Molecular mechanisms of pruritus in cholestasis
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Serum Autotaxin Activity correlates with Pruritus in Pediatric Cholestatic Disorders

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ATX in paediatric cholestatic disorders
**ABSTRACT**

**Objective:** Pruritus is a common symptom of cholestatic liver disorders. The present study aimed at evaluating autotaxin, a lysophospholipase recently identified as potential cause for cholestatic pruritus, and total serum bile salt (TBS) levels in pediatric cholestatic diseases presenting with or without itching.

**Methods:** A cohort of 48 children consisting of patients with Alagille syndrome (n=12), biliary atresia (n=2), neonatal sclerosing cholangitis (n=1), progressive familial intrahepatic cholestasis type 2 (n=1), bilhemia (n=1), bile salt-synthesis defects (3β-hydroxy-C27-steroid-oxidoreductase (n=7) and D4-3-oxosteroid-5β-reductase deficiency (n=2)) and healthy children (n=22) were studied. Serum ATX activity and TBS were determined enzymatically, ATX protein content was semi-quantified by western blotting.

**Results:** Pruritus was present in most children (14 out of 17) with pediatric cholestatic disorders, but not reported in those with bile salt-synthesis deficiencies. Serum ATX activity was increased in pruritic children with Alagille and other pruritic cholestasis syndromes (mean±SD: 16.1±4.3 nmol mL⁻¹ min⁻¹) compared to non-pruritic cholestatic children with bile salt synthesis defects (10.4±4.7 nmol mL⁻¹ min⁻¹; p<0.01) and healthy controls (7.6±2.3 nmol mL⁻¹ min⁻¹; p<0.001). ATX protein levels closely correlated with serum ATX activity. Serum ATX activity showed a linear correlation with itch intensity (r=0.66, p<0.001). No correlation was observed between ATX activity and TBS or bilirubin. ATX mRNA expression in HepG2 cells was not induced by FXR ligands.

**Conclusions:** Serum ATX activity correlated with itch intensity in cholestatic children. Bile salts neither correlated with presence of pruritus nor increased ATX expression in vitro. ATX inhibitors may be useful antipruritic agents in paediatric cholestatic disorders.
INTRODUCTION

Chronic pruritus is a frequent and often agonizing symptom accompanying various cutaneous and systemic disorders. It is frequently observed in patients with various hepatobiliary disorders, particularly those with cholestatic features. Beside cholestatic disorders of adulthood associated with pruritus e.g. primary biliary cirrhosis or primary sclerosing cholangitis, itching is also commonly reported in children suffering from pediatric cholestatic disorders such as Alagille syndrome (AGS), biliary atresia (BA), or progressive familial intrahepatic cholestasis (PFIC). Conversely, pruritus is not observed in children with bile salt synthesis defects (BASD) despite chronic cholestasis. Pruritus may be mild and tolerable, but it may also dramatically reduce quality of life by causing severe sleep deprivation, by preventing the child from focusing on activities such as game or school and by resulting in depressive mood. It is often regarded by parents as the most incapacitating symptom.

Various substances among which are histamine, bile salts, endogenous opioids and progesterone metabolites have been proposed with limited evidence as pruritogens. None of these substances is likely to represent the unique causal pruritogen as serum and/or tissue levels could not be correlated with itch intensity. By functional screening of sera of cholestatic patients suffering from pruritus on neuronal cells we recently identified lysophosphatidic acid (LPA) as a potent neuronal activator. LPA levels were raised in cholestatic patients suffering from pruritus. Intradermal injection of LPA caused a dose-dependent scratch response in mice. Circulating LPA is synthesized by the lysophospholipase autotaxin (ATX) which hydrolyses the choline group from lysophosphatidylcholine. ATX is considered the main source of circulating LPA. In line with the observed increase in LPA, ATX activity was higher in sera of cholestatic patients with pruritus compared to those without pruritus. Furthermore, ATX activity strongly correlated with itch intensity and response to therapy.

ATX is a secreted large glycoprotein and represents the second member of the family of ectonucleotide pyrophosphatases / phosphodiesterases (ENPP1–7). Initially identified as cell motility factor secreted from melanoma cells, ATX (= ENPP2) has been
implicated in various (patho)physiological processes including cell survival, proliferation, differentiation, and migration.\textsuperscript{14,15} It is also involved in vascular and neural development, lymphocyte homing, wound healing, and neuropathic pain.\textsuperscript{14,15} The effects of ATX are largely mediated by the enzymatic formation of LPA which can bind to at least six different G-protein-coupled receptors (LPA\textsubscript{1-6}).\textsuperscript{10,16}

Here, we studied ATX levels in pediatric cholestatic disorders and correlated ATX activity with itch intensity as well as other laboratory parameters. The effect of lacking bile salts was analyzed in children with bile salt synthesis defects. Furthermore, we tested the effect of FXR ligands on ATX expression \textit{in vitro}.

\section*{Materials and Methods}

\textbf{Human subjects.} Peripheral venous whole blood samples were collected from children with chronic cholestatic diseases (n=26) and healthy children (n=22) who were seen at the Pediatric Department of Bicêtre Hospital, University Paris-Sud, France. Patients with chronic cholestatic diseases consisted of 9 children with BASD (3\textbeta-Hydroxy-C27-steroid-oxidoreductase (n=7) and \Delta4-3-oxosteroid-5\textbeta-reductase deficiency (n=2)) which are not/never associated with pruritus, 16 children suffering from chronic cholestatic diseases (AGS n=12, BA n=2, neonatal sclerosing cholangitis n=1 and PFIC2 n=1) and 1 liver transplanted child with bilhemia not suffering from pruritus. Out of 16 patients with chronic cholestatic diseases 14 children exhibited itching while two patients with AGS did not experience pruritus. Out of 16 patients with chronic cholestatic diseases received oral UDCA at a daily dose of 600 mg m\textsuperscript{-2} d\textsuperscript{-1} whereas sera from the 9 children with BASD were collected prior to start of cholic acid therapy (bile salt supplementation). Those 14 children suffering from pruritus additionally received rifampicin therapy up to 20 mg kg\textsuperscript{-1} d\textsuperscript{-1}. Blood drawing was performed during ongoing treatment. Children with liver disorders and healthy volunteers were only enrolled after receiving informed consent by a parent or legal representative. The study was conducted according to the guidelines of the local Medical Ethical Committee.
Blood samples were allowed to clot for an hour before they were centrifuged for 10 minutes at 1000 g and 4°C. The serum supernatant was aliquoted and cryopreserved at -20°C until measurements were performed. Itch intensity was quantified by the medical staff and the parents using a 3-point scale ranging from 0 to 2; 0 indicating no pruritus, 1 indicating mild pruritus and 2 indicating severe pruritus.

**Materials.** Choline oxidase, horseradish peroxidase, homovanillinic acid, dimethylsulfoxide (DMSO), chenodeoxycholate (CDC) and GW4064 were purchased from Sigma-Aldrich (St. Louis, MO); myristoyl-lysophosphatidylcholine (LPC 14:0) was from Avanti Polar Lipids (Alabaster, AL).

**Cell culture.** Human HepG2 hepatoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Lonza BioWhittaker, Cologne, Germany) supplemented with 10% fetal calf serum, 4 mM of L-glutamine, and a mixture of antibiotics (5 mg/mL of penicillin and 5 mg/mL of streptomycin). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. For studying the effect of FXR ligands, cells were seeded in six-well plates at a density of 8 x 10⁵ cells/well until reaching 80% confluence. After brief washing, cells were incubated for 4 hours in DMEM/0.2% bovine serum albumin containing solvent control (0.1% Dimethylsulfoxide), 100 μM CDC or 1 μM GW4064.

**RNA isolation and quantification of transcript levels.** Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized from 2 μg total RNA with an oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). Real-time PCR were performed in a Lightcycler apparatus (Roche, Mannheim, Germany) using Lightcycler Faststart DNA Master Plus CYBR Green I (Roche). Transcript levels were normalized to housekeeping gene 36b4 (acidic ribosomal phosphoprotein P0). The following primer sequences were used: ATX forward: TGCAATAGCTCAGAGGACGA; ATX reverse: AGAAGTCCAGGCTGGTGAGA; 36B4
forward: TCATCAACGGTACAAACGA; 36B4 reverse: GCCTTGACCTTTTCAGCAAG. SHP forward: CGCCCTATCATTGGAGATGT; SHP reverse: TGTCTATACAGGCTTGCCCC.

**Autotaxin activity assay.** ATX activity was quantified as recently described.\(^{17}\) 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 as the amount of liberated choline which was assayed using an enzymatic fluorimetric method. Samples were added to a buffer containing choline oxidase (2 U/mL), horseradish peroxidase (1.6 U/mL), and homovanillnic acid as substrate for peroxidase. The increase in fluorescence was monitored at 37°C on a Novostar analyzer.

**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.

**Determination of FGF19.** Serum FGF19 levels were determined using a sandwich enzyme-linked immunosorbent assay specific for FGF19 as described.\(^{18}\)

**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)\(^{11}\) bound to protein G-sepharose for 4 hours at 4°C. After washing, 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 antibody (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). Spearman’s correlation coefficient and corresponding p-values were calculated to assess the relationship between tested parameters. All data are expressed as means ± standard deviations (SD).

**RESULTS**

**ATX level is increased in patients with pediatric cholestatic diseases suffering from pruritus**

Serum ATX activity (mean ± SD) was markedly higher in children with cholestatic diseases suffering from pruritus (16.1±4.3 nmol mL⁻¹ min⁻¹, n=14, p<0.0001) than in cholestatic children with bile salt synthesis defects (10.4±4.7 nmol mL⁻¹ min⁻¹, n=9), or healthy children (7.6±2.3 nmol mL⁻¹ min⁻¹, n=22) of comparable age (Figure 1A, for patient characteristics, see Table 1). ATX activity was comparable in children with either 3β-hydroxy-C27-steroid-oxidoreductase (n=7) and Δ4-3-oxosteroid-5β-reductase deficiency (n=2; Figure 1B). Our cohort of pediatric cholestatic disorders consisted of Alagille syndrome, biliary atresia, progressive familiar intrahepatic cholestasis type 2, neonatal sclerosing cholangitis and bilhemia. Irrespective of the underlying disorder, ATX activity was increased only in children suffering from pruritus (Figure 1C). Enhanced ATX activity correlated with increased ATX protein content in sera from children with pediatric cholestatic diseases (Figure 1D). Taken together, ATX levels are increased in those children with pediatric cholestatic disorders suffering from pruritus.
Figure 1: Increased serum ATX activities and protein levels in children with pediatric cholestatic disorders associated with pruritus. (A) ATX activities were specifically increased in children suffering from pruritus but not in children with bile salt synthesis syndromes or healthy children. ***p<0.001 (ANOVA); n.s. = not significant. (B) Children with the either 3β-hydroxy-C27-steroid-oxidoreductase (3β-HSD; n=7) or Δ4-3-oxosteroid-5β-reductase deficiency (Δ4-3oxo-R, n=2) had comparable ATX activities. (C) Increased ATX activity was observed in children with pruritus with cholestatic disorders irrespective of the underlying disease. (D) ATX protein levels paralleled ATX activity and was increased in three children with Alagille syndrome suffering from pruritus compared to three children with bile acid deficiency and three healthy children. Recombinant ATX (rATX) was used as a positive control. HC: healthy controls; BSD: bile salt synthesis defects, PFIC2: progressive familiar intrahepatic cholestasis type 2, NSC: neonatal sclerosing cholangitis.

ATX activity correlates with itch intensity

Itch intensities on a 3-point-scale for children were correlated with ATX activity measured in serum of these children by linear regression analysis. A linear correlation between enzymatic activity and itch intensity was found (r=0.66, p<0.001; Figure 2A). Even after exclusion of non-itching children with bile salt synthesis defects, a linear correlation was still observed between ATX activity and itch intensity (r=0.49, p<0.05; Suppl. Figure 1A). A correlation could also be observed for TBS and itch intensity which is not surprising as all bile salt synthesis defect children did not suffer from pruritus (r=0.58, p<0.01, Supp. Figure 1B). This correlation was, however, lost if these children were excluded (Suppl. Figure 1C). No correlation was observed between itch intensity and total bilirubin levels (data not shown).
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Table 1: Clinical data and serum chemistry of children with bile salt synthesis defects and children with pediatric cholestatic disorders suffering from pruritus. All values are expressed as mean ± SD. Abbreviations: GGT = gamma-glutamyltransferase, TBS = total serum bile salts. Abbreviations: GGT = gamma-glutamyltransferase, TBS = total serum bile salts, n.d. = not determined; p-values: healthy children vs. bile salt synthesis defects: *p<0.01; healthy children vs. Alagille syndrome and other cholestasis syndromes: †p<0.01; bile salt synthesis defects vs. Alagille syndrome and other cholestasis syndromes: ‡p<0.01.

Furthermore, ATX activity showed no correlation with extent of cholestasis indicated by total serum bile salts or total bilirubin levels (Figure 2B+C). As expected, children with bile salt synthesis defects presented with low total serum bile salts, whereas these levels were strongly increased in other pediatric cholestatic diseases independent of the presence of pruritus (Figure 2D). Similarly, FGF19 levels were low in children with bile salt synthesis defects as compared to children with pediatric cholestatic disorders with or without pruritus (Figure 2E), indicating that no oral bile salt substitution took place.
ATX expression is not induced by FXR ligands

Bile salts have long been discussed as potential pruritogen in pruritus of cholestasis.\textsuperscript{3,7} To further elucidate the effect of bile salts on ATX mRNA expression, we quantified ATX mRNA in HepG2 cells treated with the FXR ligands CDC (100 μmol/L) and GW4064 (1 μmol/L) for 4 hours (Figure 3A) and 24 hours (data not shown). ATX mRNA expression was unaltered by FXR agonists, whereas the established FXR target short heterodimer partner (SHP) showed an increased expression (Figure 3B). This suggests that bile salts do not directly contribute to increased ATX expression, although FXR-independent effects of bile salts cannot be ruled out.
Figure 3: FXR agonists do not induce expression of ATX. (A) mRNA expression of ATX in HepG2 cells was unaltered by addition of 100 μM CDCA (an endogenous FXR agonist) or 1 μM of the FXR agonist GW4064. (B) mRNA expression of SHP, used as a positive control for FXR-mediated gene induction, was increased after addition of both compounds. Results of three independent experiments are shown. *p<0.05; n.s.: not significant.

**DISCUSSION**

Pruritus is a well-known feature of various, mainly cholestatic hepatobiliary disorders.3,7 Beside adult patients, children with paediatric cholestatic diseases such as the Alagille syndrome, biliary atresia, or progressive familial intrahepatic cholestasis frequently suffer from itching which in more severe cases may dramatically reduce their quality of life.4,19,20 In contrast, children with bile salt synthesis defects do not report itching despite ongoing cholestasis.5 Many parents have reported that pruritus is the most incapacitating symptom of their child’s chronic liver disorder.6 Treatment of cholestatic pruritus in these children is often not fully effective and unsatisfactory which is mainly due to limited therapeutic options as well as the incomplete understanding of the underlying mechanisms. The present study provides new insights into the role of ATX in pediatric cholestatic disorders. Although cholestatic children with low TBS levels due to inborn errors of bile salt synthesis did not report itching, the presence of pruritus in other pediatric cholestatic disorders was independent of increased TBS levels. In contrast, increased levels of ATX were associated with the presence of pruritus and correlated with its severity. In vitro, ATX mRNA expression was not directly affected by activation of the bile salt receptor FXR.
We have previously reported that rifampicin decreased serum ATX activity in adults suffering from cholestatic pruritus.\textsuperscript{12} In the present study, all children suffering from pruritus received rifampicin. Despite therapy, ATX activity was increased in these children compared to those children without pruritus. It remains to be determined whether rifampicin decreases ATX activity in pediatric cholestatic diseases and whether the decrease in ATX activity correlates with rifampicin doses used in cholestatic children.

Bile salts have been controversially discussed as potential pruritogens in cholestasis for many decades.\textsuperscript{3,7} So far, no correlation between the concentration of any naturally occurring bile salt in the circulation, urine or skin and severity of pruritus could be demonstrated.\textsuperscript{3,7,21} The G-protein coupled receptor for hydrophobic bile salts, TGR5, was recently suggested to be involved in the development of cholestatic pruritus.\textsuperscript{22} TGR5 was shown to be expressed on murine sensory neurons in dorsal root ganglia (but not in the skin) and TGR5 agonists such as deoxycholate (DCA) were capable of activating these neurons. Indeed, intradermal injection of high concentrations of DCA evoked scratch responses in wild-type mice which was attenuated in TGR5\textsuperscript{−/−} mice and augmented in TGR5 transgenic mice. However, the applied concentrations of these hydrophobic bile salts were far beyond the pathophysiological levels observed in cholestatic disorders such as primary biliary cirrhosis, intrahepatic cholestasis of pregnancy or progressive familiar intrahepatic cholestasis which are associated with pruritus. These disorders are characterized by a depleted DCA pool size\textsuperscript{23} with barely detectable concentrations of unconjugated DCA in serum and bile. Still, other agonists of TGR5 such as neurosteroids might be capable of activating this receptor leading to itch sensation. Notably, progesterone has recently been shown to activate TGR5 in placental tissue in a dose-dependent manner.\textsuperscript{24}

The lysophospholipase D autotaxin belongs to the family of ectonucleotide pyrophosphatases / phosphodiesterases (ENPP2). This enzyme plays a critical role in diverse physiological conditions including vascular and neuronal development, during pregnancy or lymphocyte migration and pathophysiological states such as cholestatic pruritus, neuropathic pain, cardiovascular diseases, pulmonary fibrosis, cancer development and formation of metastases.\textsuperscript{15,25} Significant levels of ATX mRNA was detected in various tissues and organs such as brain, adipose tissue, lung, liver, intestine, kidney, ovary and
high endothelial venules.\textsuperscript{26} The source(s) of circulating ATX has so far not been established. ATX seems to be cleared from plasma by scavenger receptors of liver sinusoidal endothelial cells,\textsuperscript{27} but is not secreted into bile.\textsuperscript{8} However, if the enterohepatic circulation is interrupted by nasobiliary drainage, circulating levels of ATX rapidly dropped concomitant with relief of pruritus,\textsuperscript{6,10} indicating that a factor in bile is responsible for the increased autotaxin levels. Recently, we could show that conditions associated with a strong increase in female steroid hormones such as hormone treatment or regular pregnancy result in increased circulating ATX levels. In contrast, neither the regular menstrual cycle, nor oral food intake or day-night rhythm did affect serum ATX activity. In the present study we show that activation of the bile salt sensor FXR did not directly alter ATX expression \textit{in vitro}. Thus, further studies on the direct or indirect induction of ATX expression by compound(s) present in the enterohepatic circulation are warranted.

In summary, our present study gives new insights in the role of ATX in pruritus related to pediatric cholestatic diseases. Serum ATX activity but not TBS levels correlate with presence and intensity of pruritus in these children. These data provide further clinical and experimental evidence that LPA receptor blockers and ATX inhibitors may represent future therapeutic agents for this often insufficiently treatable symptom.

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Supplementary Figure 1: (A) ATX activity showed a linear correlation with itch intensity on a 3-point scale developed for children even if children with bile salt synthesis defects were excluded. Spearman correlation coefficient: $r=0.49$, $p<0.05$; $n=15$ (2 children were excluded due to age of 2 months). (B+C) TBS showed a linear correlation with itch intensity (Spearman correlation coefficient: $r=0.58$, $p<0.01$) which was lost if children with bile salt synthesis defects were excluded.