression analysis significantly improved the diagnostic performance to diagnose ICP from uncomplicated pregnancies and pruritic dermatoses of pregnancy, the most interesting control group seen from a clinical perspective (Supplementary Figure 4).

Changes of ATX activity during gestation

To further elucidate the changes of ATX activity during pregnancy and prior to the development of symptoms such as pruritus, serum samples of pregnant women with a previously uncomplicated pregnancy and those with a history of ICP were collected in a longitudinal protocol. During uncomplicated pregnancy ATX activity is constantly rising from low levels during the first trimester to the highest levels in the third trimester (Figure 2A) which was in line with a previous report. In a following pregnancy, women with a history of ICP had serum ATX activities before onset of itching that were comparable to women with uncomplicated pregnancy (grey dots in Figure 2A). In these women serum ATX activity rose after onset of pruritus (black dots in Figure 2A). When serum samples before onset of pruritus (within 20.8 ± 8.0 gestational weeks), were compared with the first samples after onset of pruritus (within 29.3 ± 7.9 gestational weeks), a marked rise in serum ATX activity was observed (p<0.01; Figure 2B). In our cohort of patients, two pregnant women presented with ICP symptoms in the 10th and 12th gestational week, respectively. ATX activities were the highest (34.2 and 49.5 nmol mL⁻¹ min⁻¹, respectively) compared to all other pregnant women of that gestational age, indicating that this biomarker could also be helpful to diagnose ICP in rare early cases.
Figure 2: ATX activities during gestation in maternal and fetal blood. (A) Comparison of serum ATX activities in women during uncomplicated pregnancy (open circle) and pregnant women with a previous history of ICP before onset of pruritus (grey dots) and after onset of pruritus (black dots). Longitudinal studies during gestation revealed a marked rise in ATX activity in women with ICP particularly during the last trimester of pregnancy. (B) ATX activity markedly rose with the onset of pruritus in women with ICP. (C) Blood sampling months to years after child birth (41.6 ± 54.5 months) revealed increased serum ATX activity in women with a history of ICP compared to those with uncomplicated pregnancy. (D) The increased ATX activity in serum obtained from umbilical cord blood was comparable in newborn babies from women with uncomplicated pregnancy, HELLP-syndrome / pre-eclampsia and ICP. (E) No differences in ATX activity were observed in serum derived from arterial and venous umbilical cord blood.

Notably, ATX activity analyzed in non-pregnant women with a history of ICP (41.6 ± 54.5 months after child birth) remained at higher levels compared to non-pregnant women with previous uncomplicated pregnancies, indicating that genetic factors could be responsible for the increased ATX activities (Figure 2C). No correlation between length of period post-pregnancy and ATX activity was observed (data not shown). No differences could be observed in ATX activities from umbilical cord blood of newborn babies from women with uncomplicated pregnancy, pre-eclampsia complicated by HELLP-syndrome or ICP (Figure 2D).
ATX clearance by the fetus could be excluded as arterial and venous umbilical cord blood presented with comparable ATX activities (Figure 2E). Taken together, serum ATX activity increases markedly more in women with ICP during the course of pregnancy than in pregnant controls, independent of fetal ATX activity, and also remains elevated long after pregnancy.

**Oral contraception increases ATX activity**

In healthy, non-pregnant women, as well as men, serum ATX levels are tightly controlled and remain in a narrow range. Only very few conditions such as pregnancy have been reported in which ATX levels are increased.\(^\text{17}\) Thus, female sex hormones could be involved in the up-regulation of ATX. In order to investigate this aspect, we studied serum ATX activity in a group of 199 healthy volunteers. ATX activity was significantly higher in healthy females compared to age-matched healthy men (3.1 ± 1.6 nmol mL\(^{-1}\)min\(^{-1}\) vs. 2.5 ± 0.7 nmol mL\(^{-1}\)min\(^{-1}\), p<0.0001; Figure 3A) in agreement with a previous report.\(^\text{21}\) Intriguingly, however, this rise in ATX activity was caused by the subgroup of female controls that were using oral contraceptives (Figure 3B).
Figure 3: Increased ATX activity in female patients using oral contraceptive pills (OCP). (A) ATX activity was increased in healthy women compared to healthy men (p<0.001). (B) This increase in ATX activity was largely associated with the intake of oral contraceptive pills in women (p<0.001). (C) Hormonal changes during menstrual cycle did hardly influence circulating ATX levels in healthy, reproductive-age women.

When women taking oral contraceptives were excluded from the group of healthy females, the ATX activity was comparable to men (2.5 ± 0.7 nmol mL⁻¹ min⁻¹ vs. 2.6 ± 1.0 nmol mL⁻¹ min⁻¹, n.s.; Figure 3B). To exclude the possibility that hormonal changes during the menstrual cycle could have been responsible for differences in healthy females as well as in post-natal women with a history of ICP compared to women with previously uncomplicated pregnancy we sampled blood during the menstrual cycle of eight healthy women who were not using oral contraception. ATX activities remained unaltered during the menstrual cycle in all these women (Figure 3C). Thus, increased levels of female sex
hormones as seen during pregnancy or caused by oral hormone supplementation are associated with increased serum ATX activities.

**Increased ATX levels during ICP are not derived from placental tissue**

The source of increased serum ATX levels during ICP remain a matter of debate. High mRNA expression in human tissues has been described for brain, placenta, small intestine and ovary.\(^{23}\) We quantified ATX mRNA in placental tissue derived from women with uncomplicated pregnancies, pre-eclampsia complicated by HELLP-syndrome and ICP. Placental ATX expression was comparable between these groups (Supplementary Figure 3A). Thus, placental tissue does not contribute to increased ATX activity during ICP. Furthermore, intake of UDCA had no influence on ATX expression in placental tissue of women suffering from ICP (Supplementary Figure 3B). Still, ATX activity in serum dropped after start of 1-3 weeks of UDCA treatment (Supplementary Figure 3C).

**ATX is not influenced by food intake**

Serum fasting TBS levels are used as gold standard for the diagnosis of ICP as increased levels represent the earliest sign of a cholestatic liver disorder. However, this sensitive marker easily causes false positive test results upon oral food intake due to gallbladder contraction and re-uptake of bile salts from the gut lumen. Indeed, serum TBS levels rose markedly after food intake (Figure 4A) as described extensively in the past. In contrast, ATX activity had no circadian rhythm and was not increased after food intake as shown in healthy controls (Figure 4B). Hence, also in this context ATX is a more reliable marker for ICP than serum TBS.
The lysophospholipase D autotaxin represents the secreted form of ectonucleotidpyrophosphatases (ENPP2) and plays a critical role in diverse physiological conditions such as vascular and neuronal development, during pregnancy or for lymphocyte migration. The present study provides new insights into the role of ATX in normal pregnancy and pregnancy-related liver disease. Conditions that raise female steroid
hormones such as intake of oral contraceptives or uncomplicated pregnancy are associated with increased circulating ATX levels in healthy controls. In contrast, neither the regular menstrual cycle, nor oral food intake or day-night rhythm did affect serum ATX activity. In intrahepatic cholestasis of pregnancy (ICP), elevated serum ATX represents an accurate biomarker to differentiate ICP from other pregnancy-related disorders. Unexpectedly, the marked rise of ATX observed during ICP is derived from other sources than placental tissue.

The importance of ATX in fetal development is underlined by the fact that ATX-deficient mice are embryonically lethal due to vascular malformation and neuronal abnormalities. During pregnancy ATX serum levels have been shown to increase and correlate positively with gestation. Placental trophoblasts and syncytiotrophoblast were assumed to be the source of the increased ATX levels. However, we were unable to identify the increased serum ATX activities in fetal blood stream even in newborn babies of women suffering from ICP (see Figure 2E), despite the high levels in the maternal circulation. Thus, ATX secretion from trophoblasts may represent a unidirectional process towards the maternal, but not the fetal circulation. Alternatively, the marked increase in serum ATX activities during ICP may be derived from other tissue than placenta as no differences in mRNA and protein level could be observed in placental tissue from patients with ICP and healthy mothers. Thus, a yet to be defined source is responsible for increased ATX levels during ICP. As ATX levels are also increased in serum of patients with other cholestatic disorders, particularly in those suffering from pruritus, we hypothesize that a factor capable of increasing ATX expression (or reducing its clearance) accumulates in cholestatic patients. Further studies are warranted to identify this factor and the source of circulating ATX levels.

The present study indicates elevated ATX activity as a highly sensitive and specific biomarker to differentiate intrahepatic cholestasis of pregnancy from other pregnancy-related liver disorders or pruritic dermatoses. In contrast to the current gold standard for diagnosis, total fasting serum bile salt levels, ATX appeared as a very robust biomarker not being influenced by food intake nor by circadian rhythm. Thus, a serum ATX activity assay represents a potentially powerful test to reliably diagnose ICP, a disease that is associated
with increased risk of adverse fetal outcomes and can be effectively treated with UDCA and close obstetric monitoring.⁹

Here we also show that female steroid hormones contribute to enhanced activities of circulating serum ATX as indicated by marked differences between healthy women with or without hormone treatment. The observation that female steroid hormones modulate serum ATX levels is further supported by the increased serum ATX activities during the course of pregnancy. Further studies are required to identify the underlining molecular mechanisms.

Our study certainly needs confirmation in independent patient cohorts as our results are based on a limited number of patients. A number of other issues need to be addressed. First, the diagnostic value of serum ATX activity as a biomarker for ICP should be compared to that of serum ATX protein levels after development of a reliable quantitative test. Second, total fasting serum bile salt levels are currently regarded as the gold standard for diagnosis of ICP, but our data suggest that the determination of serum ATX activity is more accurate as an early marker of ICP. Thus, a combination of enhanced ATX activity and raised total fasting serum bile salt levels further improved the diagnostic accuracy for ICP in contrast to women with uncomplicated pregnant controls and pruritus gravidarum. In a small subset of samples UDCA reduced serum ATX activity shortly after start of treatment. However, the effect on pruritus could not be analyzed in the current cohort due to lack of quantification of itch intensity and remains to be evaluated in future prospective studies. Third, a cut-off value of 27 nmol mL⁻¹min⁻¹ (3.2 x upper limit of normal) showed optimal sensitivity and specificity as a biomarker for ICP using receiver operating characteristics curve analysis. This cut-off value requires extramural validation in subsequent large-scale studies. Fourth, our serum ATX activity assay may differ from those used in other studies. Thus, comparative studies of different ATX activity assays are needed. Finally, general application of such an assay would require the development of a simple enzymatic test, enzyme-linked immunosorbent assay (ELISA) or dye-coupled autotaxin antibodies. Similar to the common pregnancy test using the detection of human chorionic gonadotrophin in urine one could think of a technique using a drop of blood to detect a threshold level of ATX thereby diagnosing ICP.
Pruritus is a common symptom during pregnancy and is estimated to occur in up to 18% of all pregnancies.\textsuperscript{27} Itching is most frequently caused by specific dermatoses of pregnancy\textsuperscript{5} such as atopic eruption of pregnancy and polymorphic eruption of pregnancy, which in contrast to ICP are not associated with increased risk for adverse fetal outcomes.\textsuperscript{3,28} In addition, itching may also be caused by other skin and systemic disorders which coincide with pregnancy. A simple test which could distinguish ICP from these differential diagnoses could help general practitioners and gynaecologists to adequately treat these women. UDCA is regarded as first-line therapy of ICP and has been shown to improve maternal symptoms, serum liver tests, and placental abnormalities,\textsuperscript{1,2,8,29-31} although improvement of pruritus by UDCA seemed not to be clinically meaningful in a recent trial.\textsuperscript{32} About 80% of affected women develop pruritus after 30 weeks of gestation, but ICP has been reported as early as after 8 weeks of gestation.\textsuperscript{2,33} In our cohort there were two cases of ICP that presented already during weeks 10 and 12 of gestation. These unusual cases were characterized by a serum ATX activity above 31 nmol mL\textsuperscript{-1} min\textsuperscript{-1} as cut-off value for 100% specificity. Thus, particularly in atypical cases, ATX may represent a suitable biomarker to diagnose ICP.

In summary, our present study gives new insights in the role of ATX during pregnancy and pregnancy-related pathophysiological conditions. Raised female steroid hormones are associated with increased circulating ATX activities in healthy controls and during regular pregnancy, whereas the further rise of ATX during ICP is not derived from placental tissue. Furthermore, elevated serum ATX represents an accurate biomarker to diagnose ICP. A diagnostic test for ATX could facilitate diagnosis of ICP and enable early therapeutic intervention which may attenuate fetal and maternal morbidity as well as fetal mortality.
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REFERENCES

**SUPPLEMENTARY DATA**

**Supplementary Figure 1**

*Supplementary Figure 1: The number of previous pregnancies did not affect ATX activity. (A) ATX activity in women with ICP in regard to the number of pregnancies (first pregnancy (Н=14), one prior pregnancy (Н=19), two or more previous pregnancies (Н=13)). (B) ATX activity in women with uncomplicated pregnancies in regard to the number of pregnancies (first pregnancy (Н=12), one prior pregnancies (Н=8), two or more previous pregnancies (Н=6)).*
Supplementary Figure 2: Diagnostic value of total fasting serum bile salts (TBS) and alaninaminotransferase (ALT) levels. (A) TBS levels were specifically increased in women with ICP but not pregnant women with pre-eclampsia complicated by HELLP-syndrome, other pruritic disorders and women with uncomplicated pregnancy. (B&C) Non-parametric receiver operating characteristic curves for TBS levels resulted in high areas under the curve, distinguishing between women with intrahepatic cholestasis of pregnancy and pre-eclampsia complicated by HELLP-syndrome, respectively (p<0.001) as well as between ICP and pruritic disorders of pregnancy (p<0.001). (D) ALT levels were increased in women with ICP and pregnant women with pre-eclampsia complicated by HELLP-syndrome but not pruritic disorders or women with uncomplicated pregnancy. (E&F) Non-parametric receiver operating characteristic curves for ALT levels could distinguish between ICP and pruritic disorders of pregnancy (p<0.001) but not ICP and pre-eclampsia complicated by HELLP-syndrome.
Supplementary Figure 3: Placental expression of ATX is similar during regular pregnancy and under pregnancy-related pathophysiological conditions. (A) mRNA expression of ATX in placental tissue was unaltered in women with normotensive pregnancy, HELLP-syndrome / pre-eclampsia and ICP. (B,C) Oral intake of UDCA had no influence on placental ATX expression, but serum ATX activity dropped after 1-3 weeks after start of UDCA treatment.
Supplementary Figure 4: Comparison of areas under the curves of ROC analyses (AUROCs) of ATX (continuous black line), TBS (dashed dark grey line) and the combination of both parameters (dotted light grey line), indicating the superior diagnostic accuracy of the combined use of ATX activity and TBS concentrations in the diagnosis of ICP versus pregnant controls (p<0.05) and pruritus gravidarum (p<0.05).
Supplementary Table 1.

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*Supplementary Table 1. Comparison of areas under the curve of ROC analysis (AUROC) of TBS, ATX and their combination for the diagnosis of ICP from women with uncomplicated pregnancy (PC), HELLP-syndrome and pruritic disorders of pregnancy (PG). TBS = total fasting serum bile salts, 95% CI: 95% confidence interval; 1p (AUC): difference compared to null discriminatory value (c = 0.5); 2p (ATX vs TBS): difference between AUC of TBS and ATX; 3p (ATX plus TBS vs TBS): difference between AUC of TBS and ATX plus TBS.*