Dissertation

Role of ‘Atypical’ Bile Acids in Itch

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DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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ABBREVIATIONS

AIH	Autoimmune hepatitis
ALGS	Alagille syndrome
ALT	Alanine aminotranferase
AMCA	α-muricholic acid
AP	Alkaline phosphatase
ASBT	Apical Na^+ -dependent bile salt transporter
ASC	Autoimmune sclerosing cholangitis
AST	Aspartat aminotransferase
ATX	Autotaxin
BA	Bile acid(s)
BACS	BA-CoA synthetase
BAT	BA-CoA amino acid N-acetyltransferase
BMCA	β-muricholic acid
BRIC	Benign recurrent intrahepatic cholestasis
BSEP	Bile salt export pump
CA	Cholic acid
CAR	Constitutive androstane receptor
CCLD	Chronic cholestatic liver disease(s)
CDCA	Chenodeoxycholic acid
Cl^-	Chloride
cPA	Cyclic phosphatic acid
CUO	Cholestasis of unknown origin
CYP27A1	Sterol-27-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol-7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
DCA	Deoxycholic acid
DRG	Dorsal root ganglia
EHC	Enterohepatic circulation
ELISA	Enzyme-linked Immunosorbent Assay
EOS	Early onset sepsis

ERCP	Endoscopic retrograde cholangiopancreatography
ESI	Electrospray ionization
F	female
FDF15	Fibroblast growth factor 15
FGF19	Fibroblast growth factor 19
FGFR4	Fibroblast growth factor receptor 4
FXR	Farnesoid X receptor
G	Glycine
GGT	γ-glutamyltranspeptidase
GMCA	γ-muricholic acid
GPCRs	G-protein-coupled receptors
HCC	Hepatocellular carcinoma
HCO_3^-	Bicarbonate
HDCA	Hyodeoxycholic acid
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry
HSD3B7	3β-hydroxy-Δ5-C27 steroid oxidoreductase
I-BABP	Intestinal BA-binding protein
IBAT	Ileal bile salt transporter
IBD	Inflammatory bowel disease
ICP	Intrahepatic cholestasis of pregnancy
INR	International Normalized Ratio
IQR	Interquartile range
ISBT	Ileal bile salt transporter
LCA	Lithocholic acid
LPA	Lysophosphatidic Acid
LPC	Lysophosphatidylcholine
LTX	Liver transplantation
M	Male
MARS	Molecular Adsorbent Recirculation System
MCA	Muricholic acid(s)
MDR1	Multidrug export pump 1
MDR2	Multidrug export pump 2

MDR3	Multidrug export pump 3
Mon	months
MRCP	Magnetic resonance cholangiopancreatography
MRCP	Magnetic resonance cholangiopancreatography
Mrgprs	Mas-related G-protein-coupled receptors
MRP2	Multidrug resistance associated protein 2
MRP3	Multidrug resistance associated protein 3
MRP4	Multidrug resistance associated protein 4
N/A	Not available
Na^+	Sodium
NTCP	Na^+-taurocholate cotransporter
OATPs	Organic-anion-transporting-peptides
OMCA	ω-muricholic acid
OSTα/OSTβ	Organic-solute-transporter
PAR	Protease-activated receptors
PBC	Primary biliary cirrhosis
PCOS	Polycystic ovarian syndrome
PEBD	Partial external biliary diversion
PFIC	Progressive familial intrahepatic cholestasis
PSC	Primary sclerosing cholangitis
PT	Prothrombin time
PVAS	Visual analogue scale of pruritus
PXR	Pregnane X receptor
S1P	Sphingosine 1-phosphate
SC	Sclerosing cholangitis
sFlt-1/PlGf	Soluble Fms-like tyrosinkinase-1/placental growth factor
SHP	Small heterodimer partner
SNPs	Single nucleotide polymorphisms
SSRI	Serotonin reuptake inhibitor
St.p.	Status post
T	Taurine
tBA(s)	Total bile acid(s)
TGR5	G-protein coupled bile acid receptor 1

tMCA	Total muricholic acid(s)
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
U:T ratio	Unconjugates to total bile acid ratio
UDCA	Ursodeoxycholic acid
Y	Year

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ABSTRACT

Background: Pruritus is a common symptom in pediatric and adult patients with chronic cholestatic liver disease (CCLD) like autoimmune sclerosing cholangitis (ASC) and progressive familial intrahepatic cholestasis (PFIC), and the key clinical finding in intrahepatic cholestasis of pregnancy (ICP). Increased bile acid (BA) levels and enhanced autotaxin (ATX) activity are considered to play a pathophysiological role in cholestatic pruritus amongst others, however, the exact etiology is still unknown. Besides the typical human BA, the appearance of 'atypical' muricholic acids (MCA) in pediatric and adult CCLD patients has been observed. This study aimed to determine total BA (tBA), total MCA (tMCA) levels and profiles of BA/MCA isoforms in serum of pediatric pruritic and non-pruritic ASC and PFIC patients and of pregnant women with and without ICP. ATX antigen levels were studied in serum of all study groups. We hypothesized that a rise of specific BA/MCA correlates with a surge in plasmatic ATX levels.

Methods: We determined serum human BA, MCA, and ATX antigen levels in 27 pediatric CCLD patients aged 1-18 years (ASC n=20 [pruritus n=6, without pruritus n=14]; PFIC n=7 [pruritus n=5, without pruritus n=2]) and 23 healthy age-matched controls as well as in 19 ICP patients and 20 healthy pregnant controls. BA profiling was performed using high-performance liquid chromatography-tandem mass spectrometry. ATX antigen levels were determined using a commercial ELISA. Pediatric patients ≥ 5 years of age were asked to rate their severity of pruritus by using a visual analogue scale of pruritus (PVAS).

Results: ASC- and PFIC patients showed significantly higher tBA (ASC: median: 42.4 µmol/L, interquartile range [IQR]: 18.5-100.1; PFIC median: 262.6 µmol/L, IQR: 32.3-451.7) and tMCA levels, (ASC: median: 0.59 µmol/L, IQR: 0.16-1.00; PFIC: median: 7.39 µmol/L, IQR: 1.31-37.13) than healthy controls (tBA: median: 1.7 µmol/L, IQR: 0.9-3.6; ASC $p<0.0001$; PFIC $p<0.0001$; tMCA: (median: 0.05 µmol/L, IQR: 0.001-0.16, ASC $p=0.0006$; PFIC $p<0.0001$). ATX antigen levels were only increased in children with PFIC (median: 1650 ng/ml, IQR: 776.9-3742) when compared to control subjects (median: 315.9 ng/ml, IQR: 251.1-417.2; PFIC $p=0.0003$). In patients with pruritic ASC, tBA levels were only slightly enhanced (median: 76.5 µmol/L, IQR: 54.7-205). However, they showed a predominance of taurine (T) -conjugates (median: 16.4 µmol/L, IQR: 8.9-41.4) and tMCA levels (median: 1.15 µmol/L, IQR: 0.77-2.44) than non-pruritic ASC patients (tBA median: 24.3 µmol/L, IQR: 16.2-80.8; $p<0.0408$; T-conjugates median: 1.3 µmol/L, IQR: 0.8-4.9; $p=0.0023$; tMCA median: 0.30 µmol/L, IQR: 0.13-

0.64, p=0.0033). BA and MCA profiles were distinctly different in pruritic patients compared to non-pruritic patients. Different to PFIC patients, we found no statistically significant surge of ATX antigen levels in both, pruritic- (median: 665.8 ng/ml, IQR: 357.8-1203) and non-pruritic ASC patients (median: 391.0 ng/ml, IQR: 283.2-485.6).

In ICP patients, tBA levels were significantly enhanced compared to healthy pregnant controls (tBA-median: 3.7 µmol/L, IQR: 1.8-7.8 vs. 1.7 µmol/L, IQR: 1.2-3.1; p=0.0063). However, tBA levels in ICP patients were significantly lower than in pediatric pruritic and non-pruritic ASC and PFIC patients (ICP vs. ASC: p=0.0033; ICP vs. PFIC: p=0096). TMCA levels were comparable in ICP patients and in pregnant controls but more scattered in the latter group (tMCA-median: 0.1 µmol/L, IQR: 0.05-0.4 vs. 0.07 µmol/L, IQR: 0.03-0.11). In ICP patients and pregnant controls, ATX antigen levels were also increased, however, without a statistical significance between the groups (p=0.1771). Compared to all pediatric study groups, ATX antigen levels were significantly increased in ICP patients as well as in pregnant controls. Moreover, ATX antigen levels did not correlate with tBA or tMCA levels in ICP patients.

Conclusion: In pruritic ASC patients, BA and MCA profiles were significantly different in comparison to non-pruritic ASC patients. Different to “typical” BA, MCA show a vastly surge in pruritic ASC patients compared to non-pruritic ASC patients. Our results suggest that ATX antigen levels are only of little prognostic and diagnostic relevance have in ASC patients. Moreover, in pruritic ASC patients, not only ATX activity seems causal for pruritus genesis. Furthermore, The significant elevation of ATX antigen levels in ICP patients and healthy pregnant controls despite significantly lower tBA/tMCA levels argues for additional factors besides BA/MCA inducing a surge in ATX levels. It may be that the main source of ATX in pregnant woman is the placenta whereas in CCLD, ATX is mainly built by the liver. In ICP, tBA might not correlate with ATX activity as ATX still is the overwhelmingly source of the placenta in these states.

ZUSAMMENFASSUNG

Hintergrund: Bei Kindern und Erwachsenen sind chronisch cholestatische Lebererkrankungen (CCLD), wie autoimmun sklerosierende Cholangitis (ASC) und progressiv familiäre intrahepatische Cholestase (PFIC), aber auch die intrahepatische Cholestase der Schwangerschaft (ICP), mit Pruritus assoziiert. Vermutlich spielen erhöhte Gallensäure (GS)-Spiegel im Serum und eine gesteigerte Autotaxin (ATX)- Aktivität eine Rolle bei der Juckreizentstehung bei CCLD. Die genaue Pathophysiologie ist unbekannt. Neben den humanen GS konnten bei CCLD sog. Muricholsäuren (MCA) nachgewiesen werden. Ziel dieser Studie war es die Gesamt-GS (tGS)- und Gesamt-MCA (tMCA)-Spiegel und die GS/MCA Profile im Serum und die ATX-Antigen Spiegel von pädiatrischen ASC-/PFIC PatientInnen mit und ohne Pruritus bzw. von ICP Patientinnen zu bestimmen. Die Ausgangshypothese ist, dass der Anstieg spezifischer GS/MCA mit einer gesteigerten ATX-Aktivität korreliert.

Methodik: Wir untersuchten die tGS/tMCA und ATX Antigen-Spiegel im Serum von 27 pädiatrischen CCLD PatientInnen zwischen 1-18 Jahren (ASC n=20 [Pruritus n=6, kein Pruritus n=14]; PFIC n=7 [Pruritus n=5, kein Pruritus n=2]) plus 23 gesunde, altersgematchte Kontroll-PatientInnen, sowie von 19 ICP Patientinnen plus 20 gesunden Schwangeren. Die tGS/tMCA wurden mittels Hochleistungschromatografie plus Massenspektrometrie bestimmt. Die ATX Antigen Level wurden mittels ELISA gemessen. Die Juckreizintensität wurde bei pädiatrischen PatientInnen mit Pruritus über einen Visual Analogue Scale für Pruritus (PVAS) erhoben sofern diese ≥5 Jahre alt waren.

Ergebnisse: ASC- und PFIC PatientInnen hatten signifikant höhere tGS (ASC: Median: 42.4 µmol/L, Interquartilsbereich [IQR]: 18.5-100.1; PFIC Median: 262.6 µmol/L, IQR: 32.3-451.7) und tMCA Spiegel (ASC: Median: 0.59 µmol/L, IQR: 0.16-1.00; PFIC: Median: 7.39 µmol/L, IQR: 1.31-37.13) als die Kontrollgruppe (tGS: Median: 1.7 µmol/L, IQR: 0.9-3.6; ASC $p<0.0001$; PFIC $p<0.0001$; tMCA: (Median: 0.05 µmol/L, IQR: 0.001-0.16, ASC $p=0.0006$; PFIC $p<0.0001$). Anders als ASC PatientInnen hatten PFIC PatientInnen signifikant erhöhte ATX Antigen Spiegel (Median: 1650 ng/ml, IQR: 776.9-3742) verglichen mit der altersgematchten Kontrollgruppe (Median: 315.9 ng/ml, IQR: 251.1-417.2; $p=0.0003$). Bei ASC PatientInnen mit Pruritus zeigten sich nur ein gering ausgeprägter Anstieg der tGS (Median: 76.5 µmol/L, IQR: 54.7-205), aber deutlich erhöhte Taurin (T) -konjugierte GS (Median: 16.4 µmol/L, IQR: 8.9-41.4) und tMCA-Spiegel (Median: 1.15 µmol/L, IQR: 0.77-2.44) als bei ASC

PatientInnen ohne Pruritus (tGS Median: 24.3 µmol/L, IQR: 16.2-80.8; p<0.0408; T-konjugierte GS Median: 1.3 µmol/L, IQR: 0.8-4.9; p=0.0023; tMCA Median: 0.30 µmol/L, IQR: 0.13-0.64, p=0.0033). Die GS/MCA Profile unterschieden sich stark in Abhängigkeit von vorliegendem Juckreiz bei den ASC PatientInnen. Die ATX-Antigen Spiegel waren bei ASC PatientInnen mit (Median: 665.8 ng/ml, IQR: 357.8-1203) und ohne Juckreiz (Median: 391.0 ng/ml, IQR: 283.2-485.6) nicht signifikant erhöht.

Bei ICP Patientinnen waren die tGS im Vergleich zu gesunden Schwangeren signifikant erhöht (tGS-Median: 3.7 µmol/L, IQR: 1.8-7.8 vs. 1.7 µmol/L, IQR: 1.2-3.1; p=0.0063). Im Vergleich zu pädiatrischen ASC-/PFIC PatientInnen waren die tGS-Spiegel der ICP PatientInnen allerdings signifikant niedriger (ICP vs. ASC: p=0.0033; ICP vs. PFIC: p=0096). Die tMCA-Spiegel zwischen ICP Patientinnen und den gesunden Schwangeren unterschieden sich nicht signifikant (tMCA-Median: 0.1 µmol/L, IQR: 0.05-0.4 vs. 0.07 µmol/L, IQR: 0.03-0.11). Bei den ICP Patientinnen und bei der Schwangeren-Kontrollgruppe zeigten sich erhöhte ATX Antigen-Spiegel ohne signifikante Unterschiede innerhalb der Gruppen (p=0.1771). Verglichen mit den pädiatrischen PatientInnen, waren die ATX Antigen-Spiegel bei den ICP Patientinnen und den gesunden Schwangeren signifikant erhöht.

Schlussfolgerung: Trotz derselben zugrundeliegenden Erkrankung zeigten sich bei ASC PatientInnen mit Juckreiz signifikant unterschiedliche BA-/MCA Profile als ASC PatientInnen ohne Juckreiz. Im Gegensatz zu „typischen“ GS, kam es bei ASC PatientInnen mit Pruritus zu einem erheblichen Anstieg der MCA, welcher bei ASC PatientInnen ohne Pruritus nicht auftrat. ATX-Antigen Spiegel scheinen bei ASC PatientInnen mit Juckreiz nur von geringer diagnostischer und prognostischer Bedeutung zu sein. Eine erhöhte ATX Aktivität allein scheint nicht kausal für die Entstehung von cholestatischem Pruritus zu sein. Die Erhöhung der ATX Antigen-Spiegel bei ICP Patientinnen und auch gesunden Schwangeren trotz signifikant niedrigerer tGS-/tMCA-Spiegel spricht für zusätzliche Faktoren, die zu einer erhöhten ATX-Aktivität. Eine mögliche Erklärung ist, dass die ATX-Hauptquelle bei Schwangeren Frauen die Plazenta bildet und bei chronischer Cholestase die Leber. Bei Cholestase könnte die zusätzliche ATX-Quelle zu Juckreiz bei ICP PatientInnen führen.

1 INTRODUCTION

1.1 BILE ACIDS

With more than 60 species, bile acids (BA) are a group of structurally diverse molecules that are products of the cholesterol metabolism (2). Chemically, they are composed of a steroid nucleus and a short aliphatic side chain that terminates in a carboxyl group (2–4). In the pericentral hepatocytes of the liver, primary BA are synthesized through a complex, multienzyme process converting insoluble, uncharged cholesterol-molecules to soluble, amphipathic BA-molecules (2,5).

Four main steps are essential for BA-synthesis: initiation (7α-hydroxylation), sterol ring modification, oxidation and shortening of the side chain, and conjugation (6). Chenodeoxycholic acid (CDCA) and cholic acid (CA) with hydroxyl groups at C7 or C7 and C12, respectively, are the predominating primary BA in humans (3). Different in rodents, an additional C6 hydroxylation is leading to the formation of primary muricholic acids (MCA) which contributes to species-specific differences in the physiology of BA metabolism (2,7).

1.1.1 BA FORMATION

1.1.1.1 PRIMARY BA FORMATION

The majority of primary BA (75%) is formed via the classical pathway (8). Cholesterol is hereby converted by cholesterol 7α-hydroxylase (CYP7A1) into 7α-hydroxycholesterol by introducing a hydroxyl group at position C7 of the steroid nucleus. CYP7A1 is a cytochrome P450 enzyme and the rate-limiting enzyme of the classical pathway which is only expressed in the endoplasmic reticulum of the liver (6,8). Additionally, 25% of the primary BA synthesis occurs through the alternative pathway. In this, cholesterol is hydroxylated at position 27 by sterol-27-hydroxylase (CYP27A1), which is also a cytochrome P450 enzyme but is found in mitochondria of many tissues. Subsequently, 7α-hydroxylation occurs through oxysterol-7α-hydroxylase (CYP7B1) (8).

Sterol ring modifications take place in both pathways equally by 3β-hydroxy-Δ5-C27 steroid oxidoreductase (HSD3B7). Afterward, the activity of sterol 12α-hydroxylase (CYP8B1) is

crucial for the composition of the BA-pool. A hydroxyl group at position 12 introduced by CYP8B1 leads to generation of CA, whereas the absence of C12-hydroxylation leads to formation of CDCA (6,8). In rodents, additional C6-hydroxylation by Cyp2c70 (2,7) is leading to the formation of the primary MCA, α-muricholic acid (AMCA), and β-muricholic acid (BMCA) (2,7).

The bulk of the synthesized primary BA and MCA are conjugated to either taurine (T) or glycine (G) at the terminal carbon of the side chain (8). In the human liver, especially two enzymes, BA-CoA amino acid N-acetyltransferase (BAT) and BA-CoA synthetase (BACS) are involved in the conjugation of BA (9,10). Conjugation permits higher concentrations of BA in bile and intestinal content and makes them less permeable to cell membranes (5,11). After conjugation, primary BA are excreted from the hepatocytes into bile where they are stored in the gallbladder together with other bile constituents unless they are secreted into the small intestine (2,5).

1.1.1.2 Secondary BA Formation

In the distal intestine and colon, primary BA undergo several modifications by the action of bacterial enzymes such as deconjugation and dehydroxylation, amongst others. Those actions arise the secondary BA, lithocholic acid (LCA), through C7-dehydroxylation of CDCA, and deoxycholic acid (DCA), through C7-dehydroxylation of CA (11). Another secondary BA, ursodeoxycholic acid (UDCA), is present in trace amounts physiologically. As UDCA is not synthesized in the liver, it is likely originated by bacterial 7β-epimerization of CDCA. The β-configuration leads to greater hydrophilicity of UDCA compared to the other BA, which possess an α-configuration (12). Secondary MCA are γ-muricholic acid (GMCA), ω-muricholic acid (OMCA) and hyodeoxycholic acid (HDCA) (2,7,13).

Both, primary and secondary BA/MCA, are absorbed largely in the distal part of the intestine and recycled to the liver (5). In the hepatocyte, secondary BA are also conjugated with G or T. In humans, DCA is secreted into bile after conjugation without undergoing 7-rehydroxylation. As LCA is cytotoxic, it receives a double conjugation during hepatocyte transport. To be more precise, LCA is amidated at C24 and esterified with sulfate at C3. Sulfate groups at C3 prevent intestinal absorption which leads to rapid elimination of LCA (11).

1.1.2 Enterohepatic Circulation (EHC) of BA

Under physiological conditions BA cycle between liver and small intestine in a dynamic process (14). After conjugation, BA are secreted from the liver into bile and are stored in the gallbladder during fasting. Gallbladder contraction, and thus BA secretion to the small intestine, is induced through ingestion of a meal and mediated by the gut hormone cholecystokinin (2,14). Although a small amount of BA is absorbed passively in the colon (15), most of the BA (95%) are absorbed actively in the terminal ileum (11). BA return via the portal vein back to the liver where they are extracted efficiently from the portal venous blood before they are secreted into bile again (11).

In humans, the BA pool comprises about 3 gramm and recycles between 4 - 12 times per day (95%) (5,15). This highly efficient process is called enterohepatic circulation (EHC) which guarantees an adequate distribution of BA concentrations (4,5). Each BA has its own EHC which varies from the others and is therefore responsible for the differing BA composition of serum BA and biliary BA (5,16). Only 5% of the circulating BA are eliminated by the fecal route and replaced by BA-synthesis de novo in the liver. The amount of new synthesized BA comprises 0.2 - 0.6 g daily (5,15).

Physiologically, the concentration of BA in human plasma is low due to efficient first-pass extraction of the liver. Serum BA are considered as measure of the spillover which occurs physiologically in trace amounts during EHC. During fasting states, the BA concentration in systemic venous plasma is less than 5 µmol/L. In comparison, portal venous plasma has a concentration of 20-50 µmol/L (5). However, the concentration of serum BA rises severalfold during digestion (17).

1.1.3 Physiological Functions of BA

The elimination of cholesterol is the most important function of BA. The conversion of insoluble cholesterol molecules to BA enables the secretion of cholesterol from the hepatocytes to the biliary tract (5). Phospholipids, mostly phosphatidylcholine, which are present in high concentrations in bile, form mixed micelles with BA (4). Through micellar solubilization in bile, movement of cholesterol to the intestinal lumen is facilitated, ultimately leading to fecal elimination (5).

Another important function of BA is dietary lipid absorption in the small intestine by formation of mixed micelles, promoting diffusion through the unstirred layer (4). Furthermore, the presence of BA enables the absorption of fat-soluble vitamins (A, D, E, and K). In the duodenum, BA solubilize polyvalent metals like iron and calcium and therefore promote their absorption (5).

BA are transported actively from the hepatocytes into biliary canaliculi. Since BA are osmotically active membrane-impermeable molecules, they stimulate bile flow due to an increased secretin-stimulated bicarbonate secretion by the cholangiocytes (5). Moreover, BA are stimulating biliary phospholipid secretion from the canalicular membrane into bile. A greater proportion of phospholipids in bile is leading to a higher fraction of BA in the form of mixed micelles (18). This is beneficial as lower monomer concentrations of BA prevent BA from damaging the bile duct epithelium (4,5).

Besides their classic functions BA are also involved in a lot of other activities. BA are controlling the expression of many genes encoding for proteins which are involved in BA synthesis, transport, and metabolism (5,19). Furthermore, BA are known to operate hormone-like functions in the control of glucose, lipid, and energy metabolism specifically (5).

1.1.4 BA TRANSPORT

1.1.4.1 HEPATOCELLULAR BA TRANSPORT

BA-uptake from the portal venous blood is initiated at the basolateral hepatocytes via the sodium (Na^+) -taurocholate cotransporter (NTCP) or by Na^+-independent organic anion transporting proteins (OATPs). NTCP accounts for >80% of conjugated BA and is, therefore, the most important BA-uptake-system (20). OATPs are highly specific transporter systems which substrate preferences include conjugated as well as unconjugated BA (21,22). The exact mechanisms of intracellular BA transport from the basolateral to the canalicular membrane of the hepatocytes are hardly known. Under physiological conditions, most BA are bound predominantly to intracellular binding proteins. In conditions of increased BA levels, especially hydrophobic BA can be detected within intracellular organelles like e.g. Golgi apparatus or endoplasmic reticulum (23).

Canalicular BA secretion is the rate-limiting step in the formation of bile. Monovalent BA are excreted by the bile salt export pump (BSEP). Divalent BA, together with various amphipathic conjugates, are excreted by multidrug resistance-associated protein 2 (MRP2). Furthermore, the canalicular membrane contains a chloride/bicarbonate (Cl^-/HCO_3^-) exchanger (AE2) for $HCO3^-$ excretion, a multidrug export pump (MDR1), a phospholipid flippase for phosphatidylcholine (MDR3 in humans, MDR2 in rodents) and a P-type ATPase called FIC1, which is crucial for the distribution of lipids between the apical membranes two leaflets. BA-independent bile flow is mediated predominantly via MRP2 and AE2 whereas BSEP drives BA-dependent bile flow (24–26).

1.1.4.2 Cholangiocellular BA Transport

In biliary epithelia, BA are taken up by the apical Na^+-dependent bile salt transporter (ASBT) [synonym: ileal bile salt transporter IBAT]) (27). Furthermore, MRP2, located in the apical membrane, and multidrug resistance associated protein 3 (MRP3), located in the basolateral membrane, were identified in gallbladder epithelium of humans and are considered to modify BA pool composition (28).

1.1.4.3 Intestinal BA Transport

The uptake of conjugated BA into the enterocyte occurs mainly in the terminal ileum via ASBT (29). Substrates of ASBT are primary and secondary conjugated BA (30). The Na^+-independent BA uptake occurs by OATPs, which is particularly located at the apical surface of jejunal epithelial cells (31). Passive diffusion of unconjugated BA takes place in small and large intestine (32). The intestinal BA-binding protein (I-BABP) probably represents the most important protein for intracellular BA transport, although there is only limited information of intracellular BA movement mechanisms in enterocytes (33).

Several active export pumps at the basolateral as well as at the apical membrane of enterocytes are known. Exporter proteins moving BA from enterocytes in the portal circulation include e.g. the organic-solute-transporter ($OST\alpha/OST\beta$) and MRP3, predominantly expressed in the terminal ileum and colon (22,34,35). MRP2 and MRP4 are mainly expressed in the apical membrane of the proximal intestine moving BA into the intestinal lumen (36,37).

1.1.4.4 Placental BA Transport

In utero, BA are synthesized by the fetal liver (38). However, due to immaturity of the fetal liver, BA are mainly eliminated by the maternal liver after translocation into maternal circulation via the placenta (29). Placental BA uptake takes place at the basolateral trophoblast membrane by OATP (29,39). Furthermore, a potential role of MRP3 is suggested (40). In the apical trophoblast membrane, ATP-dependent and -independent BA transport systems seem to be the predominant (29,41).

1.1.5 Regulatory BA Receptors

The identification of multiple BA-responsive nuclear receptors led to growing interest in the mechanisms by which BA are influencing signal transduction in various tissues. The nuclear farnesoid X receptor (FXR) and the Takeda G-protein receptor 5 (TGR5) are the best researched BA-responsive receptors (2).

1.1.5.1 FXR

FXR is predominantly expressed in organs that are involved in EHC like liver and intestine (42,43). Through FXR, the expression of genes encoding for proteins involved in BA-synthesis, -transport and -metabolism is directly under the control by BA themselves (44,45). However, not all BA are equally effective in FXR-activation, the order of potency is CDCA > DCA = LCA > CA (43). Moreover, AMCA, BMCA and its G and T conjugates as well as UDCA have antagonistic effects on FXR (8,46).

FXR activation leads to downregulation of BA uptake systems and increased expression of exporter proteins like BSEP, OSTα, and OSTβ in liver as well as in intestine (15,47,48). Furthermore, the small heterodimer partner (SHP), a nuclear orphan receptor, is a key target gene of FXR. Through SHP activation, many of the suppressive effects of FXR are mediated. SHP functions as a transcriptional repressor by binding directly to other nuclear receptors to control e.g. the expression of CYP7A1 and therefore BA synthesis (49,50).

Within ileal enterocytes, BA induce the expression of fibroblast growth factor 15 (FGF15) in mice and FGF19 in human through FXR in the terminal ileum (49,51). FGF15/19 is excreted

from the enterocytes in the portal circulation and travels to the liver via the portal circulation. Through binding to FGF receptor 4 (FGFR4), FGF15/19 additionally suppresses CYP7A1 (52,53). However, FXR signaling could have been shown to regulate CYP7A1 mRNA stability independently of SHP and FGF19/15 (54). Moreover, the genes encoding for BACS and BAT, which are mediating BA conjugation, are direct targets of FXR (10).

1.1.5.2 TGR5

While FXR is known as important regulator of bile formation and flow, TGR5 complements these functions by coordinating mechanisms of the biliary physiology. Different from FXR, TGR5 is highly expressed in cholangiocytes and gallbladder epithelium but not in hepatocytes (2). TGR5 is activated through both, conjugated- and unconjugated BA, though T-conjugated BA could have been shown to activate TGR5 more potent than G-conjugated- or unconjugated BA (55). The rank of potency in which TGR5 is activated by BA is LCA>DCA>CDCA>CA (2).

The activation of TGR5 leads to cell-specific consequences comprising e.g. gallbladder relaxation, increase of intestinal motility, and improvement of glucose metabolism (56,57). Furthermore, TGR5 activation promotes Cl^- secretion (58). Due to the generated Cl^- gradient, HCO_3^- can be secreted into bile through the apical membrane by anion exchanger 2 (57). Moreover, TGR5 activation by LCA is promoting smooth muscle relaxation of the gallbladder and therefore excretion (59).

1.1.5.3 Other Nuclear Receptors

Other nuclear receptors including pregnane X receptor (PXR) and constitutive androstane receptor (CAR), amongst others, usually require higher BA concentrations for being activated. Therefore it is assumed that their functions become especially relevant in cholestasis (2,60). PXR coordinates inhibition of BA-synthesis and detoxification in conditions that require protection from LCA excess (61,62). Furthermore, synergistic effects of PXR and CAR are described in terms of bilirubin detoxification (63).

1.1.6 BA AND CYTOTOXICITY

BA are cytotoxic, either intracellularly or extracellularly, when their concentrations reach abnormally high levels. The extent of BA cytotoxicity depends on its structure. The general rule is: The higher the hydrophobicity of a BA, the higher the cytotoxicity (5). The hydrophobicity of a BA is depending on the number, position, and orientation of their hydroxyl group and the amidation at C24. Conjugation to G or T before secretion into bile is leading to more hydrophilic amidated BA forms (64). The order from less to most hydrophobic BA is UDCA<CA<CDCA<DCA<LCA (8). Although having two hydroxyl groups, UDCA is considered as hydrophilic BA due to its β-configuration at C7 (5). UDCA is used as therapeutic agent in hepatobiliary disorders (see chapter 1.3.3.5) (12).

1.1.7 BA IN THE NEONATAL PERIOD

Various studies showed that the BA pool in neonates differs from that in adults. CA accounts for 40% of the BA pool in adults, CDCA 37%, DCA 20%, and LCA 3%. Similarly in newborns, CA is predominating, however, exceeds CDCA concentrations 2.5-fold (65). Furthermore, neonates only show primary BA in bile after birth. The presence of DCA in bile signals the development of an anaerobic microbiome within the first year of life (5,66).

Whereas in adults G-conjugated BA is predominating, T-conjugates are prevailing in neonates (65). As T is the most common free amino acid in breast milk, these observations seem to be diet-related (67). T has been shown to be important for various biological functions like brain development, retinal photoreceptor activity, normal growth development, and antioxidant activity (65). Furthermore, lack of T has been shown to lead to impaired fat absorption, BA secretion, and renal function in preterm neonates (67). Moreover, T-conjugated BA seem to be better fat emulsifiers than G-conjugates (68). Yung *et al.* evaluated T intake of pregnant women during late gestational period. They found that newborns of pregnant women with high T intake had significantly more body weight and a greater length compared to pregnant women with low T intake (69).

The existence of “usual” BA, also called “atypical” BA or MCA, has been described earlier in literature in amniotic fluid and umbilical cord blood (65). These tri-hydroxylated BA are typically present during liver development, as hydroxylation is hepato-protective (13,38). Significantly higher MCA levels could have been demonstrated in full-term- compared to preterm neonates

(70). Significantly increased TOMCA levels could have been shown in preterm infants with early-onset sepsis compared to age-matched controls (70). In this context, a potential as biomarker was assumed for TOMCA. Besides neonates, the existence of MCA have been described in cholestatic patients. Those findings lead to the suggestion of a deviating BA metabolism in both: fetal liver as well as cholestatic liver (13).

1.1.8 BA IN CHILDREN AND ADOLESCENTS

Due to different measurement methods, serum BA studies are often not comparable and show inconsistent results. In 1985 Niijima *et al.* found high levels of total BA (tBA) in serum of healthy neonates measured by high-performance liquid chromatography (HPLC) which continuously decreased with increasing age (71). Furthermore, primary BA predominated within the first 12 months of life (71). High BA concentrations in serum of neonates were measured by Polkowska *et al.* by an enzymatic-colorimetric test (72). In this study, tBA decreased and reached nearly adult standard values after the first 12 months of life (72).

In 2015 Jahnel *et al.* defined reference values of serum BA in children and adolescents (73). In 194 healthy subjects of different age groups, serum tBA and BA profiles were determined using HPLC combined with high-resolution mass spectrometry (HPLC-HR-MS). High serum tBA within the first 24 months of life could have been observed in this study. After the first two years of life, tBA continually decreased. Children ≥11 years of age showed tBA levels corresponding to those in adults (73). In the same study, BA profiles were found to be age dependent as well. In accordance with other studies, T-conjugates were predominating in neonates. After 6 months of age G conjugated noticeably outweighed (73).

In pediatrics, serum BA can be determined as biomarkers for inborn but also for acquired cholestatic disorders including the different types of progressive familial intrahepatic cholestasis (PFIC) amongst others (73–75).

1.2 Chronic Cholestatic Liver Diseases (CCLD)

An impairment of bile formation and/or bile flow leading to a retention of biliary substances defines the clinical picture of cholestasis. The origin of cholestasis can be distinguished in biliary or hepatocellular. Whereas biliary cholestasis is obstructive and affects large extrahepatic and small intrahepatic bile ducts, the causes of hepatocellular cholestasis may be a defect in membrane transport, embryogenesis, or metabolic function (76). Although the etiology of cholestatic jaundice in infants is often unknown, the most common sources in young infants are biliary atresia (25-40%) and uncommon genetic disorders e.g., PFIC (25%). Further diseases associated with neonatal cholestasis include extrahepatic obstruction, metabolic disorders, such as tyrosinemia type I or galactosemia, Alagille syndrome (ALGS), infection or parenteral nutrition-associated liver disease amongst others (76). In older children, diseases like autoimmune sclerosing cholangitis (ASC), amongst others, present with chronic cholestasis (77,78).

Patients usually present with fatigue, jaundice, and pruritus in some cases. For correct evaluation of jaundice, measurement of conjugated bilirubin in serum is indispensable as it counts as a reliable cholestasis indicator. Basic laboratory testing should also include alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), gamma glutamyl transpeptidase (GGT), prothrombin time (PT), the International Normalized Ratio (INR), glucose, and albumin. Furthermore, it is important to examine for hypothyroidism, galactosemia, tyrosinemia, and cystic fibrosis. Evaluation should further include family history, gestational history of the mother, physical examination, and inspection of stool/urine color (76). Abdominal ultrasound is useful in excluding extrahepatic bile duct obstruction (79). However not diagnostic for biliary atresia, an absent or abnormal gallbladder can be indicative (80,81). Liver biopsy is often key of the diagnostic workup of cholestatic jaundice. The correct diagnosis can be provided by an experienced pathologist in 90-95% of cases after biopsy (82,83). In the following only those chronic cholestatic liver diseases (CCLD) will be described more precisely, which were investigated in this research project.

1.2.1 Autoimmune Sclerosing Cholangitis (ASC)

Sclerosing cholangitis (SC) is typified by inflammation and obliterative fibrosis of intra- and extrahepatic bile ducts. SC is known as chronic CCLD which terminates in biliary cirrhosis and may end with liver failure (78). Importantly, SC is a heterogeneous condition that results from

different chronic hepatic diseases. SC may be secondary to Langerhans cell histiocytosis, cystic fibrosis or psoriasis amongst others. Inherited forms of SC are typically diagnosed in the neonatal period (neonatal SC) (78,84). In cases where the etiology of SC is unknown, the term primary SC (PSC) is widely used especially in adults (85). Different in pediatrics, SC typically presents with additional features like those of autoimmune hepatitis (AIH). This led to the introduction of the term PSC/AIH-Overlap or ASC, respectively (77,78). The terms PSC/AIH-overlap and ASC are used inconsistently depending on the center where the diagnosis is made. In our study, all included patients with SC had features of autoimmunity. Therefore, we will use hereinafter the term ASC.

The prevalence of ASC varies between 0.6 to 4/100.000 children and differs geographically (86). The exact pathogenesis of ASC is unknown. On the one hand, damages in endothelium and bile duct epithelium due to toxic biliary lipids has been suspected (87). On the other hand, an altered microbiota (88) has been discussed or changes in cystic fibrosis transmembrane conductance regulator (89). An association between ASC and inflammatory bowel disease, especially ulcerative colitis, is known (90). Symptoms in context with ASC are variable and range from fatigue and hepatomegaly to jaundice and pruritus in some cases (90,91).

In ASC, elevated biomarkers indicating hepatobiliary injury and increased IgG levels are typical findings. Furthermore, autoantibodies like ANA, SMA, and pANCA are detectable in most cases (90,92). The final diagnosis of ASC should be made based on liver biopsy and endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP), respectively (93). UDCA is considered the treatment of choice in ASC. However, there is no proof of effectiveness in preventing progression of bile duct disease (78). The use of early immunosuppressive therapy in children with ASC was shown to reduce liver parenchymal liver damage and is leading to normalization of biochemical parameters (84,94). However, progression of bile duct disease stops only in every second child (90).

1.2.2 Progressive Familial Intrahepatic Cholestasis (PFIC)

The term PFIC is an umbrella term that comprises a heterogeneous group of autosomal recessive disorders of childhood presenting with cholestasis. The prevalence varies between 1/50.000 and 1/100.000. In PFIC, cholestasis is caused by underlying mutations in genes, which are involved in hepatocellular transport and the formation of bile (95). To date, five types

of PFIC have been described in literature from which PFIC1-3 are the most common forms. PFIC1, also known as Byler disease, and PFIC2 typically appear in the first months of life. Different in PFIC3, onset of the disease is also possible in childhood or even young adulthood (96). Furthermore, the existence of PFIC 4 and 5 has been described in a few cases (97). Diagnosis is based on clinical manifestations (e.g., cholestasis, pruritus, jaundice), laboratory testing, liver ultrasound, cholangiography, and liver histology. Of course, a thorough exclusion of other causes of childhood cholestasis is necessary (96). The main characteristics of PFIC1-3 are illustrated in **Table 1.** Only PFIC 1-3 are described in the following in more detail.

	PFIC1	**PFIC2**	**PFIC3**
Mutated gene	*ATP8B1*	*ABCB11*	*ABCB4*
Protein	FIC1	BSEP	MDR3
Hepatocyte location	Canalicular membrane	Canalicular membrane	Canalicular membrane
Functional defect	ATP-dependent amino-phospholipid transport	ATP-dependent bile acid transport in bile	ATP-dependent phosphatidylcholine translocation in bile
Pruritus	severe	severe	moderate
Serum GGT activity	Normal	Normal	High
Serum primary bile acid concentration	Very high	Very high	High
Bile composition	Low primary bile acid concentration	Very low primary bile acid concentration	Low phospholipid concentration
Characteristic histology at diagnosis	Bland canalicular Cholestasis; Coarsely granular canalicular bile	Giant cell transformation	Cholangiocytic changes
Typical clinical outcome	Moderate rate of Progression; Post-transplant hepatic steatosis & diarrhea	Moderate to rapid Progression; Allo-antibody formation after transplant in some	Highly variable rate of progression

Table 1: Typical features of PFIC 1-3 associated with different genetic etiologies (95,98)

1.2.2.1 PFIC1 - FIC1 Deficiency

The underlying cause of PFIC1 is FIC1 deficiency. FIC1, encoded by ATP8B1, is an ATP-dependent membrane transporter with phospholipid flippase function (99). FIC1 is expressed in the liver and intestine but in a variety of other tissues as well (100). In the absence of FIC1, normal distribution of lipids between the 2 membranes of the lipid bilayer is disturbed, which probably makes the canalicular membranes more vulnerable to damage by BA (101). Patients with an entirely deficit of FIC1 function are usually diagnosed with PFIC1 whereas milder phenotypes with mutations in ATP8B1 are typically termed as benign recurrent intrahepatic cholestasis (BRIC) (102).

Patients with PFIC1 are usually presenting with jaundice and severe pruritus within the first months of life. However, due to the broad tissue distribution of FIC1, extrahepatic manifestations, e.g. diarrhea or pancreatic disease are frequent (98,103). Laboratory and histologic findings in FIC1 are listed in **Table 1**. Initially, PFIC1 is commonly managed with medication like UDCA or non-transplant surgical intervention like partial external biliary diversion (PEBD) (98,104). However, in end-stage liver disease liver transplantation (LTX) is necessary. Development of graft steatosis and inflammation after LTX in patients with FIC1 deficiency are common. As FIC1 is expressed in many tissues, LTX is not curative of PFIC1 (98,104,105).

1.2.2.2 PFIC2 - BSEP Deficiency

BSEP-deficiency is causal for PFIC2. BSEP is encoded by ABCB11 (106). Significant reduction in BSEP function is the most frequent form of all types of PFIC. Patients generally present with jaundice, high serum BA, and transaminases but normal GGT in early childhood (98,103). Histological findings are illustrated in **Table 1**. To date, most drug treatments show no significant impact on the disease in patients with severe BSEP deficiency. An improvement through supplementation of UDCA was described in some cases (98). Furthermore, PEBD might help in some cases but seems to show a better response in those patients with some residual BSEP function (104). Due to severe pruritus, patients with significant BSEP deficiency, and hence PFIC2, are often transplanted before they reach end-stage liver disease. By 5 years of age, 15% of the patients have already developed hepatocellular carcinoma (HCC) clinically or at explant, despite the early use of LTX (107). LTX is considered as treatment of PFIC2 as

BSEP is only expressed in the liver (95). However, in some patients, a recurrence of the same symptoms that they have had before LTX is described due to allo-reactive antibodies, which are considered to block the BSEP protein of the transplanted liver (108). Most of those patients respond to immunosuppression whereas in some cases B-cell-depletion for years is necessary (109,110).

1.2.2.3 PFIC3 - MDR3 DEFICIENCY

The underlying cause of PFIC3 is MDR3 deficiency. MDR3 is encoded by ABCB4 (95). Due to lack of PC, which is usually transported by MDR3 into bile, large quantities of free BA are present in bile which injures the membrane of the cholangiocytes. MDR3 deficiency is strictly speaking a cholangiopathy as it does not lead to BA retention in the hepatocyte and therefore does not directly cause cholestasis (111). Symptoms occur as a result of cholangiopathy caused by BA, however, even in complete MDR3 deficiency, it can take several years before clinical presentation of the patients (112). In **Table 1** clinical and histologic characteristics are mentioned. However, the improvement in long-term clinical outcome is not clear yet, the supplementation with UDCA shows significant biochemical improvement especially in milder forms of MDR3 deficiency (113). In end-stage liver disease, LTX applies as an effective treatment (95).

1.2.3 INTRAHEPATIC CHOLESTASIS OF PREGNANCY (ICP)

Intrahepatic cholestasis of pregnancy (ICP) the most frequent cholestatic liver disease unique to pregnancy, characterized by pruritus and elevated serum BA levels (114). The incidence in Europe ranges from 0.5-1.5% (highest rates in Scandinavia). For unknown reasons, ICP occurs usually in the winter months as observed in Sweden, Finland, and Chile (115).

The exact pathophysiology of ICP remains unknown. A genetic susceptibility is assumed and supported by an enhanced risk in first-degree relatives and a high recurrence rate up to 70% (116). In addition, heterozygous mutations in the ABCB4 gene, encoding for MDR3, were described in literature in woman with ICP (prevalence in Caucasian ICP-patients 16%) (117–119). Furthermore, a role of estrogen and progesterone is supposed in the etiology of ICP, as the disease develops typically in the late second or third trimester where female sex hormones reach peak levels (120). Levels of estrogen in serum and urine do not differ during ICP compared to normal pregnancies at the same gestational age (121,122). However, ICP is seen

more often in multiple gestations in which estrogen levels are known to be higher than in single pregnancies (123,124). In the 1970s, Sjövall *et al.* reported for the first time that ICP patients have higher serum levels of sulfated metabolites of progesterone than healthy pregnant women (125). Abu-Hayyeh *et al.* discovered that sulfated progesterone metabolites are a prognostic indicator for ICP. Furthermore, concentrations of progesterone sulfates were associated with itch severity (126). After delivery, pruritus in ICP patients disappears, correlating with a decrease of the steroid hormone levels (127,128). This, however, does not explain why not all pregnant women are suffering from ICP despite comparable progesterone levels.

Increased serum BA levels are the key laboratory finding which is present in >90% of ICP patients (124). Increased levels of the primary BA CA and CDCA could have been shown in ICP (CA>CDCA) (129,130). BA levels >40µmol/L are acritical cut-off for complications. Furthermore, the risk of fetal demise was shown to be associated with higher tBA levels in serum, especially >100µmol/L (131). In rat liver, a glucoronidated estrogen metabolite (estradiol 17β-D-glucuronide) and a sulfated progesterone metabolite (5α-pregnan-3α-ol-20-one sulfate) have been shown to be secreted into bile, to decrease bile flow and to inhibit BSEP (132). A cholestatic effect in humans has not been reported so far. ICP is resolving after delivery of the placenta, which produces high levels of estrogen/progesterone especially towards the end of pregnancy (124).

Besides enhanced BA levels, serum aminotransferases are also enhanced in 60% of ICP patients. Total bilirubin levels seldom exceed 6mg/dL and AP may be elevated but is not specific for ICP. Interestingly, GGT serum concentrations are normal or modestly elevated which is unusual in most cholestatic states (124,133). Ultrasonography is not associated with abnormalities and histopathology is characterized by cholestasis without inflammation. In most cases, liver biopsy is not essential for the diagnosis (134). UDCA is the preferred treatment of maternal pruritus. In refractory cases other drugs like rifampicin or cholestyramine can be added (135).

1.3 PRURITUS

In the clinical context, pruritus is considered either acute or chronic. Whereas acute pruritus is defined as sensation of pruritus lasting less than 6 weeks, chronic pruritus is persisting 6 weeks or longer (136). Besides the distinction in acute or chronic, pruritus can be assigned to different

subgroups: neurogenic, neuropathic, pruritoceptive, or psychogenic pruritus. Whereas neurogenic itch is defined as induced by mediators without signs of neural damage, neuropathic pruritus is associated with damage of neurons (e.g., in post-herpetic neuralgic itch). Pruritoceptive itch is associated with pruritogen activation of sensory fibers. As the mediators of neurogenic itch are defined, it is likely that neurogenic pruritus will fall under pruritoceptive itch. Psychogenic pruritus is based on a psychosomatic or psychiatric origin (136–138).

1.3.1 Neurophysiology of pruritus

The neurophysiology of pruritus is not completely understood. It is known that pruritus-related signals are transmitted by unmyelinated, histamine-sensitive, and non-histamine-sensitive peripheral C-fibers (139–142). The sensory nerve endings of itch-specific neurons are predominately located in the epidermis and the dermal-epidermal junctions of the skin (136). Whereas histamine-sensitive C-fibers own histamine-receptors in their nerve endings, histamine-independent C-fibers possess G protein-coupled receptors (GPCRs), cytokine receptors, and transient receptor potential (TRP) ion channels. Subgroups of GPRCs include e.g. protease-activated receptors (PAR) and Mas-related GPCRs (Mrgprs) (136,143).

The primary nerve endings related to pruritus terminate in the dorsal horn of the spinal cord. From here, the itch stimulus travels via secondary transmission neurons to the contralateral spinothalamic tract which reaches the thalamus or the parabrachial nucleus (136). The itch signal is finally projected to various brain areas including the primary and secondary cerebral cortex, cingulum and insula amongst other (143,144). Histaminergic and non-histaminergic itch signals are partially activating similar but also specific brain areas, which elucidates the multimodality of pruritus mediation (136).

Both, histamine-sensitive and -insensitive peripheral C-fibers were shown to be insensitive to mechanically induced pain stimuli indicating that itch and pain are transmitted via different pathways (143,145). However, both pathways are closely connected as similar mechanisms are used for transducing signals from peripheral nerve endings to integration centers (136,146). Furthermore, pruritus- and pain-related neurons are sharing many common receptors (e.g., PAR, TRP) and mediators (e.g., substance P and prostaglandins, serotonin, opioids) (147–149). Admittedly, pruritus and pain are known to interact with each other as

scratching leads to activation of pain neurons, which inhibit the sensation of itch through the release of endogenous opioids. Inversely, analgesia can induce itch (140,143) (**see 1.3.3.2**).

1.3.2 Pruritus in Childhood

To our knowledge, no epidemiological study concerning pruritus in infancy has been published. In children, pruritus is predominately caused by dermatological diseases, especially atopic dermatitis (150). Systemic reasons for pruritus in children and adolescents are much rarer including terminal renal failure, neoplastic diseases, and hepatic diseases (**see 1.3.3**) (151). As itching is subjective, diagnosis and assessment of pruritus can be difficult, especially in younger infants (152). A Visual Analogue Scale of Pruritus (PVAS) (**see 2.3.6**) or other itch-severity scales are the most frequently used tools for pruritus assessment in children but also in adolescents and adults (138,153). However, they are not validated in children under 5 years of age due to non-verbality and therefore lack of reliability (151,154).

1.3.3 Pruritus in Cholestatic Liver Diseases

Various CCLD such as ASC, PFIC, ALGS, BRIC, primary biliary cirrhosis (PBC) and ICP, amongst others, are associated with chronic pruritus (155–157). However, the exact pathogenesis underlying is not well established, and the frequency and severity of pruritus is varying greatly according to the underlying CCLD (158).

Cholestatic pruritus can be generalized or localized (typically over the soles and palms), showing typically a circadian rhythm with a peak in the evening and early night (135). The intensity of pruritus can transiently aggravate in association with psychological stress, heat or premenstrual states (159,160). Furthermore, pruritus in CCLD is observed more often in women. A worsening of pruritus in the progesterone phase of the menstrual cycle or in late pregnancy is described by some female patients, suggesting a role for female sex hormones (128,135).

Typically, no primary skin lesion is detectable, but scratching may lead to cutaneous complications like excoriations, folliculitis, or lichenification (135,160). The severity of pruritus during cholestasis can range from mild over moderate (sleep deprivation) to extreme (completely disrupted lifestyle, and even suicidal ideation) (158). Therefore, severe, refractory

pruritus in chronic cholestasis is an accepted indication for LTX, even in the absence of liver failure (161).

Acute pruritus typically arises from degranulating mast cells in the skin and is therefore considered to be mediated by histamine-sensitive neurons (139). As antihistamines do not alleviate itching in most chronic cholestatic conditions, it is assumed that pruritus sensation is mediated via non-histamine-sensitive fibers in that cases (162). However, the exact pathophysiology of cholestatic pruritus is poorly understood (163). Several hypotheses concerning specific mediators implicated in pruritus in CCLD have been proposed. Those include elevated BA levels, increased endogenous opioids, enhanced progesterone metabolites, and elevated lysophosphatidic acid (LPA) levels amongst others, which will be discussed in the following in more detail.

1.3.3.1 The Role of BA

In various studies, enhanced BA levels have been assumed to act to induce pruritus in cholestatic states (164,165). The bile acid sequestrant, cholestyramine, has been shown to ameliorate pruritus in different cholestatic conditions (159). Moreover, a reduction of BA concentrations by nasobiliary drainage has led to an improvement of itching sensation in CCLD patients (166). Furthermore, the BA mediated activation of TGR5, expressed in the peripheral neurons of the dorsal root ganglia, has been shown to induce a release of neuropeptide transmitters of pruritus by *Alemi et al.* (165). However, they also determined that cholestatic mice do not itch (165). DCA treatment led to a scratch response in $Tgr5^{+/+}$ mice but not in $Tgr5^{-/-}$ mice, speaking also for a TGR5 dependency (165). The excitatory effects through TGR5 were shown to be mediated by activation of the TRP ankyrin 1 (TRPA1) channel (167).

However, neither correlations between itch severity and BA levels in serum or in tissue have ever been established (164,165) and despite predominantly low BA levels, severe itching is often the initial symptom in PBC but also in ICP (115,135). Moreover, a reduction of pruritus is described by some patients despite progress of their underlying CCLD and persistence of increased BA levels (168). Furthermore, the absence of pruritus in many patients suffering from cholestatic disorders and enhanced serum BA levels questions the role of BA as a key mediator in genesis of cholestatic pruritus (158,169). Of note is also that TGR5 can be activated by an opioid-dependent mechanism in the dorsal root ganglia (165). Additionally, for the selective TGR5 agonist, SB-756050, no itching in humans is reported as adverse event

(170). Kuiper *et al.* described that colesevelam, also a bile acid sequestrant but with a 7 fold-higher capacity compared to cholestyramine, lowered BA levels significantly, but was not effective in alleviating itch severity (171).

1.3.3.2 The Role of Endogenous Opioids

Endogenous opioids have also been considered in the pathophysiology of itching in CCLD. It is known that pain and itch neurons are separate but interact. Physiologically, itch neurons are inhibited through pain neurons in the spinal cord area. In states of inhibited pain sensation, by endogenous opioids or administrated anesthetics with agonist activity at the μ-opioid receptor, activation of itch neurons can provoke a reversible itch sensation (**Figure 1**) (172).

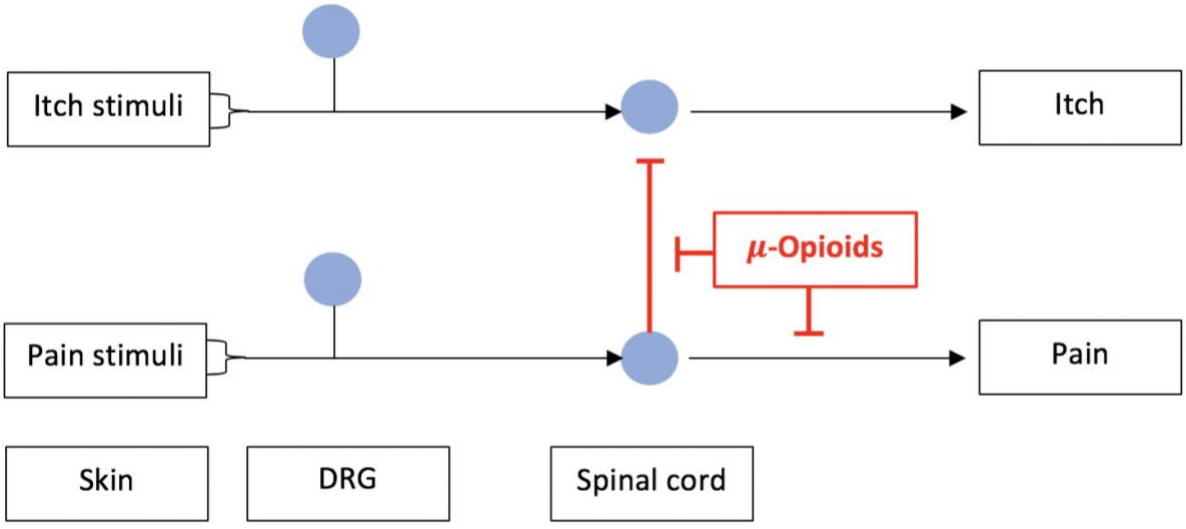

Figure 1: Neuronal pathway for Itch. The blue dots with the black lines indicate neurons. DRG= dorsal root ganglia (172)

Enhanced levels of endogenous opioids have been reported in plasma of cholestatic rats (158,173). Likewise, in human CCLD patients, enhanced endogenous opioid levels have been determined in serum (174,175). Since treatment with opioid-antagonists is leading to a reduction of itch in some patients with cholestatic pruritus, a correlation between pruritus and opioid levels was suggested (174,176). However, in humans, no significant correlation between the extent of cholestatic itch and serum μ-opioids has ever been established (175,177). Therefore, the activation of the pruritus pathway in association with opioids is still unclear (168).

1.3.3.3 The Role of Serotonin

The neurotransmitter serotonin is modulating neural signaling (178,179). A connection between the serotoninergic system and pruritus is discussed in literature as intradermal injection of serotonin has been shown to provoke itching (137). Furthermore, the selective serotonin reuptake inhibitor (SSRI), sertraline, has been used with some success cholestatic itching (180). In accordance, odansetron, a highly specific serotonin 5-HT3 receptor agonist is used as medication of cholestatic pruritus (181). However, no correlation between serum serotonin levels and pruritus scale scores could have ever been established which questions the role of serotonin as a mediator of pruritus (182,183).

1.3.3.4 The Role of LPA and Autotaxin (ATX)

ATX, also called ENPP-2 (ectonucleotide pyrophosphatase/phosphodiesterase 2), is an extracellular secreted enzyme with lysophospholipase D activity (184,185). In literature, three isoforms of ATX have been described in mouse and human (186,187). Besides minor amounts of S1P (sphingosine 1-phosphate) and cPA (cyclic phosphatidic acid), ATX generates predominately the bioactive LPA from lysophosphotidylcholine (LPC), which is present in plasma at high concentrations (186,188,189). The hydrolysis of nucleotides and lysophospholipids by ATX is mediated by a catalytic site (184,189). In mice, mutations of this site have been shown to be lethal (186,190) as LPA production is essential for formation and stability of embryonic/extraembryonic blood vessels and for neural tube closure (186,191–193). LPA acts through GPCRs (LPA 1-6) as neuronal activator (186,192) and is present in blood, urine, saliva, seminal and cerebrospinal fluids (185,194). ATX stimulates tumor cell motility and is upregulated in melanoma, glioblastoma, breast- and lung carcinoma, amongst others (185,186,188,195,196). Besides, it is considered that ATX is connected to the pathophysiology of neuropathic pain (185,194). Furthermore, since ATX is cleared by the liver, it has been shown to be elevated in various liver diseases (194,197).

Kremer *et al.* have been determined significantly increased LPA levels in patients with CCLD with pruritus. Furthermore, ATX activity has been described to be significantly enhanced in pruritic CCLD patients and to correlate positively with the intensity of pruritus (177). In another study by the same research group, ATX activity has been measured in serum after nasobiliary drainage, which is known to reduce pruritus in CCLD patients dramatically. Shortly after the intervention, Kremer *et al.* reported significantly decreased ATX activity which, however,

increased back to pretreatment levels when pruritus reoccured. Interestingly, this effect appeared not to be caused by direct ATX clearance as neither ATX activity nor ATX protein could have been detected in bile (198). Furthermore, ATX has been found to be significantly elevated in pruritic children with ALGS and in adult patients with PBC and pruritus (199,200).

Serum ATX levels are known to be slightly higher in females compared to males but to be highest in pregnant women (185,197). Already in 1996, high levels of ATX mRNA have been determined in human placenta as ATX is essential for angiogenesis and neuronal development (201). Similar findings were also reported by Macias *et al.* (202), whereas Kremer *et al.*, however, found no difference in ATX mRNA levels in placental tissue of ICP patients compared to healthy pregnant controls (203). Moreover, increased lysophospholipase D levels have been reported in pregnant women, correlating positively with gestational age (204). In ICP patients, significantly enhanced ATX activity has been reported compared to healthy pregnant controls correlating with pruritus intensity (177). In a recent study, no relation between ATX levels and pruritus severity in ICP patients could have been found (205).

In a mouse model, intradermal injection of LPA could induce a scratch response in mice (177). However in contrast to human, ATX activity was only mildly elevated arguing for no impairment of ATX clearance (206). This might be the reason why cholestatic mice in contrast to cholestatic human do not itch. Furthermore, it could have been shown, that ATX features a tunnel in which steroids like BA can be selectively bound. C7-hydroxylated BA like CDCA, including their G- and T-conjugates, were shown to inhibit ATX activities by tunnel-binding. C12-hydroxylated BA, however, like CA or DCA abrogated the inhibitory effect (207). However, literature concerning MCA in the context of ATX activation or inhibition is lacking. Still, the pruritogenic factor in bile causing the ATX surge in humans has not been found yet.

1.3.3.5 Management of Pruritus in Cholestatic Liver Disease

Treatment of pruritus in patients with CCLD represents a clinical challenge as the available therapeutic options are largely empiric and inconsistently effective (**Table 2** and **3**). Moreover, most of the recommended medications to treat cholestatic itching have an “off-label use” character. Generally, all patients should be recommended to use moisturizing and cooling ointments once or twice daily (163). Although antihistamine drugs are prescribed widely, they usually do not alleviate itching in most chronic cholestatic conditions (162).

UDCA is widely prescribed for children suffering from cholestatic pruritus. Due to its hydrophilicity, UDCA enhances bile secretion and lowers absorption of hydrophilic BA by modification of BA distribution. However, UDCA usage in cholestatic pruritus is highly controversial. On the one hand, UDCA was shown to ameliorate pruritus in women with ICP and in some patients with PFIC (98,208,209). On the other hand, it did not alleviate itching significantly in chronic cholestatic disorders like PBC or PSC (210,211).

The PXR-agonist rifampicin is increasing both, the metabolism and secretion of pruritogenic substances (212). Usually, rifampicin is well tolerated and its efficacy has been proven in children with cholestatic pruritus. Therefore, it is often used as first-line therapy (213,214).

Anion exchanger resins, e.g., cholestyramine or colesevelam, capture predominately BA, but probably other potential pruritogens as well, in the intestine. Therefore, ileal reabsorption of BA is reduced, whereas fecal elimination is increased (163). However, in cholestatic states in which the secretion of BA in bile as well as in intestine is low (e.g. biliary atresia, ALGS or PFIC) cholestyramine or comparable drugs have low efficacy (171,215). Moreover, they are often poorly tolerated due to associated nausea, diarrhea, or obstipation (163).

Novel therapeutic options in children and adult patients with severe cholestatic pruritus include substance like odevixibat (ASBT-inhibitor), through inhibiting ileal BA reabsorption. Odevexibat has been shown to reduce pruritus severity in pediatric patients with CCLD like PFIC and ALGS amongst others (163,216). The chaperone 4-phenylbutyrate is another therapeutic option. 4-phenylbutyrate has been examined in children suffering from PFIC2. An association with re-expression of BSEP and improvement of pruritus has been described in those patients (217). Moreover, bezafibrate, a broad peroxisome proliferator-activated receptor agonist, has been shown to reduce moderate and severe pruritus in adult PSC and PBC patients (218).

The opioid antagonists, naloxone, and naltrexone, as well as SSRI are therapeutic option which modulate central pruritus-transmission. However, those medications only showed improvement of pruritus sensation in little pediatric series (180,219,220)

As EHC introduces potential pruritogenic substances into the systemic circulation. Stapelbroek *et al.* described that an interruption of EHC by endoscopic nasobiliary drainage relieves cholestatic pruritus drastically in BRIC (169). Beuers *et al.* confirmed that pruritus is relieved by nasobiliary drainage in PBC as well (221). Also in PFIC1 and PFIC2, encouraging results

have been reported (222). Furthermore, the Molecular Adsorbent Recirculation System (MARS), which is an albumin-based extracorporeal dialysis, has been shown to reduce refractory pruritus in adult PBC-, ICP-patients and in children with CCLD and pruritus (223,224).

Potential antipruritic therapy options	
Medications affecting BA metabolism	
Rifampicin	Inducement of hepatic microsomal oxidizing enzymes and biotransformation transporters; promotion of metabolism and/or secretion of potential pruritogens
Anion exchange resins (Cholestyramine, colestipol, colesevelam)	Binding of potential pruritogens (particularly BA); enhancement of excretion by interruption of EHC
ASBT inhibitors (Odevixibat)	Decrease of enteric BA uptake
Medications modulating central pruritus transmission	
Opioid antagonists (Naltrexone, naloxone)	Decrease of opioidergic tone
Serotonin reuptake inhibitors (SSRI) (Sertraline)	Decrease of serotonergic signals in the central nervous system; inhibitory signals to itch pathway
Invasive therapeutic options	
Nasobiliary drainage, MARS, plasmapheresis	Removal of potential pruritogens by interruption of EHC

Table 2: Potential antipruritic therapy options of cholestatic pruritus (158)

Anticholestatic therapy options	
UDCA	Generally ineffective; except in PBC, PFIC and ICP
4-phenylbutyrate	Appropriate for specific cases of PFIC

Table 3: Anticholestatic therapy options of cholestatic pruritus (158)

1.4 Aim of the Study

The overall goal of this study was to prospectively investigate if a rise of specific Bas or MCA correlate with a surge in plasmatic ATX levels in children with ASC/PFIC and pruritus or adults with ICP and pruritus.

We aimed to investigate the following patient groups:

1) Pediatric patients pruritic ASC-/PFIC
2) Pediatric patients non-pruritic ASC-/PFIC
3) Healthy age-matched controls
4) Patients with ICP
5) Healthy pregnant controls

- We hypothesized increased tBA/tMCA levels and altered BA/MCA profiles in pediatric pruritic ASC-/PFIC patients in comparison to pediatric non-pruritic ASC-/PFIC patient. Moreover, we hypothesized increased tBA/tMCA levels and altered BA/MCA profiles in ICP patients compared to healthy pregnant controls.

  We aimed to determine serum tBA/tMCA levels and BA/MCA profiles in pediatric patients with pruritic ASC-/PFIC and in pediatric patients with non-pruritic ASC-/PFIC and ICP-patients. Furthermore, we aimed to compare the results with data from healthy age-matched individuals.

- We hypothesized that a rise of specific rise BA/MCA induces a surge in plasmatic ATX antigen levels.

  We aimed to determine serum ATX antigen levels in pediatric pruritic and non-pruritic ASC-/PFIC patients and ICP patients. Furthermore, we aimed to compare the results with data from healthy age-matched individuals and to prove a potential correlation between ATX antigen levels and tBA/tMCA via statistical analysis in each study group.

2 PATIENTS AND METHODS

2.1 STUDY DESIGN AND PATIENT CHARACTERISTICS

We planned a prospective, observational case-control study at the First Department of Pediatrics of the Semmelweis University Hungary, the Department of Pediatrics and Adolescents Medicine of the Medical University of Graz and the Department of Gynecology and Obstetrics of the Medical University of Graz. The clinical study was approved by the Hungarian ethics committee (Egészségügyi Tudományos Tanács) (43477-/2018/EKU) and the Medical University Graz ethics committee (31-337 ex 18/19 and 30-372 ex 17/18).

2.1.1 OVERVIEW OF GROUPS

Our study included overall 89 patients. Serum samples of in total 50 children (21 females, 29 males) aged 1-18 years were collected. We included 27 patients with CCLD (ASC n=20 [pruritus n=6, without pruritus n=14] and PFIC n=7 [pruritus n=5, without pruritus n=2]). Moreover, 23 healthy age-matched controls were included. Moreover, 39 adult female patients were included aged 18-45 years (**Figure 2**).

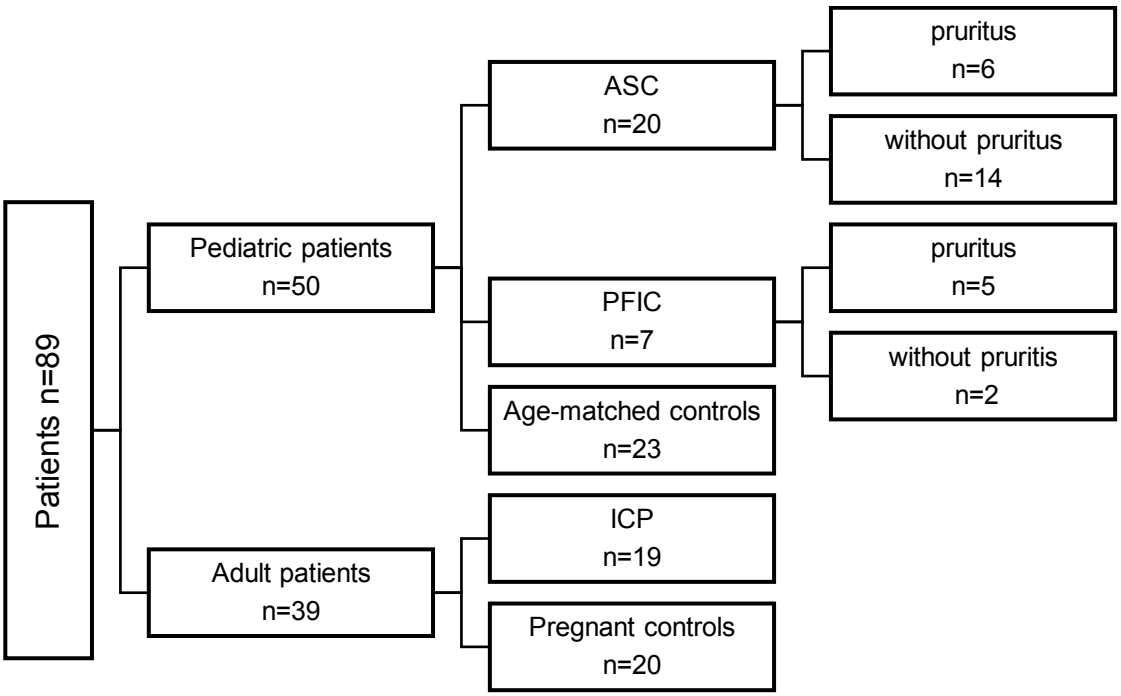

Figure 2: Overview of groups and number of all participants

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Serum samples of pediatric pruritic and non-pruritic CCLD patients (ASC/PFIC) were collected at the First Department of Pediatrics of the Semmelweis University, Budapest from August 2018 to November 2019. For each patient, parental consent was obtained previously.

Inclusion criteria pruritic/ non-pruritic ASC/PFIC patients:

- Male or female subjects between the ages of 0 and 19 years
- Diagnosis ASC/PFIC
- Available information concerning date of birth, sex, date of blood collection

Exclusion criteria pruritic/ non-pruritic ASC/PFIC patients:

- Interruption of EHC
- Chronic diarrhea requiring specific intravenous fluid/ nutritional intervention
- Liver transplant
- Decompensated cirrhosis (INR $\geq$ 1.5 (not due to vitamin K deficiency), albumin < 3.0 mg/dl, ascites, hepatic encephalopathy)
- Known diagnosis of immunodeficiency
- Any pregnant or lactating female
- Diabetes mellitus
- Chemotherapy

Serum samples of healthy age-matched controls were collected at the Department of Pediatrics and Adolescent Medicine, Medical University Graz, from December 2019 prospectively until March 2020. For each patient, parental consent was obtained previously.

Inclusion criteria pediatric control group:

- Male or female subjects under the ages of 0 and over 19 years
- Available information concerning date of birth, sex, date of blood collection

Exclusion criteria pediatric control group:

- Diagnosis of liver diseases
- Interruption of EHC
- Chronic diarrhea requiring specific intravenous fluid/ nutritional intervention
- Known diagnosis of immunodeficiency
- Any pregnant or lactating female
- Diabetes mellitus
- Chemotherapy

Serum samples of patients with ICP and healthy pregnant controls were collected at the Department of Gynecology and Obstetrics of the Medical University of Graz prospectively from July 2018 until March 2020. Consent was obtained for each participant.

Inclusion criteria ICP patients:

- Female subjects between the ages of 18 and 45 years
- Single and multiple pregnancy
- Pregnancy week 28+0 – 40+0
- Pruritus
- Fasting total serum bile acid >3x ULN and/or elevated transaminases (ALT/AST)

Exclusion criteria ICP patients:

- Female subjects under the ages of 18 and over 45 years
- Pruritus not due to ICP
- Preeclampsia, HELLP syndrome
- Dermatologic disease associated with pruritus
- Pregnancy-induced hypertension
- Previous operation at gall bladder, bile ducts, or liver

Inclusion criteria pregnant control group:

- Female subjects between the ages of 18 and 45 years
- Single and multiple pregnancy
- Pregnancy week 28+0 – 40+0
- Presentation at the delivery ward/ obstetric ambulance because of premature labor, fetal complication

Exclusion criteria pregnant control group:

- Female subjects under the ages of 18 and over 45 years
- Pruritus
- Evidence of cholestasis in laboratory testing
- Preeclampsia, HELLP syndrome
- Dermatologic disease
- Pregnancy-induced hypertension
- Previous operation at gall bladder, bile ducts, or liver

2.1.2 CCLD Group with and without Pruritus

Pediatric patients with confirmed pruritic and non-pruritic ASC and PFIC were included. As recommend by current guidelines, ASC and PFIC were diagnosed through liver biopsy and biliary imaging (76). Biomarkers indicating hepatobiliary injury were obtained for each subject. Pruritus was diagnosed by using the PVAS in children ≥ 5 years of age (**see 2.3.6**). Children <5 years of age were stratified to the pruritus group when pruritus existent (pruritus: 'yes' or 'no'). Bilirubin, ALT, AST, GGT, IgG levels as well as autoantibodies in serum were calculated by well-established laboratory methods.

2.1.3 ICP Group

In the ICP study group, only patients with proven ICP were included. Diagnosis was made in pregnant women suffering from pruritus not explainable by any other condition and fasting total serum bile acid levels >3x ULN and/or elevated transaminases (ALT/AST). Parameters in serum were calculated by well-established laboratory methods..

2.1.4 Control Groups

Healthy age-matched children were recruited as control group for our pediatric CCLD patients with and without pruritus. Those children were visiting our hospital for scheduled MRI or heart catheter. Furthermore, we were including a control group of healthy pregnant women without pruritus or any other medical condition. Those women were presenting at the delivery ward or the obstetric ambulance, respectively because of premature labor or fetal complication. No medication has been taken in both study groups.

2.2 Sample Collection

All serum samples were taken within routine investigations of children and adolescents (0-18 years). Sampling of serum of patients with ICP as well as of healthy pregnant controls was performed at the Department of Gynecology and Obstetrics of the Medical University of Graz.

As 20 µl of serum is needed to determine ATX activity and 10 µl of serum for measurement of tBA/tMCA concentrations and composition, we needed 30 µl of serum in total per patient. Serum of all patients was centrifuged after extraction for 10 min at 3000 U/min before it was frozen at -80°C within 4 hours and stored until analysis.

2.3 BA Analysis

Using High-Performance Liquid-Chromatography High-resolution Mass-Spectrometry (HPLC-HR-MS), T-/G- conjugated- and unconjugated BA were measured. We included in our measurement tBA (CA, CDCA, LCA, DCA, UDCA) and their G- and T-conjugates and furthermore tMCA (AMCA, BMCA, GMCA, OMCA, HDCA) and their G- and T-conjugates (**Table 4**).

Unconjugated BA	G- conjugated BA	T- conjugated BA
Cholic acid (CA)	GCA	TCA
Chenodeoxycholic acid (CDCA)	GCDCA	TCDCA
Deoxycholic acid (DCA)	GDCA	TDCA
Lithocholic acid (LCA)	GLCA	TLCA
Ursodeoxycholic acid (UDCA)	GUDCA	TUDCA
α-muricholic acid (AMCA)	GAMCA	TAMCA
β-muricholic acid (BMCA)	GBMCA	TBMCA
γ-muricholic acid (GMCA)	GGMCA	TGMCA
ω-muricholic acid (OMCA)	GOMCA	TOMCA
Hyodeoxycholic acid (HDCA)	GHDCA	THDCA

Table 4: Unconjugated BA and MCA and their G- or T-conjugates determined in this study. Reproduced from (1) with permission from Frontiers Media SA.

2.3.1 Sample Preparations and BA Standards

For identification and quantification in MS analysis, we used unconjugated BA, and their T- and G-conjugates (**Table 4**), and the internal standards d4-DCA, d4-LCA, d4-GLCA and d4-GCDCA (all Sigma Aldrich, Taufkirchen, Germany). All MCA used as external standards were synthesized by the protocol of Benno Amplatz *et al.* at the Clinical Institute of Medical and Chemical Laboratory Diagnostics of the Medical University of Graz in 2014 (225). We prepared plasma samples according to the protocol of Humbert *et al*. (226).

2.3.2 Chromatography

Through an autosampler (Accela Open AS, Thermo Fisher Scientific), each sample (10 µl) was introduced into the chromatographic system. For HPLC of BA samples, a nucleoshell C18 reversed-phase column (2.7µm, 50 x 2.0 mm) (Macherey-Nagel, Düren, Germany) set to 25°C was used. A 1250 Accela (Thermo Fisher Scientific) was used as HPLC pump. Different for measurements of MCA, a kinetex pentafluorphenyl column (2.6 µm, 100 x 6.0 mm Phenomenex, Aschaffenburg, Germany) was used. To separate and elute samples, a gradient of mobile phase A (aqua dest. With 1.2% v/v formic acid and 0.38% w/v ammonium acetate) and eluent B was used. Gradient setting for eluents A/B (in % v/v) were 60/40 (0 min), 60/40 (1 min), 30/70 (9 min), 35/65 (11 min), 0/100 (12 min), 0/100 (15 min), 60/40 (19 min, re-equilibration start) and 60/40 (23 min). Flow rate was 500 µl/min. The lower limit of detection was at 0.012 nmol/mL (225).

2.3.3 Mass-spectrometry

We used a Q Exactive hybrid quadrupole-orbitrap MS (Thermo Fisher Scientific) with a heated electrospray ionization ion source. Full scan was set between m/z = 370 to m/z = 570 in negative ion mode, with a resolution of 70,000 (225).

2.3.4 Calibration

Through correlation of peak area ratios (natural targets vs. internal standards) linear calibration was done. Therefore, 13 diluted standard concentrations of tBA CA, CDCA, DCA, LCA, and UDCA were used with known amounts in a range of 0.024 to 100 nmol/ml. We used the Xcalibur 2.3 software (Thermo Fisher Scientific) for setting up the calibration curve (225).

2.3.5 Human ENPP-2/ATX Immunoassay

The determination of human ATX/ENPP-2 antigen concentrations in serum samples of our included study subjects was done by using the Quantikine® ELISA (Enzyme-linked Immunosorbent Assay) Human ENPP-2-/ATX Immunoassay (227). The 4.5-hour solid-phase ELISA was done according to manufacturer's instruction.

2.3.5.1 Materials

The materials provided by the Quantikine® ELISA Human ENPP-2-/ATX Immunoassay (227) were stored at 2-8°C.

2.3.5.2 Reagent and Sample Preparation

All reagents were brought to room temperature before use. Initially, Human ENPP-2 Standard was reconstituted with distilled water. Therefore, a stock solution of 100ng/ml was reconstituted which was used to produce a dilution series.

Serum samples of study groups were initially 20-fold diluted. However, the high ATX antigen levels in some of our serum samples lead to the necessity for a 50-fold or 200-fold dilution, respectively, and a repetition of the measurements.

2.3.5.3 Assay Procedure

The assay procedure was performed according to manufacturer's instruction (227). We worked with duplicates for each standard/sample. The optical density of each well was determined right away after the assay procedure using a microplate reader set to 450 nm. As wavelength correction was available, we determined each well furthermore at 540 nm and 570 nm.

2.3.5.4 Calculation of Results

We averaged the duplicate readings for each standard/sample and subtracted the average zero standard optical density. We created a standard curve (**Figure 3**) by using Microsoft Excel. As our samples were diluted, the concentrations read from the standard curve were multiplied by the dilution factor.

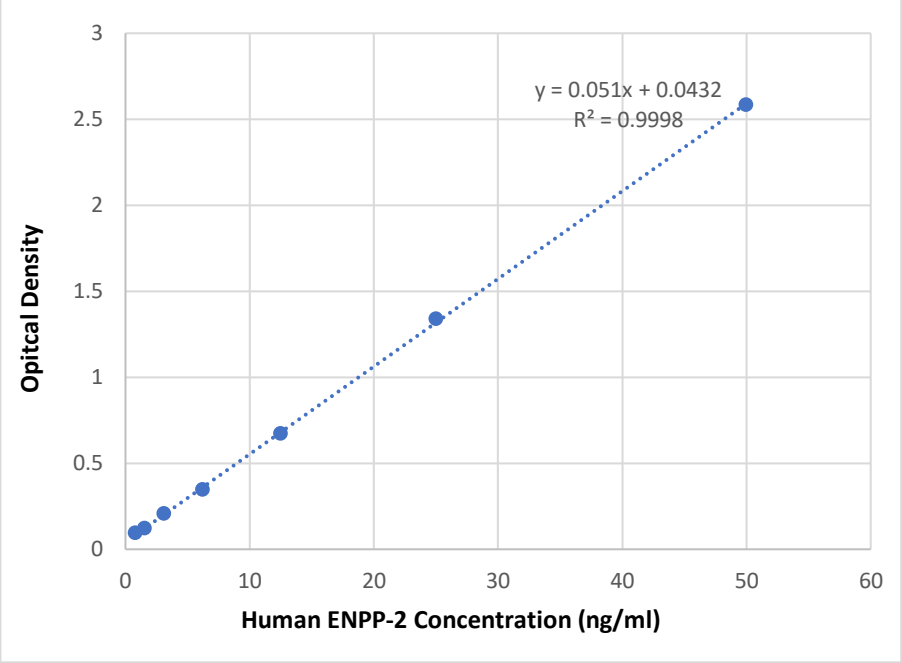
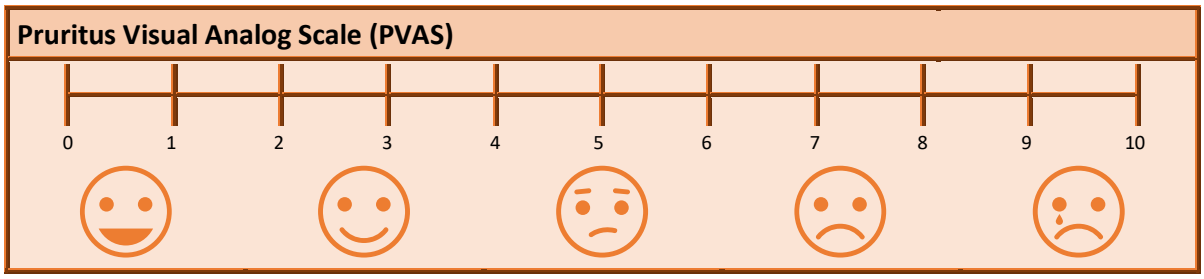


Figure 3: Standard curve ATX Immunoassay

2.3.6 PVAS

The PVAS (**Figure 4**) is showing the numbers 0 (“no itch”) to 10 (“worst imaginable itch”). All Patients are requested value their individual pruritus severity based on this scale. The advantages of the PVAS are a high validity and reliability but also a simple format. For that reason the PVAS is often used especially for children (228). All patients who named a PVAS of 0 were assigned to the study group of patients with non-pruritic ASC/PFIC. However, due to non-verbality, the application of the PVAS is not validated in children <5 years of age. Children < 5 years of age were stratified to the pruritus study group if itching, displayed by scratching behavior, was present.



0-1 No itch
2-5 Mild itch
6-8 Moderate itch
9-10 Severe itch

Figure 4: PVAS for children ≥ 5 years of age

2.3.7 Clinical and Laboratory Data

Clinical data including age, sex, height, and weight were raised. Moreover, we collected data concerning past medical history and current medical therapies in our pediatric CCLD patients and in the pediatric control group. Using well established, standard laboratory methods, the absolute concentrations of GGT, AP, bilirubin, ALT, AST, and lactate dehydrogenase (LDH) were determined.

In our ICP study group and pregnant healthy controls, we collected data concerning age, gestational week, comorbidities, and current medication. Concentrations of bilirubin, ALT, AST and GGT were determined. Preeclampsia was excluded by the sFlt-1/PlGf Ratio (soluble Fms-like tyrosinkinase-1/placental growth factor).

2.4 Statistical Analysis

We included clinical and laboratory findings in our statistical analysis. Initially a Kruskal-Wallis analysis was performed for comparison of ASC-/PFIC patients and the control subjects. The data of pruritic and non-pruritic ASC patients was compared through a Man-Whitney U-test. The profiles of BA and MCA were calculated through determination of the proportion of every single BA when expressed as a percentage of the tBA level. For investigation of a potential correlation between the examined variables, Spearman's correlation coefficient with corresponding p-values were calculated. In case of categorical data, we performed a Fisher's exact test or a Chi-Square test. The whole statistical analysis as well as the graphic visualizations of this study was calculated using GraphPad Prism software. In all figures significances were indicated with bars and stars within the diagram. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns=not significant. In the tables, significant differences are shown in bold.

3 RESULTS

3.1 DEMOGRAPHIC DATA

3.1.1 DEMOGRAPHIC DATA OF ALL PEDIATRIC ASC- AND PFIC PATIENTS AND CONTROLS

Table 5 summarizes the clinical characteristics and baseline demographic data of pediatric ASC- and PFIC patients and the control group. Compared to ASC patients, PFIC patients were significantly younger smaller and lighter than ASC patients.

In pediatric PFIC patients, serum levels of ALP (p=0.0013) and LDH (0.0160) were significantly higher whereas GGT (p=0.0358) and AST levels(p=0.0316) were significantly lower compared to pediatric ASC patients (**Table 5**). Most children with ASC and every PFIC patient received oral treatment with UDCA. Intractable pruritus was treated with rifampicin additionally in PFIC patients (n=4).

	ASC (n=20)	PFIC (n=7)	p value*	Pediatric ctrls (n=23)	p value**	p value***
Age (years)	16 (15.0-17.8)	1.5 (1.2-3.0)	**<0.0001**	14.0 (12.0-16.0)	**0.0279**	**<0.0001**
Sex, female, n (%)	9 (45)	2 (29)	0.6618	10 (43)	>0.9999	0.6693
Height (cm)	164.0 (156.3-173.8)	86.0 (83.0-101.0)	**0.0004**	165 (155.0-173.9)	0.5997	**0.0010**
Weight (kg)	56.1 (46.9-67.1)	12.3 (11.1-16.1)	**0.0001**	57.0 (42.0-65.0)	0.9664	**0.0002**
PFIC subtype, n:			-		-	-
PFIC-I	0	1		0		
PFIC-II	0	6		0		
Comorbidities, n:			-		-	-
IBD	8	0		0		
Diabetes mellitus I	1	0		0		
Protein S deficiency	1	0		0		
Serum ALP (U/l)	203.0 (133.0-409.0)	496.0 (409.0-568.0)	**0.0013**	78.0 (53.0-141.0)	**0.0003**	**<0.0001**
Serum GGT (U/l)	62.0 (33.0-127.0)	25.0 (8.0-50.0)	**0.0358**	16.0 (14.0-36.0)	**<0.0001**	0.4928
Serum ALT (U/l)	43.5 (20.0-102.0)	116.0 (18.0-184.0)	0.1760	17 (13.0-21.0)	**0.0007**	**0.0038**
Serum AST (U/l)	32.5 (29.3-67.5)	122.0 35.0-182.0)	**0.0316**	24.0 (19.0-27.0)	**<0.0001**	**<0.0001**
LDH (U/l)	177.0 (155.5-218.8)	255.0 (410.0-473.0)	**0.0160**	189.0 (157.0-226.0)	0.6339	**0.0131**
Total bilirubin (umol/l)	12.0 (8.0-22.0)	12.0 (8.3-63.3)	0.9627	0.4 (0.3-0.6)	**<0.0001**	**<0.0001**
UDCA, n	16	7	0.5453	0	-	
Additional medication, n:			**<0.0001**	0	-	
Azathioprine	16	0				
Mesalamine (5-ASA)	6	0				
Rifampicin	0	4				
Prednisolone	4	0				
Furosemide	1	0				
Spironolactone	2	0				
Tacrolimus	0	1				

Table 5: Demographic data and biochemical characteristics of pediatric study cohorts

Normal ranges: ALP 125-515 U/l, GGT 0-38 U/l; ALT 0-50 U/l; AST 0-43 U/l; LDH 120-240 U/l; ***PFIC patients compared to ASC patients; **Control group compared to ASC patients; ***Control group compared to PFIC patients.**

3.1.2 Individual demographic data of pruritic ASC- and PFIC Patients

In **Table 6**, the demographic data of pediatric pruritic ASC-/PFIC patients is lined-up according to their underlying disease. Patients 1-6 suffered from pruritic ASC and were noticeably older compared to patients 7-11 who had PFIC with pruritus. Patients 7-10 received treatment with rifampicin, indicated by the starred tBA and tMCA values.

Patient	Sex (f/m)	Age (y/mon)	Weight (kg)	Height (cm)	Disease	PVAS	tBA µmol/L	tMCA µmol/L
1	f	15 y	51	156	ASC	7	175,3	3,59
2	f	15 y	57,2	145,5	ASC	4	59,2	0,80
3	m	19 y	58,4	174	ASC	6	76,7	0,66
4	f	15 y	46,9	162	ASC	3	41,1	1,07
5	f	15 y	52,7	149	ASC	4	294,7	1,24
6	m	17 y	67,5	189	ASC	8	76,2	2,05
7	m	5 y	16,1	101	PFIC II	5	401,8*	1,76*
8	m	14 mon	11,1	84	PFIC II	N/A	311,2*	7,39*
9	f	16 mon	11,4	83	PFIC II	N/A	262,7*	31,21*
10	m	3 y	14	98	PFIC II	N/A	451,7*	51,76*
11	m	2 y	12,3	86	PFIC II	N/A	10,8	0,18

Table 6: Demographic data of pediatric pruritic CCLD patients lined-up by their underlying CCLD and corresponding PVAS value (children ≥ 5 years of age)

***Patients who received Rifampicin.**
Abbreviations: f, female; m, male; mon, months; N/A, not available; y, year

3.1.3 Individual demographic data of non-pruritic ASC- and PFIC Patients

In **Table 7**, the demographic data of pediatric ASC-/PFIC patients without pruritus is listed according to their underlying disease. Patients 1-14 suffered from non-pruritic ASC. Patients 15 and 16 were diagnosed with PFIC without pruritus. Patient 15 was noticeably younger, smaller and lighter compared to all other included patients without pruritus. None of the patients included in this study group received rifampicin.

Patient	Sex (f/m)	Age (y/mon)	Weight (kg)	Height (cm)	Disease	PVAS	tBA µmol/L	tMCA µmol/L
1	f	18 y	51,4	157	ASC	0	27,6	0,12
2	m	16 y	58,3	164	ASC	0	6,6	0,04
3	m	15 y	46,6	164	ASC	0	43,6	0,71
4	m	18 y	107	178	ASC	0	5,1	0,16
5	m	13 y	46,5	149	ASC	0	18,1	0,54
6	m	16 y	70	185	ASC	0	19,6	0,15
7	m	14 y	45	159,5	ASC	0	133,4	0,66
8	f	10 y	35,8	149	ASC	0	37,1	0,37
9	f	13 y	47	163	ASC	0	108,4	5,56
10	f	18 y	67,8	169	ASC	0	15,3	0,47
11	m	18 y	59,6	172	ASC	0	71,6	0,12
12	f	17 y	66	168	ASC	0	309,6	0,63
13	m	17 y	55	173	ASC	0	16,5	0,09
14	m	16 y	74,1	181	ASC	0	21,0	0,23
15	m	6 mon	4,3	52	PFIC II	N/A	240,2	37,13
16	m	18 y	51,3	166	PFIC I	0	32,2	1,31

Table 7: Demographic data of pediatric CCLD patients with without pruritus lined-up by their underlying CCLD

Abbreviations: f, female; m, male; mon, months; N/A, not available; y, year

3.1.4 Demographic data of pruritic and non-pruritic ASC Patients

We only stratified ASC patients in groups with and without pruritus due to the small quantity of PFIC patients.

Table 8 summarizes the clinical characteristics and baseline demographic data of pediatric pruritic and non-pruritic ASC patients and the control group (1). Age, height, and weight did not differ significantly between the study groups. In pruritic ASC patients, ALT- and AST levels were significantly enhanced than in non-pruritic ASC patients (**Table 8**). Likewise, bilirubin levels were significantly enhanced in pruritic ASC patients compared to non-pruritic ASC patients. UDCA was taken orally by 5/6 pruritic ASC patients (**Table 8** shows the mean daily dose) (1).

	Pruritic ASC (n=6)	Non-pruritic ASC (n=14)	p value*	Pediatric ctrls (n=23)	p value **
Age (years)	15 (15.0-17.3)	16 (13.8-18.0)	0.8228	14 (12.0-16.0)	0.1907
Sex, female, n (%)	4 (53)	5 (31)	0.3359	10 (43)	0.3898
Height (cm)	159.0 (148.1-177.8)	166.0 (158.9-174.3)	0.4324	165 (155.0-173.9)	0.8852
Weight (kg)	54.9 (50.0-60.7)	56.7 (46.6-68.4)	0.9044	57.0 (42.0-65.0)	0.7634
Comorbidities, n:			0.2865	0	-
IBD	3	5			
Diabetes mellitus I	1	-			
Protein S deficiency	1	-			
PVAS	5 ± 1.9	0	**<0.0001**	0	**<0.0001**
Serum ALP (U/l)	343.0 (227.3-437.3)	192.0 (112.0-212.5)	0.0549	78.0 (53.0-141.0)	**<0.0001**
Serum GGT (U/l)	126.5 (50.5-285.3)	50.5 (26.8-109.3)	0.0507	16.0 (14.0-16.0)	**<0.0001**
Serum ALT (U/l)	85.5 (42.3-135.8)	28.5 (14.3-59.3)	**0.0489**	17 (13.0-21.0)	**<0.0001**
Serum AST (U/l)	84.5 (45.8-136.8)	32.0 (27.3-48.5)	**0.0322**	24.0 (19.0-27.0)	**<0.0001**
LDH (U/l)	211.0 (175.0-248.0)	170.0 (148.3-198.5)	0.1093	189.0 (157.0-226.0)	0.3086
Total serum bilirubin (umol/l)	26.0 (13.5-79.0)	10.0 (6.5-17.0)	**0.0049**	0.4 (0.3-0.6)	**<0.0001**
UDCA, n	5	11	>0.9999	-	-
Dosage 10 mg/kg/d (Maximum 500 mg)	500	500	0.8404	-	-
Additional medication, n:			0.6717	-	-
Azathioprine	5	11			
Mesalamine (5-ASA)	2	4			
Prednisolone	1	3			
Furosemide	1	0			
Spironolactone	1	1			

Table 8: Demographic data and biochemical characteristics of pruritic and non-pruritic ASC patients and controls.

Normal ranges: ALP 125-515 U/l, GGT 0-38 U/l; ALT 0-50 U/l; AST 0-43 U/l; LDH 120-240 U/l**; After the median values, interquartile ranges are shown in brackets. *Compared to pruritic ASC patients; **Compared to non-pruritic ASC patients.**

3.1.5 Demographic data of ICP Patients and ICP controls

The baseline demographic and clinical characteristics of ICP patients and their control group are summarized in **Table 9**. In ICP patients, preeclampsia was excluded by the sFlt-1/PlGf Ratio. Basic laboratory tests were not available in the control group. All blood samples were taken from ICP patients before therapy with UDCA was started.

	ICP (n=19)	**Pregnant ctrls (n=20)**	**p value***
Sex, female, n (%)	19 (100)	20 (100)	-
Gestational week	36.0 (34.0-38.0)	34.5 (30.3-35.0)	0.0618
Comorbidities, n:			-
Hyperthyroidism	1	0	
Hypothyroidism	3	0	
PCOS	1	0	
Bronchial asthma	1	0	
PVAS	N/A	0	-
Serum GGT (U/l)	13 (7-21)	N/A	-
Serum ALT (U/l)	23 (12-57)	N/A	-
Serum AST (U/l)	27 (15-45)	N/A	-
Total serum bilirubin (mg/dl)	0.3 (0.2-0.5)	N/A	-
sFlt-1/PlGf Ratio	9.7 (2.8-28.9)	N/A	-
UDCA, n	0	0	-
Additional medication, n:			-
L-Thyroxin	3	0	

Table 9: Demographic data and biochemical characteristics of ICP patients and ICP control group.

Normal ranges: ALP 125-515 U/l, GGT 0-38 U/l; ALT 0-50 U/l; AST 0-43 U/l; LDH 120-240 U/l; ***Significant differences are shown in bold.**
Abbreviations: N/A, not available; PCOS, polycystic ovarian syndrome; sFlt-1/PlGf, soluble Fms-like tyrosinkinase-1/placental growth factor

3.2 tBA Levels in ASC-, PFIC- and ICP Patients

Independently of the presence of pruritus, ASC patients, tBA levels were significantly increased (median: 42.4 µmol/L, IQR: 18.5-100.1) compared to the control group (median: 1.7 µmol/L, IQR: 0.9-3.6; ASC p<0.0001; PFIC p<0.0001). Same accounts for PFIC patients (median: 262.6 µmol/L, IQR: 32.3-451.7) in comparison to the control group (p<0.0001). TBA levels did not differ significantly between ASC and PFIC patients (p>0.9999) (**Figure 5**) (1).

In ICP patients, tBA (median: 3.7 µmol/L, IQR: 1.8-7.8) were significantly higher than in ICP control group of healthy pregnant women (median: 1.7 µmol/L, IQR: 1.2-3.1, p=0.0063). Both, pediatric ASC- and PFIC patients had significantly higher tBA levels compared to ICP patients (ASC: p=0.0033; PFIC: p=0096) (**Figure 5**) (1).

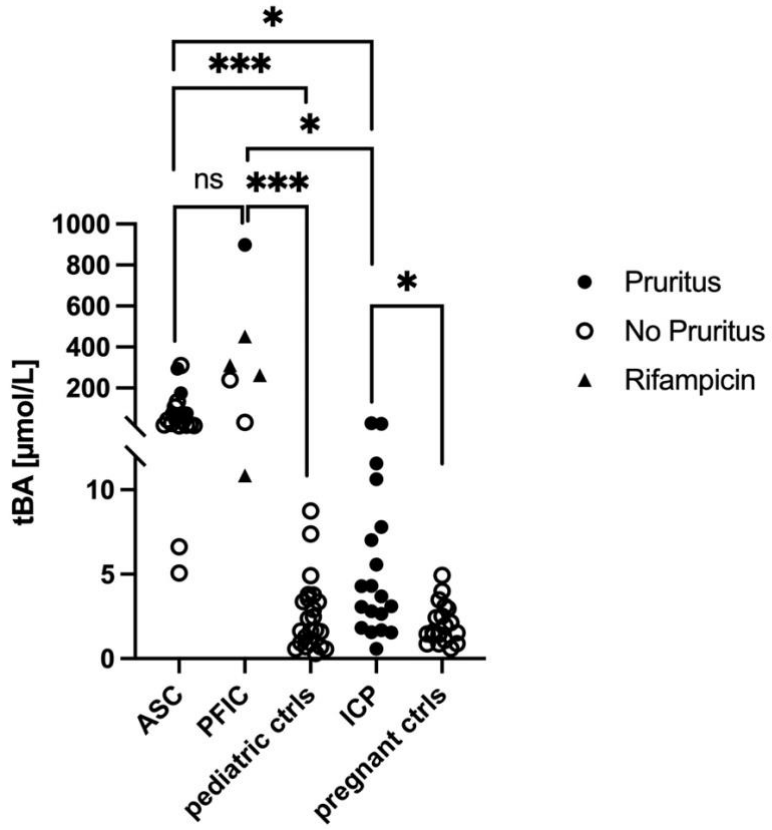


Figure 5: tBA levels in the different study groups

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3.3 Relative BA Levels in ASC-, PFIC- and ICP Patients

BA profiling was done in all our study groups. In PFIC, the relative contribution of unconjugated BA to the BA pool tended to be lower compared to ASC patients and healthy age-matched controls. Moreover, unconjugated UDCA was not detectable in PFIC patients whereas in ASC patients, UDCA represented the most frequent unconjugated BA. In all pediatric study groups, G-conjugation predominated the BA pool, however, with distinct different proportions of the single BA: GCDCA and GUDCA outweighed in ASC patients, whereas in PFIC, GCA predominated. T conjugates were comparable in ASC and PFIC patients (**Figure 6**) (1).

Likewise, in ICP patients most unconjugated BA generally tended to be lower compared to pregnant controls. There was a similar relative contribution of G-conjugated BA levels to the BA pool in both study groups. Different to pediatric patients, T-conjugation, especially TCA and TDCA, predominated in ICP patients compared to pregnant controls (**Figure 6**) (1).

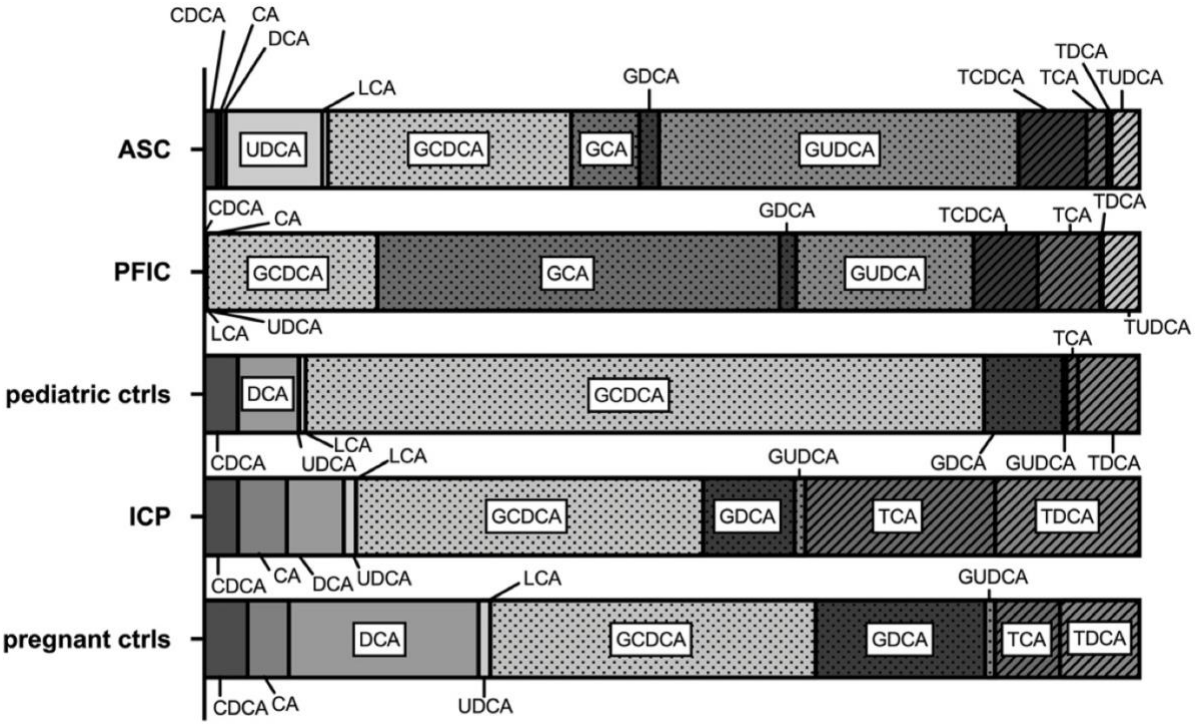

Figure 6: Relative distribution of bile acids in the different study groups

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3.4 Unconjugated to tBA Ratio in ASC-, PFIC- and ICP Patients

For comparison of the BA pool composition between the study groups we measured the ratio of unconjugated to tBA (U:T), which was significantly lower in PFIC patients (median 0.009, IQR: 0.002-0.044) compared to controls (median 0.139, IQR: 0.084-0.437, p=0.0171). The U:T ratio did not differ significantly between ASC patients (median 0.161, IQR: 0.036-0.329) and age-matched controls (p>0.9999). Moreover, there was no statistically significant difference between PFIC and ASC patients (p=0.0877) (**Figure 7**).

Furthermore, the U:T ratio differed not significantly between ICP patients (median 0.135, IQR: 0.064-0.388) and healthy pregnant controls (median 0.289, IQR: 0.197-0.405, p=0.0653). No significant difference existed between ICP- and ASC patients (p>0.9999) and ICP- and PFIC patients (p=0.0906) (**Figure 7**).

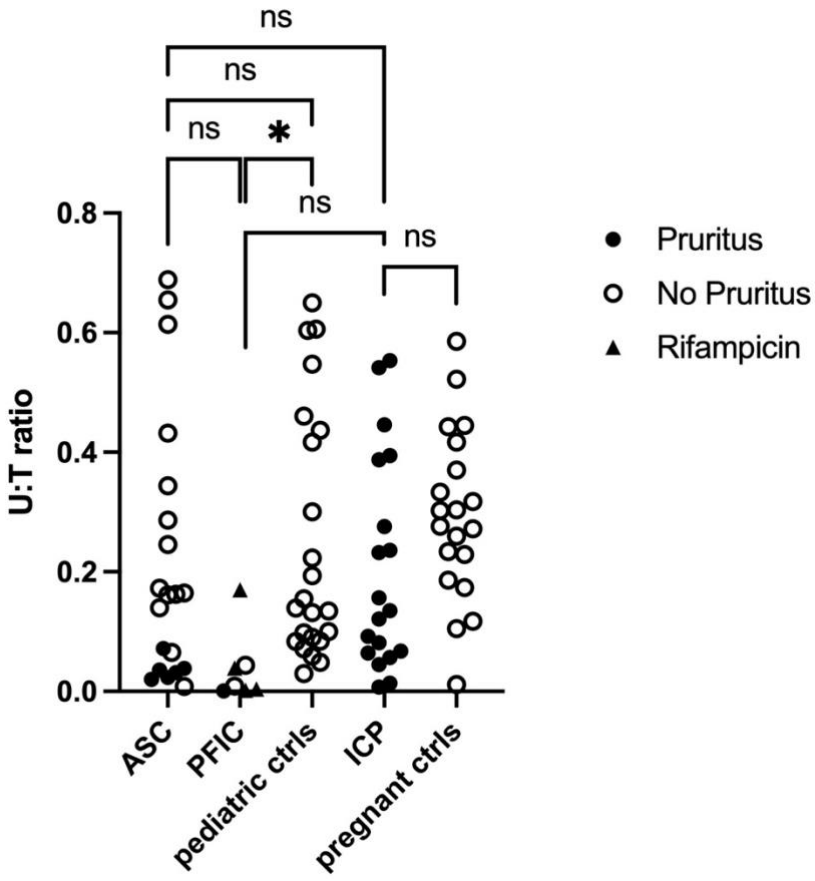

Figure 7: Comparison of the U:T ratio in the different study groups

3.5 Absolute Unconjugated- and Conjugated BA Levels in ASC-, PFIC- and ICP Patients

The unconjugated BA, as well as the G- and T-conjugates, were significantly increased in ASC- as well as in PFIC patients than in the control group (ASC: unconjugated BA p<0.0001, G-conjugates p<0.0001, T-conjugates p<0.0001; PFIC: unconjugated BA p=0.0262, G-conjugates p<0.0001, T-conjugates p<0.0001) (**Figure 8A-C, Table 10**). We could not find a statistically significant difference for unconjugated BA (p=0.5628), G-conjugates (p=0.6911) or T-conjugates (p=0.4733) between ASC and PFIC patients (**Figure 8A-C, Table 10**) (1).

In ICP patients, T-conjugates were significantly enhanced compared to pregnant controls (p=0.0294) (**Figure 8C**). No statistically difference was found for G-conjugates and unconjugated BA between ICP patients and pregnant controls (G-conjugates p>0.9999, unconjugated BA p>0.9999) (**Figure 8A-B, Table 11**) (1). Furthermore, G-conjugates were significantly enhanced in ASC patients and PFIC patients compared to ICP patients (ASC: p<0.0001; PFIC: p<0.0001) (**Figure 8B**). However, unconjugated BA and T-conjugates did not differ significantly in ASC and PFIC patients compared to ICP patients (ASC: unconjugated BA p=0.1523, T-conjugates p>0.9999; PFIC: unconjugated BA p>0.9999, T-conjugates p=0.2764) (**Figure 8A, 8C, Table 11**) (1).

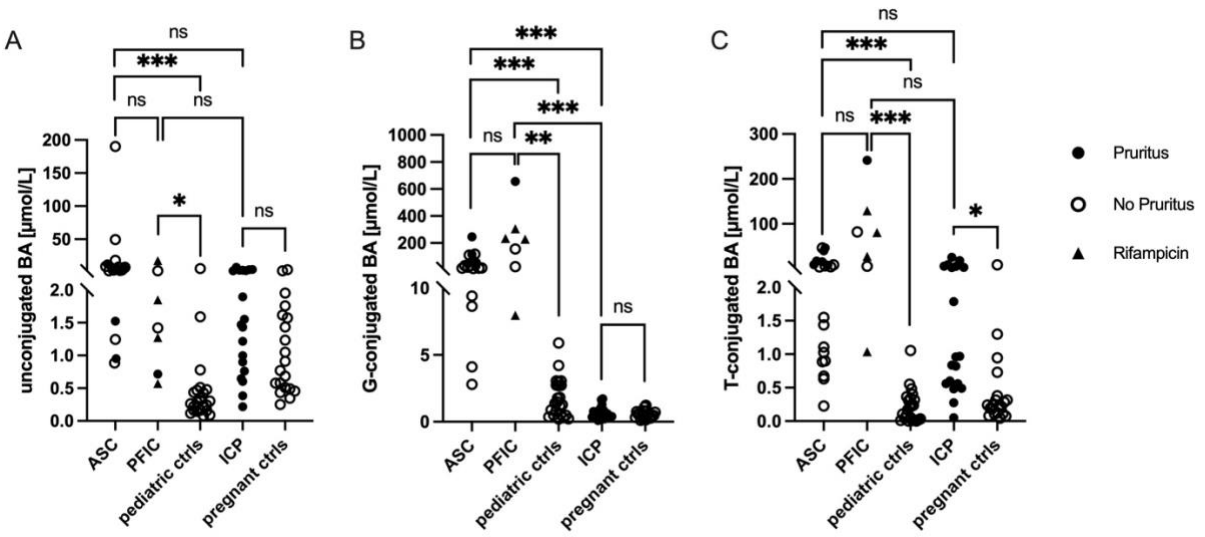

Figure 8: Unconjugated BA (**A**), G-conjugated BA (**B**) and T-conjugated BA (**C**) in the different study groups.

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Bile acid (BA) (µmol/L)	ASC (n=20)	PFIC (n=7)	p value*	Pediatric ctrls (n=23)	p value**	p value***
Unconjugated BA	3.9 (2.3-9.2)	3.9 (2.3-9.2)	0.5628	0.3 (0.2-0.5)	**<0.0001**	**0.0262**
G-conjugated BA	30.8 (11.0-60.8)	229.2 (25.3-305.1)	0.6911	1.4 (0.5-2.7)	**<0.0001**	**<0.0001**
T-conjugated BA	4.3 (0.9-13.9)	80.7 (5.5-129.0)	0.4733	0.2 (0.0-0.3)	**<0.0001**	**<0.0001**

Table 10: Absolute BA levels in pediatric ASC- and PFIC patients with and without pruritus and controls

***PFIC patients compared to ASC patients; **Controls compared to ASC patients; ***Controls compared to PFIC patients.**

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Bile acid (BA) (µmol/L)	ICP (n=19)	Pregnant ctrls (n=20)	p value*	ASC (n=20)	PFIC (n=7)	p value**	p value***
Unconjugated BA	1.5 (0.7-2.9)	0.8 (0.5-1.6)	>0.9999	3.9 (2.3-9.2)	3.9 (2.3-9.2)	0.1523	>0.9999
G-conjugated BA	0.6 (0.3-0.8)	0.5 (0.3-0.8)	>0.9999	30.8 (11.0-60.8)	229.2 (25.3-305.1)	**<0.0001**	**<0.0001**
T-conjugated BA	0.9 (0.6-5.3)	0.2 (0.1-0.4)	**0.0294**	4.3 (0.9-13.9)	80.7 (5.5-129.0)	>0.9999	0.2764

Table 11: Absolute BA levels in ICP patients and pregnant controls and ICP patients compared to pediatric ASC- and PFIC patients

***ICP patients compared to pregnant controls; **ASC- compared to ICP patients; ***PFIC- compared to ICP patients.**

3.6 Absolute Single BA Levels in ASC-, PFIC- and ICP Patients

Bile acid (µmol/L)	ASC (n=20)	PFIC (n=7)	p value*	Pediatric ctrls (n=23)	p value**	p value***
CA	0.11 [0.09-0.32]	0.09 [0.06-0.22]	0.5243	0.00 [0.00-0.09]	**0.0001**	**0.0075**
CDCA	0.41 [0.15-0.61]	0.23 [0.13-0.35]	0.2228	0.06 [0.02-0.11]	**<0.0001**	**0.0009**
DCA	0.13 [0.03-0.38]	0.00 [0.00-0.01]	**0.0032**	0.11 [0.03-0.23]	0.4878	**0.0057**
LCA	0.16 [0.09-0.61]	0.19 [0.19-0.19]	N/A	0.01 [0.00-0.05]	**<0.0001**	N/A
UDCA	2.68 [1.05-7.72]	0.79 [0.31-1.86]	0.1455	0.00 [0.00-0.05]	**<0.0001**	**<0.0001**
GCA	1.89 [0.73-10.35]	120.0 [10.4-170.1]	**0.0027**	0.00 [0.00-0.00]	**<0.0001**	**<0.0001**
GCDCA	6.74 [3.12-14.5]	50.95 [9.32-82.12]	**0.0125**	1.22 [0.39-2.36]	**<0.0001**	**<0.0001**
GDCA	0.55 [0.33-0.78]	4.90 [4.90-4.90]	N/A	0.13 [0.08-0.34]	**0.0003**	N/A
GLCA	0.03 [0.00-0.11]	0.00 [0.00-0.01]	**0.0311**	0.00 [0.00-0.00]	**<0.0001**	0.2069
GUDCA	9.96 [4.46-34.34]	52.86 [3.97-63.78]	0.2635	0.01 [0.00-0.09]	**<0.0001**	**<0.0001**
TCA	0.58 [0.12-5.39]	18.66 [2.51-84.27]	**0.0063**	0.02 [0.00-0.08]	**<0.0001**	**<0.0001**
TCDCA	1.86 [0.48-3.79]	19.18 [1.42-38.08]	**0.0358**	0.00 [0.00-0.00]	**<0.0001**	**<0.0001**
TDCA	0.12 [0.06-0.32]	0.73 [0.01-1.46]	**0.9412**	0.11 [0.02-0.27]	0.4000	0.8700
TLCA	0.00 [0.00-0.01]	0.00 [0.00-0.01]	0.4456	0.00 [0.00-0.00]	0.0325	0.1250
TUDCA	0.77 [0.21-2.87]	10.98 [0.23-26.25]	0.0813	0.00 [0.00-0.00]	**<0.0001**	**0.0025**

Table 12: Absolute serum median single BA levels in µmol/L and interquartile ranges [IQR] in pediatric ASC- and PFIC patients and healthy age-matched controls

***PFIC patients compared to ASC patients; **Controls compared to ASC patients; ***Controls compared to PFIC patients.**

Abbreviations: N/A, not available (due to not measurable amounts)

Bile acid (µmol/L)	ICP (n=19)	Pregnant ctrls (n=20)	p value*
CA	0.13 [0.05-0.19]	0.06 [0.03-0.11]	**0.0277**
CDCA	0.1 [0.06-0.22]	0.07 [0.04-0.15]	0.1639
DCA	0.16 [0.03-0.38]	0.29 [0.12-0.52]	0.1415
LCA	0.00 [0.00-0.03]	0.00 [0.00-0.00]	0.1220
UDCA	0.03 [0.00-0.05]	0.02 [0.00-0.04]	0.4734
GCA	0.00 [0.00-0.04]	0.00 [0.00-0.00]	**0.0191**
GCDCA	0.96 [0.57-1.68]	0.49 [0.29-0.92]	**0.0130**
GDCA	0.25 [0.07-0.91]	0.26 [0.13-0.56]	0.9005
GLCA	0.00 [0.00-0.02]	0.00 [0.00-0.00]	0.0995
GUDCA	0.03 [0.01-0.07]	0.01 [0.00-0.03]	0.2215
TCA	0.53 [0.26-3.33]	0.09 [0.05-19]	**0.0001**
TCDCA	0.00 [0.00-0.00]	0.00 [0.00-0.00]	>0.9999
TDCA	0.40 [0.22-1.79]	0.12 [0.06-0.23]	**0.0008**
TLCA	0.00 [0.00-0.01]	0.00 [0.00-0.00]	**0.0192**
TUDCA	0.00 [0.00-0.00]	0.00 [0.00-0.00]	>0.9999

Table 13: Absolute serum median single BA levels in µmol/L and interquartile ranges [IQR] in ICP patients and healthy pregnant controls

***ICP patients compared to pregnant controls.**

3.7 tMCA Levels in ASC-, PFIC- and ICP Patients

Same as with tBA levels, tMCA levels were significantly increased in both, ASC patients (median: 0.59 µmol/L, IQR: 0.16-1.00) and in PFIC patients (median: 7.39 µmol/L, IQR: 1.31-37.13) compared to control subjects (median: 0.05 µmol/L, IQR: 0.001-0.16, ASC p=0.0006; PFIC p<0.0001). However, tMCA levels did not differ significantly between ASC and PFIC patients (p=0.1807) (**Figure 9**) (1).

In ICP patients, tMCA levels tended to be higher than in pregnant controls but without statistically significance (ICP: tMCA median 0.1 µmol/L, IQR: 0.05-0.4; ICP control: 0.07 µmol/L, IQR: 0.03-0.11 p>0.9999). In PFIC patients, tMCA levels were significantly higher compared to ICP patients (p=0.0080). TMCA levels did not differ significantly between ASC- and ICP patients (p=0.1356) (**Figure 9**) (1).

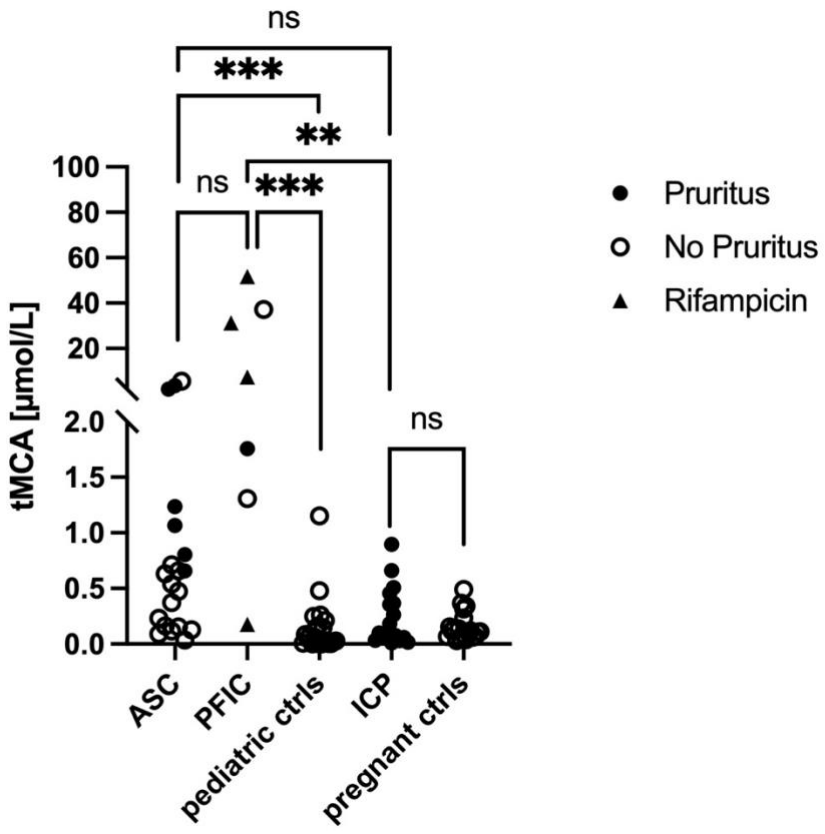

Figure 9: tMCA levels in the different study groups

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3.8 ATX Antigen Levels in ASC-, PFIC- and ICP Patients

ATX antigen levels were just in PFIC patients significantly increased (median: 1650 ng/ml, IQR: 776.9-3742) compared to the control group (median: 315.9 ng/ml, IQR: 251.1-417.2; PFIC p=0.0003). There was no significantly difference of ATX antigen levels in ASC patients (median: 435.9 ng/ml, IQR: 315.8-662.8) compared to controls subjects (p=0.0721). Furthermore, ASC- and PFIC patients did not differ significantly concerning ATX antigen levels (p=0.0713) (**Figure 10**) (1).

In ICP patients as well as in healthy pregnant controls, ATX antigen levels were elevated, however, without a statistically significant difference between the groups (ICP, median: 2388 ng/ml, IQR: 1643-3989; control ICP, median: 1791 ng/ml, IQR: 1585-2375; p=1771). In ICP patients, ATX antigen levels were significantly higher compared to pediatric ASC (p<0.0001). ATX antigen levels did not differ significantly between ICP- and PFIC patients (p>0.9999) (**Figure 10**) (1).

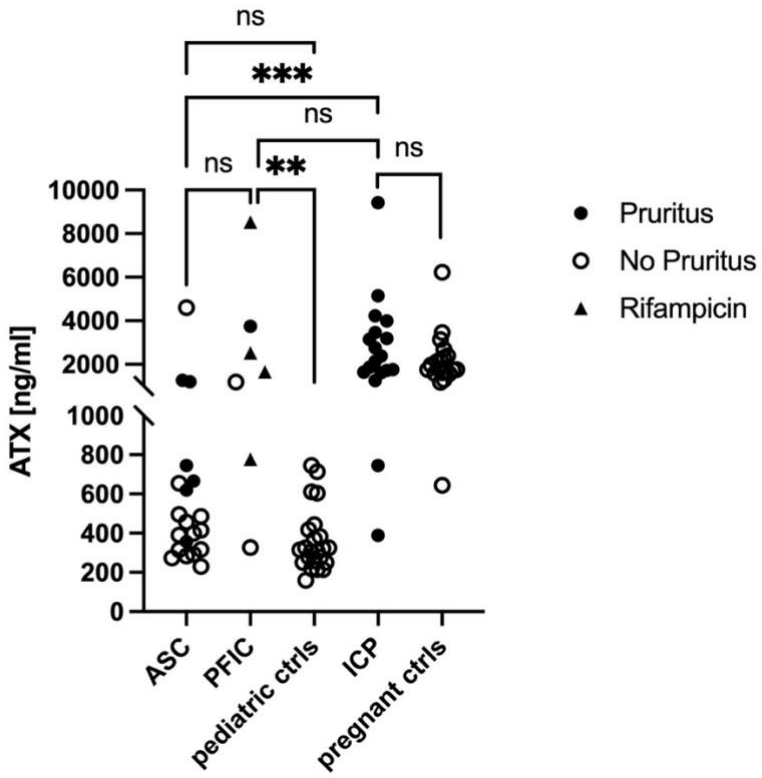

Figure 10: Serum ATX antigen levels in the different study groups

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3.9 TBA LEVELS IN PRURITIC AND NON-PRURITIC ASC PATIENTS

In the following, only pediatric ASC patients were stratified in a “pruritus” (=pruritic ASC) and “no pruritus” (non-pruritic ASC) group.

TBA levels were significantly increased in pruritic ASC patients (median: 76.5 µmol/L, IQR: 54.7-205.1) than in non-pruritic ASC patients (median: 24.3 µmol/L, IQR: 16.2-80.8; p<0.0408) (**Figure 11**) (1).

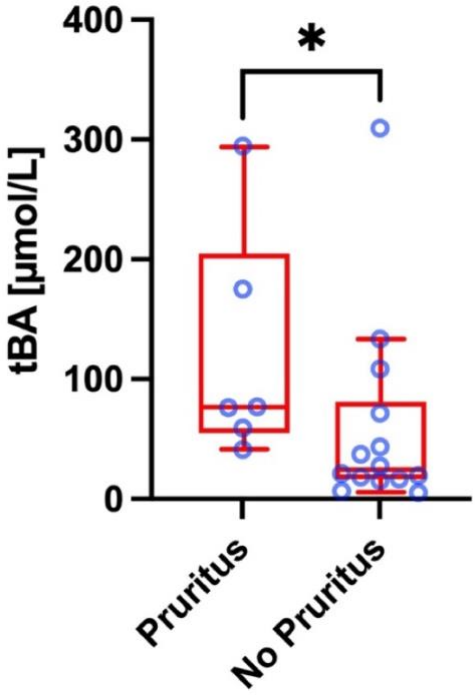

Figure 11: tBA levels in pediatric pruritic and non-pruritic ASC patients

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3.10 Relative BA Levels in pruritic and non-pruritic ASC patients

BA profiles were determined in pruritic and non-pruritic ASC patients and the control group (**Figure 12**) (1)**.**

The amount of unconjugated BA was relatively higher in non-pruritic ASC patients compared to pediatric pruritic ASC patients. Especially UDCA was relatively higher in non-pruritic ASC patients than in pruritic ASC patients. Despite G-conjugates predominated in all study groups, T-conjugates were relatively higher in non-pruritic ASC patients than in pruritic ASC patients (1).

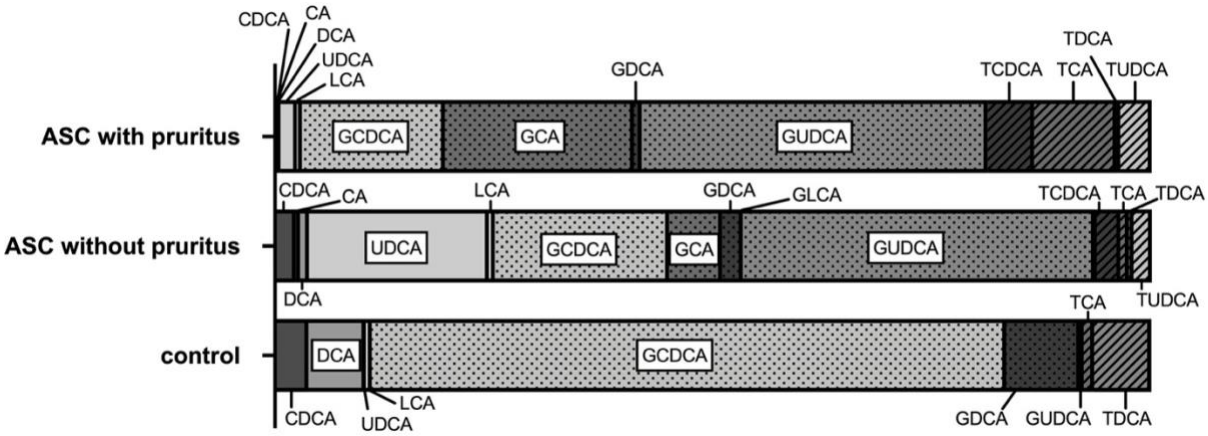

Figure 12: Relative distribution of bile acids in pruritic and non-pruritic ASC patients

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3.11 Unconjugated to tBA Ratio in pruritic and non-pruritic ASC Patients

The U:T ratio was significantly lower in pruritic ASC patients (median 0.034, IQR: 0.022-0.047) compared to non-pruritic ASC patients (median 0.209, IQR: 0.156-0.478, p=0.0015) and control subjects (median 0.139, IQR: 0.084-0.437, p=0.0082). The U:T ratio did not differ significantly between non-pruritic ASC patients and age-matched controls (p>0.9999) (**Figure 13**).

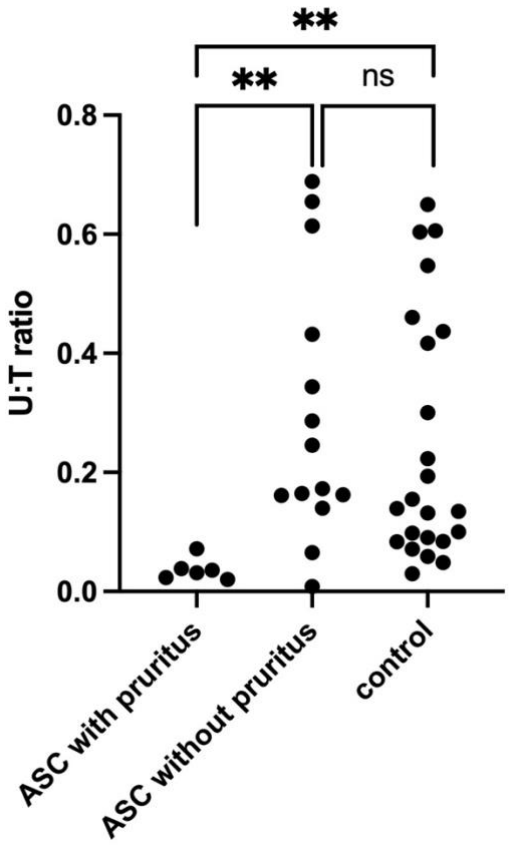

Figure 13: Comparison of the U:T ratio in pruritic and non-pruritic ASC patients

3.12 Absolute BA Levels in pruritic and non-pruritic ASC Patients

3.12.1 Unconjugated BA Levels in pruritic and non-pruritic ASC Patients

Unconjugated BA levels did not differ significantly in pruritic and non-pruritic ASC patients (p=0.4442) (**Figure 14**) (1).

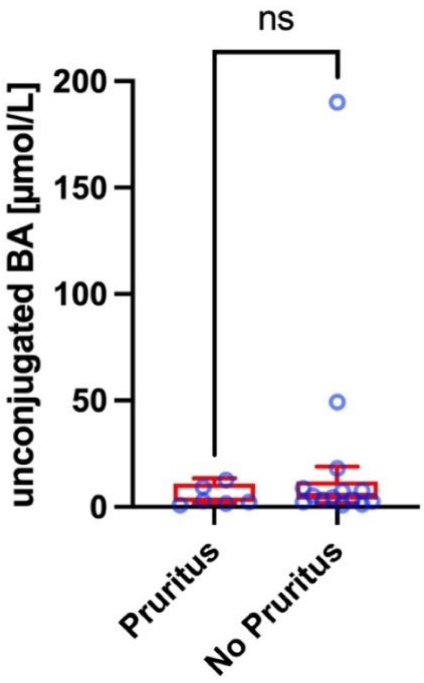

Figure 14: Unconjugated BA levels in pruritic and non-pruritic ASC patients

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3.12.2 G-CONJUGATED BA LEVELS IN PRURITIC AND NON-PRURITIC ASC PATIENTS

In pruritic ASC patients, G-conjugated were significantly enhanced (median: 57.9 µmol/L, IQR: 44.6-148.8) than in non-pruritic ASC patients (median: 14.3 µmol/L, IQR: 9.2-39.3; p=0.0200) (**Figure 15**) (1).

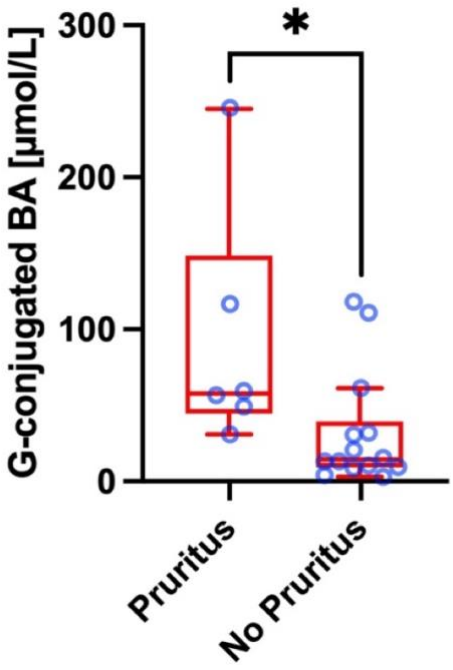

Figure 15: G-conjugated BA levels in pruritic and non-pruritic ASC patients

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3.12.3 T-CONJUGATED BA LEVELS IN PRURITIC AND NON-PRURITIC ASC PATIENTS

In pruritic ASC patients, T-conjugated BA were highly significant increased (median: 16.4 µmol/L, IQR: 8.9-41.4) compared to non-pruritic ASC patients (median: 1.3 µmol/L, IQR: 0.8-4.9; p=0.0023) (**Figure 16**) (1).

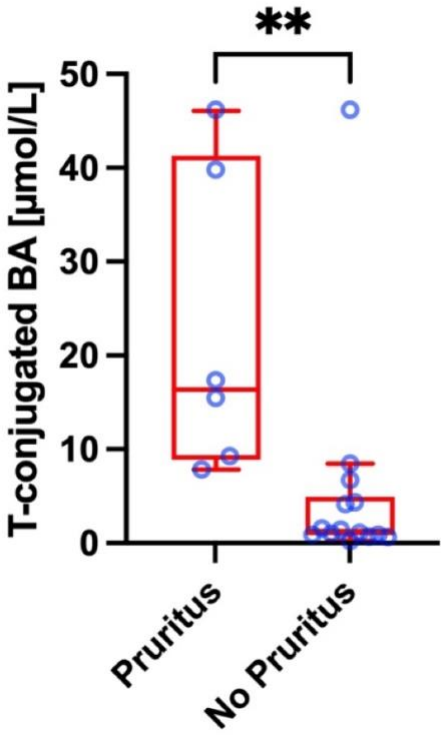

Figure 16: T-conjugated BA levels in pruritic and non-pruritic ASC patients

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3.13

3.14 Absolute Single BA Levels in pruritic and non-pruritic ASC patients

Bile acid (µmol/L)	Pruritic ASC (n=6)	Non-pruritic ASC (n=14)	p value*	Pediatric ctrls (n=23)	p value**	p value***
CA	0.09 [0.05-0.35]	0.09 [0.03-0.20]	>0.9999	0.00 [0.00-0.09]	**0.0041**	**0.0090**
CDCA	0.14 [0.07-0.45]	0.47 [0.23-0.78]	0.2291	0.06 [0.02-0.11]	0.3175	**<0.0001**
DCA	0.04 [0.02-0.28]	0.19 [0.06-0.38]	0.1335	0.11 [0.03-0.23]	0.9145	0.3972
LCA	0.46 [0.46-0.46]	0.13 [0.08-0.61]	>0.9999	0.01 [0.00-0.05]	0.2060	**0.0053**
UDCA	1.59 [0.84-6.28]	4.04 [1.49-10.22]	>0.9999	0.00 [0.00-0.05]	**0.0072**	**<0.0001**
GCA	18.21 [9.74-35.92]	1.19 [0.63-2.82]	0.3856	0.00 [0.00-0.00]	**<0.0001**	**<0.0001**
GCDCA	13.78 [10.06-39.25]	3.91 [2.33-7.95]	0.3117	1.22 [0.39-2.36]	**<0.0001**	**0.0016**
GDCA	0.73 [0.47-3.65]	0.46 [0.30-0.71]	0.8167	0.13 [0.08-0.34]	**0.0053**	**0.0219**
GLCA	0.02 [0.01-0.19]	0.04 [0.00-0.12]	>0.9999	0.00 [0.00-0.00]	**0.0020**	**0.0001**
GUDCA	33.33 [9.79-73.29]	7.90 [3.68-21.40]	0.7598	0.01 [0.00-0.09]	**<0.0001**	**<0.0001**
TCA	7.94 [4.37-16.65]	0.19 [0.09-0.68]	0.2585	0.02 [0.00-0.08]	**<0.0001**	**0.0005**
TCDCA	4.46 [3.08-11.82]	0.53 [0.36-2.40]	0.3961	0.00 [0.00-0.00]	**<0.0001**	**<0.0001**
TDCA	0.34 [0.12-1.16]	0.10 [0.04-0.24]	0.1825	0.11 [0.02-0.27]	0.1452	>0.9999
TLCA	0.01 [0.00-0.04]	0.00 [0.00-0.01]	0.9761	0.00 [0.00-0.00]	**0.0283**	0.4328
TUDCA	2.99 [1.23-11.02]	0.41 [0.11-1.05]	0.0842	0.00 [0.00-0.00]	**0.0002**	**0.0238**

Table 14: Absolute serum median single BA levels in µmol/L and interquartile ranges [IQR] in pruritic and non-pruritic ASC and healthy age-matched controls

***Pruritic ASC patients compared to non-pruritic ASC patients; **Controls compared to pruritic ASC patients; ***Controls compared to non-pruritic ASC.**

3.15 TMCA LEVELS I IN PRURITIC AND NON-PRURITIC ASC PATIENTS

In pruritic ASC patients, tMCA levels were highly significant higher (median: 1.15 µmol/L, IQR: 0.77-2.44) than in non-pruritic ASC patients (median: 0.30 µmol/L, IQR: 0.13-0.64, p=0.0033) (**Figure 17**) (1).

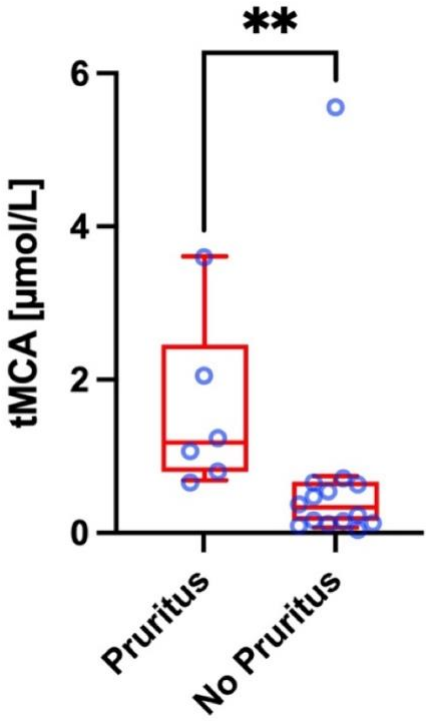

Figure 17: tMCA levels in pruritic and non-pruritic ASC patients

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3.16 Relative MCA Levels in pruritic and non-pruritic ASC patients

Pruritic ASC patients had a greater variance of the MCA. Likewise, pruritus ASC patients had lower levels of unconjugated MCA. HDCA predominated as unconjugated MCA in pruritic ASC patients. T-conjugated MCA made up the largest proportion of MCA in both study groups. Particularly TOMCA was detectable in both study groups, however, the amounts were equally. Compared to pruritic ASC patients, G-conjugated MCA, especially GAMCA und GGMCA, predominated in non-pruritic ASC patients **Figure 18** (1).

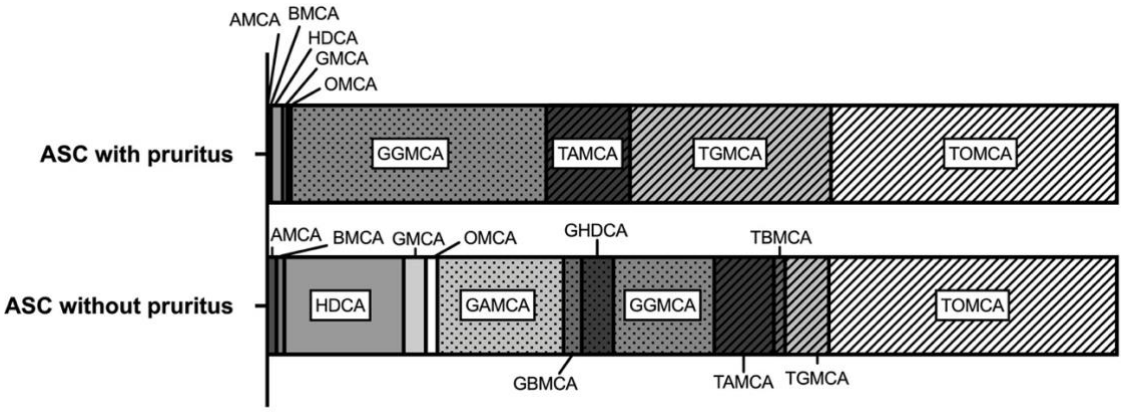

Figure 18: Relative distribution of muricholic acids in pruritic and non-pruritic ASC patients Reproduced from (1) with permission from Frontiers Media SA.

3.17

3.18 Absolute Single MCA Levels in ASC patients with and without Pruritus

Bile acid (µmol/L)	Pruritic ASC (n=6)	Non-pruritic ASC (n=14)	p value*	Pediatric ctrls (n=23)	p value**	p value***
AMCA	0.004 [0.003-0.005]	0.004 [0.003-0.007]	>0.9999	0.003 [0.002-0.003]	0.2254	0.1062
BMCA	0.004 [0.002-0.006]	0.003 [0.002-0.007]	>0.9999	0.000 [0.000-0.000]	0.1026	**0.0016**
GMCA	0.006 [0.005-0.071]	0.008 [0.004-0.021]	>0.9999	0.000 [0.000-0.000]	**0.0001**	**<0.0001**
GMCA	0.005 [0.005-0.005]	0.004 [0.003-0.023]	>0.9999	0.000 [0.000-0.002]	0.0603	**0.0016**
HDCA	0.016 [0.008-0.123]	0.044 [0.016-0.082]	>0.9999	0.064 [0.016-0.226]	0.5244	>0.9999
GAMCA	0.000 [0.000-0.036]	0.046 [0.007-0.069]	0.0586	0.000 [0.000-0.000]	>0.9999	**<0.0001**
GBMCA	0.000 [0.000-0.000]	0.007 [0.005-0.025]	**0.0014**	0.000 [0.000-0.000]	>0.9999	**<0.0001**
GGMCA	0.374 [0.216-0.459]	0.037 [0.019-0.153]	0.1766	0.001 [0.000-0.004]	**<0.0001**	**0.0016**
GOMCA	N/A	N/A	-	N/A	-	-
GHDCA	N/A	N/A	-	N/A	-	-
TAMCA	0.112 [0.043-0.228]	0.022 [0.011-0.130]	>0.9999	0.000 [0.000-0.001]	**0.0020**	**0.0010**
TBMCA	N/A	N/A	-	N/A	-	-
TGMCA	0.268 [0.138-0.641]	0.002 [0.005-0.016]	0.2538	0.000 [0.000-0.000]	**<0.0001**	**0.0005**
TOMCA	0.217 [0.217-1.133]	0.106 [0.006-0.132]	0.1221	0.000 [0.000-0.000]	**<0.0001**	0.0078
THDCA	N/A	N/A	-	N/A	-	-

Table 15: Absolute serum median MCA levels in µmol/L and interquartile ranges [IQR] in pruritic and non-pruritic ASC patients and healthy age-matched controls

***Pruritic ASC patients compared to non-pruritic ASC patients; **Controls compared to pruritic ASC patients; ***Controls compared to non-pruritic ASC.**

Abbreviations: N/A, not available (due to not measurable amounts)

3.19 ATX Antigen Levels in pruritic and non-pruritic ASC Patients

ATX antigen levels did not differ significantly in pruritic and non-pruritic ASC patients (pruritic ASC: median: 665.8 ng/ml, IQR: 357.8-1203; non-pruritic ASC: median: 391.0 ng/ml, IQR: 283.2-485.6, p=0.1061) (**Figure 19**) (1).

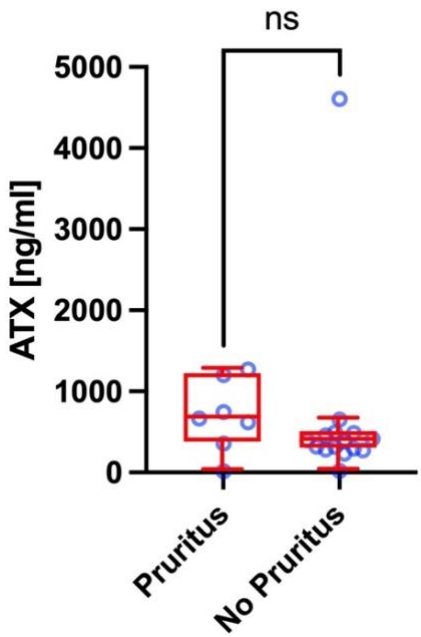

Figure 19: Serum ATX antigen levels in pruritic and non-pruritic ASC patients

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3.20 Correlations in pruritic and non-pruritic ASC Patients

3.20.1 Correlation between tBA and ATX antigen levels

In pediatric pruritic ASC patients, we found a significant correlation between ATX antigen levels and serum tBA levels but not in non-pruritic ASC patients (**Figure 20**).

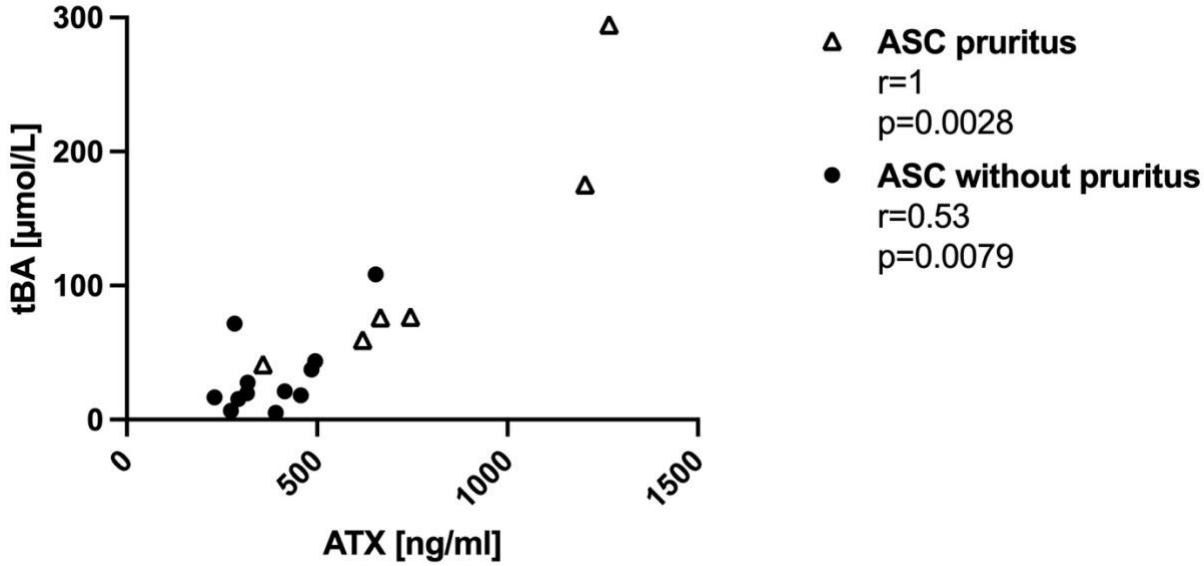

Figure 20: Correlation between tBA and serum ATX antigen levels in ASC patients

3.20.2 CORRELATION BETWEEN TMCA AND ATX ANTIGEN LEVELS

In pediatric pruritic ASC patients, we could not find a significant correlation between ATX antigen levels and serum tMCA levels but in non-pruritic ASC patients (**Figure 22**).

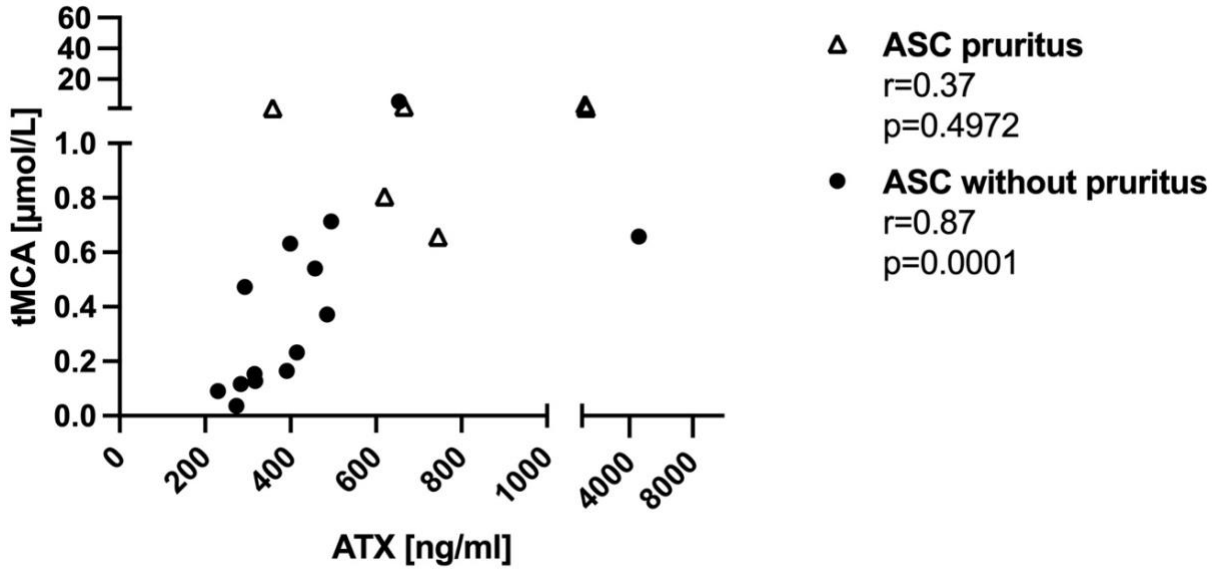

Figure 21: Correlation between tMCA and serum ATX antigen levels in ASC patients

3.20.3 CORRELATION BETWEEN TBA AND PVAS

TBA levels did not correlate significantly with PVAS values in pediatric pruritic ASC patients (**Figure 22**).

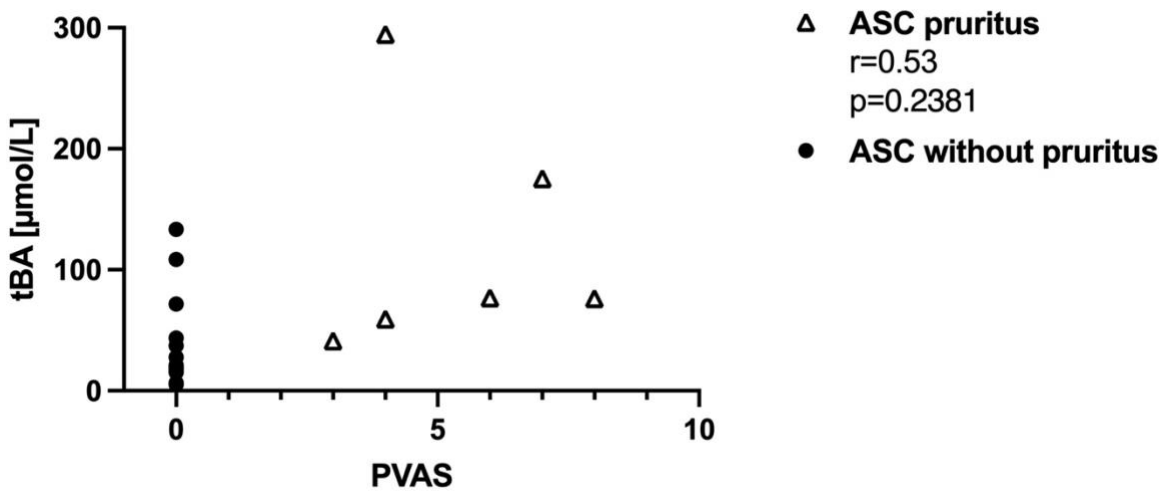

Figure 22: Correlation between tBA and PVAS in ASC patients

3.20.4 CORRELATION BETWEEN TMCA AND PVAS

TMCA levels did not correlate significantly with PVAS values in pediatric pruritic ASC patients (**Figure 23**).

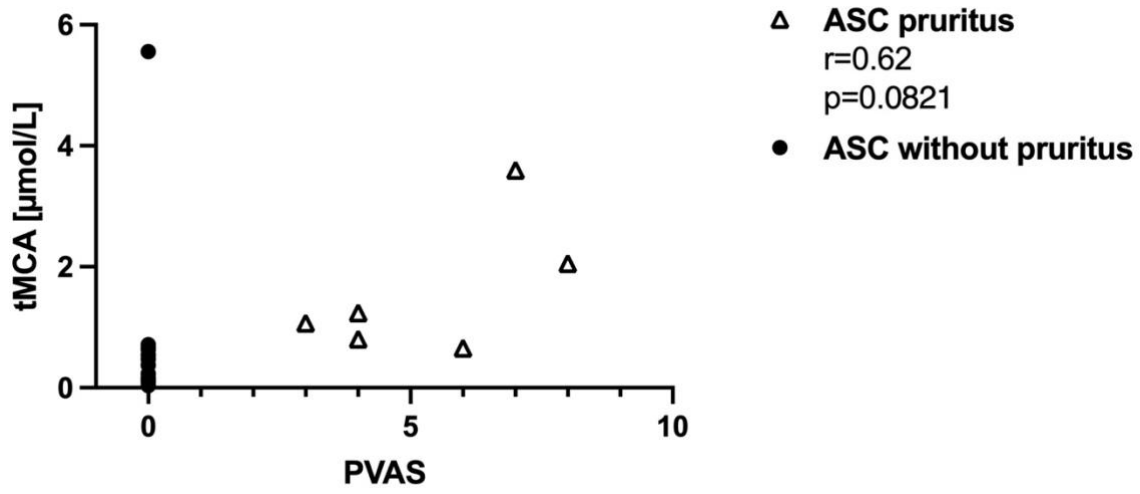

Figure 23: Correlation between tMCA and PVAS in ASC patients

3.20.5 CORRELATION BETWEEN ATX ANTIGEN LEVELS AND PVAS

In pruritic ASC patients, we found no correlation between PVAS and ATX values (**Figure 24**).

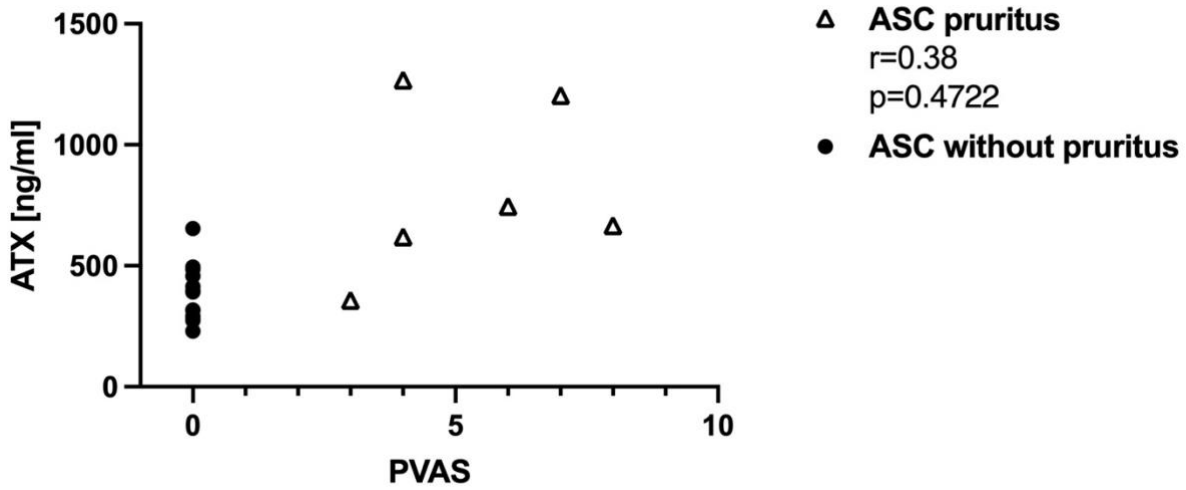

Figure 24: Correlation between serum ATX antigen levels and PVAS in ASC patients

3.20.6 CORRELATIONS LABORATORY DATA AND ATX ANTIGEN LEVELS IN PEDIATRIC PATIENTS

In both, pruritic and non-pruritic ASC patients, ATX levels did not correlate with ALP-, GGT-, total bilirubin- (**Figure 25**), ALT-, AST- or LDH levels (**Figure 26**).

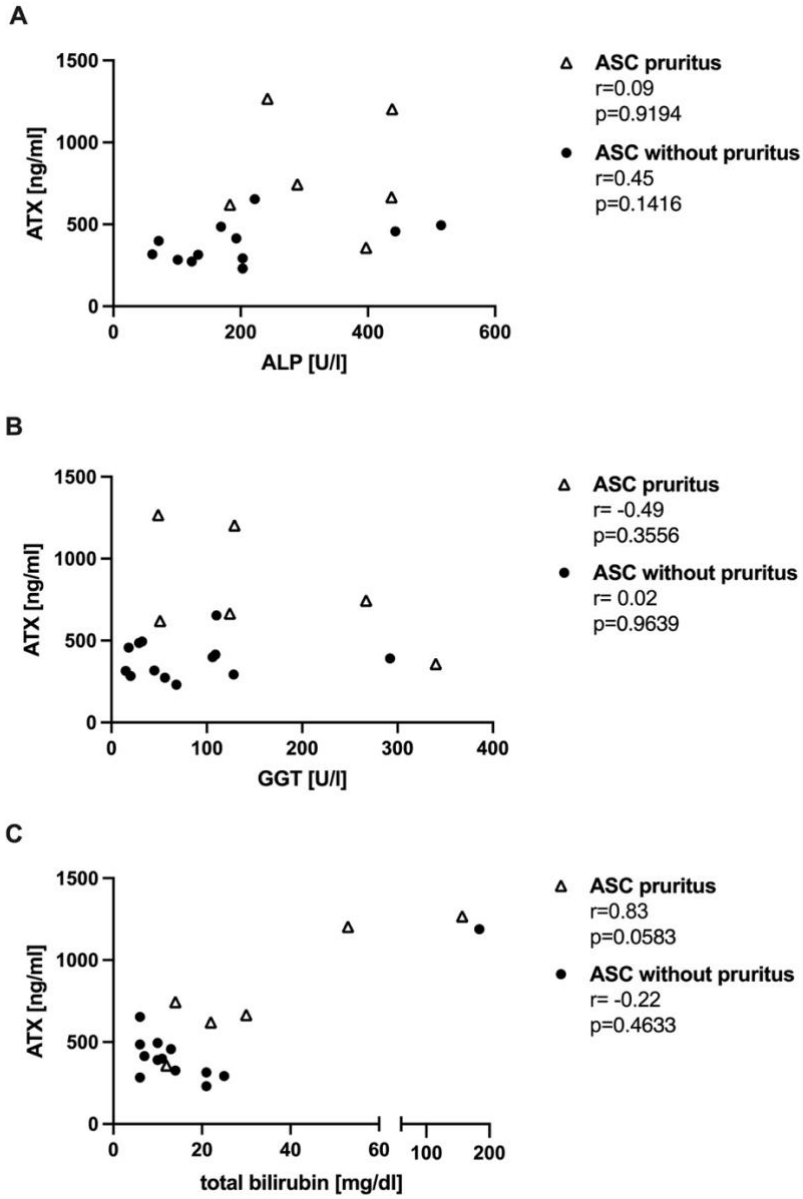

Figure 25: Correlations between laboratory data and ATX antigen levels in pruritic/non-pruritic ASC patients

Correlations between serum ATX antigen levels and ALP (**A**), GGT (**B**) and total bilirubin (**C**)

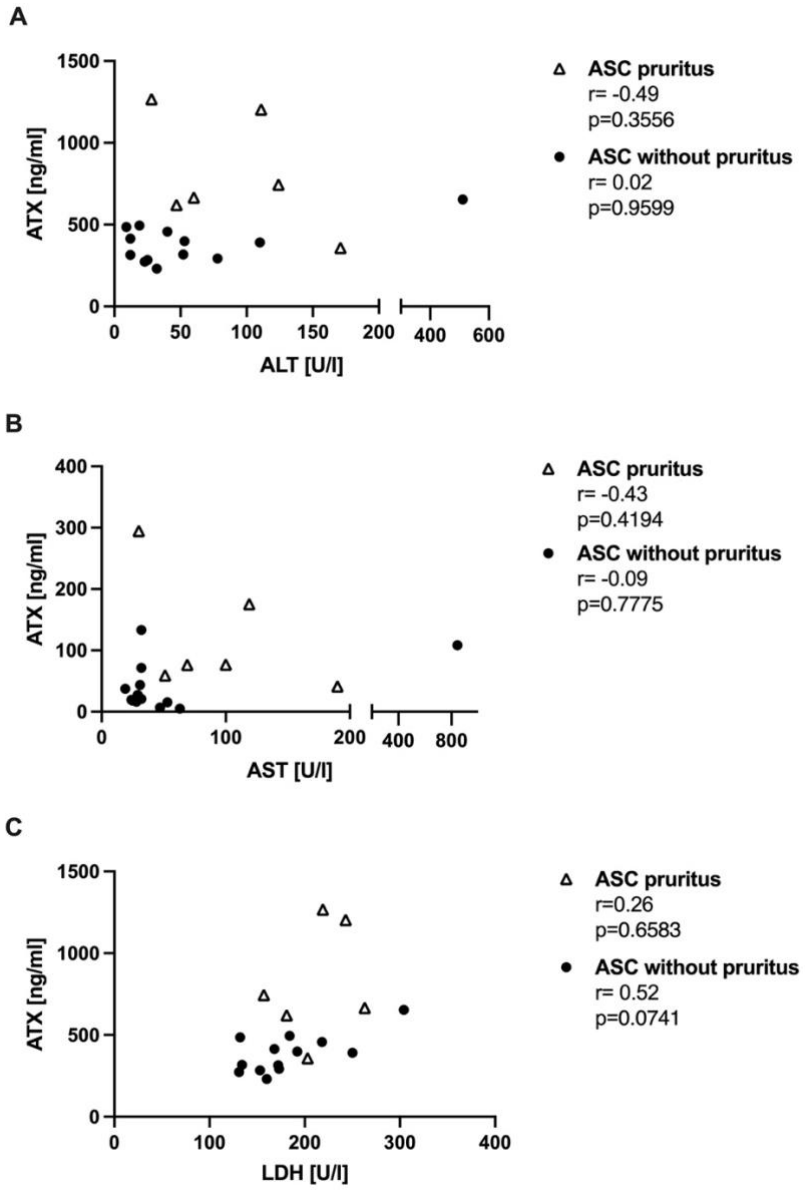

Figure 26: Correlations between laboratory data and ATX antigen levels in pruritic/non-pruritic ASC patients

Correlations between serum ATX antigen levels and ALT (**A**), AST (**B**) and LDH (**C**)

3.21 CORRELATIONS IN ICP PATIENTS

3.21.1 CORRELATION BETWEEN TBA AND ATX ANTIGEN LEVELS

Neither in ICP patients nor in healthy pregnant controls did ATX antigen levels correlate with tBA levels (**Figure 27**).

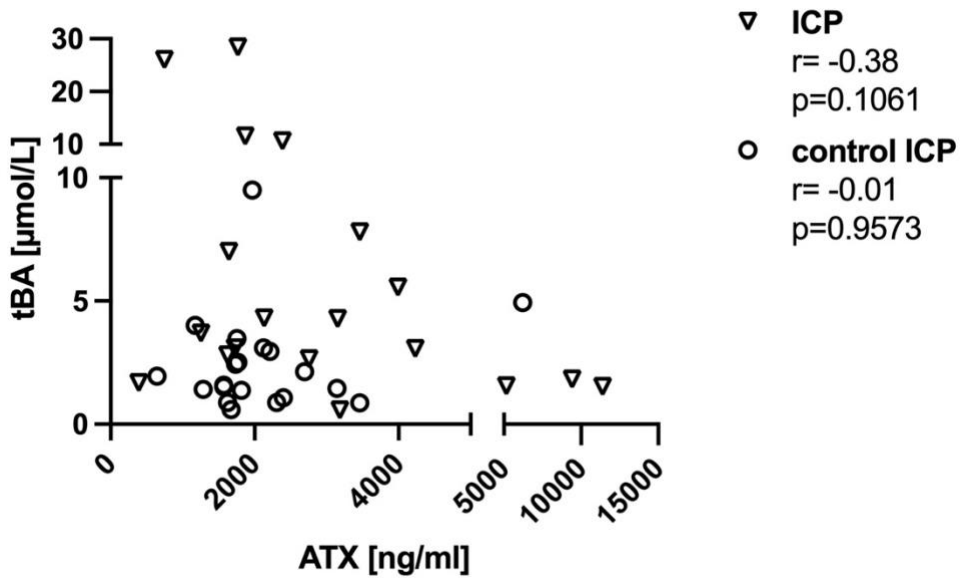

Figure 27: Correlation between tBA and serum ATX antigen levels in ICP patients/healthy pregnant controls

3.21.2 CORRELATION BETWEEN TMCA AND ATX ANTIGEN LEVELS IN ICP PATIENTS

We found no correlation between ATX antigen and tMCA levels in ICP patients and healthy pregnant controls (**Figure 28**).

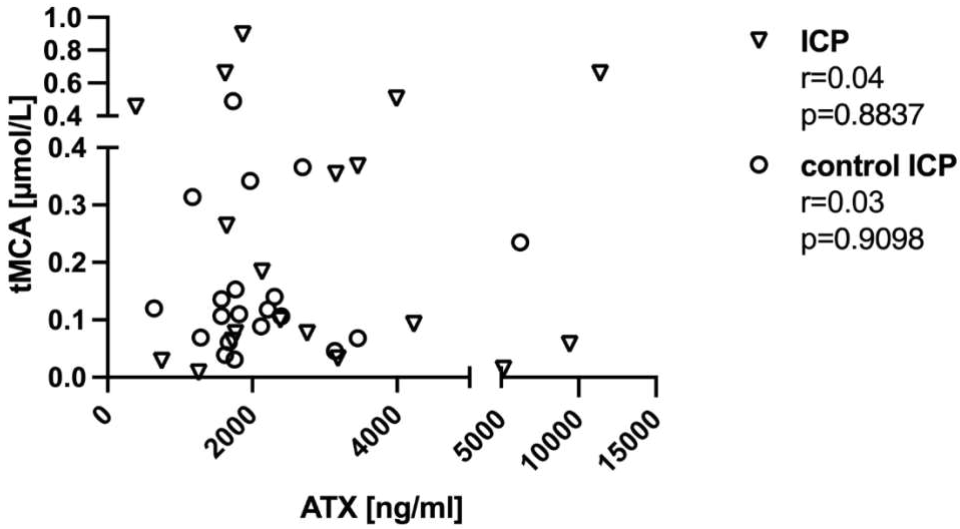

Figure 28: Correlation between tMCA and serum ATX antigen levels in ICP patients/healthy pregnant control

3.21.3 CORRELATIONS BETWEEN LABORATORY DATA AND ATX ANTIGEN LEVELS IN ICP PATIENTS

ATX levels did not correlate with GGT-, total bilirubin- ALT- and AST- levels in ICP patients (**Figure 29**).

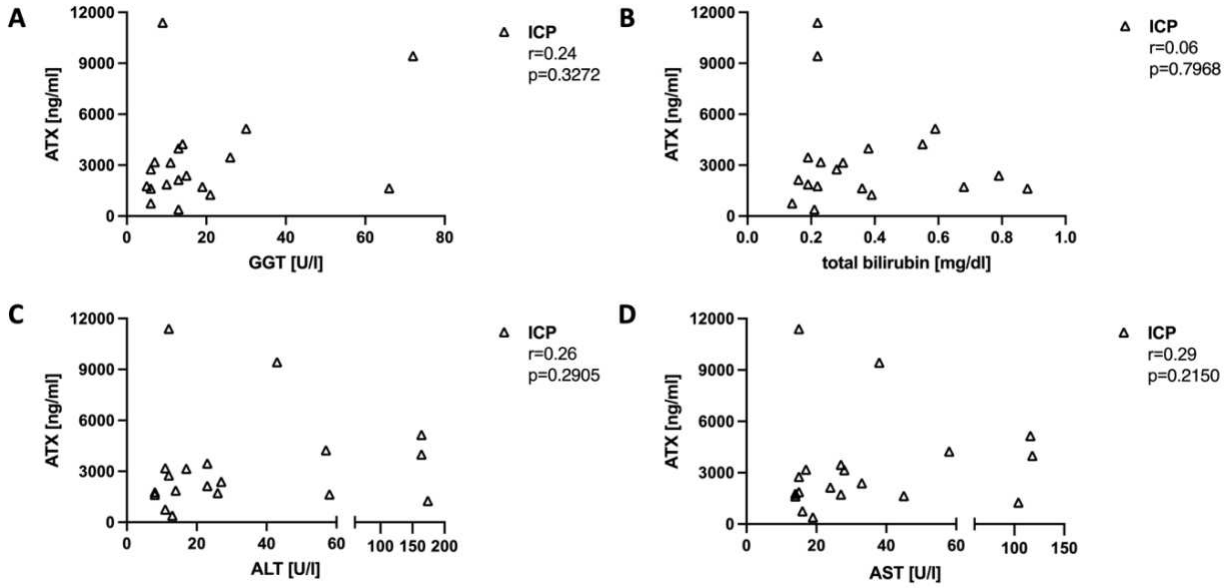

Figure 29: Correlations between laboratory data and ATX antigen levels in ICP patients

Correlations between serum ATX antigen levels and GGT (**A**), total bilirubin (**B**), ALT (**C**), and AST (**D**)

4 DISCUSSION

Various pediatric and adult CCLD (e.g., ASC, PFIC, or ICP), often are associated with chronic pruritus (155–157). However, the underlying pathophysiology is not well established. Moreover, the frequency and severity of cholestatic pruritus are often not correlating with the severity of the CCLD (158,159). Several hypotheses have been proposed, including enhanced BA levels, increased endogenous opioids, and increased ATX activity, amongst others, as underlying mechanism of pruritus in cholestasis, but still, the pruritogenic factors in cholestatic pruritus have not been found yet (1,164,165,174,175,177).

Clinically challenging in the context of cholestatic pruritus are particularly that therapeutic options (158). The PXR-agonist, Rifampicin, enhances the metabolization and secretion of potentially pruritogenic substances. However, rifampicin is only inconsistently effective in pediatric patients (213,214). Anion exchanger resins, e.g., cholestyramine, are prescribed often in CCLD. However, in cholestatic states with reduced BA secretion into bile and intestine (e.g., biliary atresia or PFIC), the efficacy of anion exchanger resins is low (171,215). In literature, peroral UDCA is discussed controversial, however, it is widely prescribed. In PSC or PBC for instance, UDCA could not ameliorate pruritus (210,211,229). Newer therapeutic approaches include odevixibat, an ASBT-inhibitor, which is reducing ileal BA reabsorption and has been shown to improve the severity of itching in pediatric PFIC patients, amongst others (163,216). The chaperone 4-phenylbutyrate was tested pediatric PFIC2 patients. Through re-expression of BSEP, 4-phenylbutyrate could improve pruritus severity (217). Furthermore, in adult PBC and PSC patients, the peroxisome proliferator-activated receptor agonist, bezafibrate, lead to improvement of even severe pruritis (1,218).

In children, adolescents and adults, new insights into the pathophysiology pruritus in CCLD indispensable due to mostly empiric and not consistently effective treatment options. Severe, refractory pruritus in chronic cholestasis is an accepted indication for LTX, even if liver failure is absent (161).

4.1 Results of Pediatric ASC- and PFIC Patients

In this study, tBA levels were significantly higher in pediatric PFIC and ASC patients compared to the control group independent of the presence of pruritus. Those findings were expected since they are characteristics of the pathophysiology of the underlying disease. In both, PFIC and ASC patients, particularly, G- and T-conjugates were significantly enhanced. However, a lower U:T ratio could have been found in our patients only in PFIC patients compared to healthy age-mateched controls (1).

Different in pruritic ASC patients, tBA levels were only slightly higher tBA levels than in non-pruritic ASC patients. Furthermore, tBA levels did not correlate with PVAS values in pruritic ASC patients. Those findings are in accordance with previous studies in which the severity of pruritus did not correlate with tBA levels or any single BA in children and adult patients with different underlying CCLD (135,164). However, a correlation of tBA and PVAS has been reported in pediatric patients with cholestatic pruritus (199), which we could not observe. However, Kremer *et al.* did not include ASC patients in their study (1).

In our study, BA profile were altered in ASC patients suffering from pruritus. T-conjugates were significantly enhanced and the proportion of unconjugated BA in the total BA pool was lower compared to non-pruritic ASC patients, which was displayed by the lower U:T ratio in pruritic ASC patients.

Makino *et al.* already described in 1969 an increase of conjugated BA in adult patients with obstructive jaundice while the unconjugated BA were only slightly altered, leading to a low U:T ratio (9). Hegade *et al.*, also reported significantly elevated tBA levels in adult patients with PBC with pruritus with a predominance of G- and T-conjugated CDCA and CA compared to adult PBC patients without pruritus (230). An upregulation of BAT and BACS due to an enhanced FXR stimulation may be one explanation the higher proportion of conjugation in our patients (9,10). However also a decrease of deconjugation by gut microbiomes reduced bile salt hydroxylase may be also a conceivable reason (231). Unfortunately, as we did not have stool samples for analysis, we could not prove this hypothesis. As BA profiles were distinctly different in our ASC patients, a clinical differentiation of pruritic and non-pruritic ASC patients seems useful in pediatric patients based on our findings

Moreover, the enhanced T-conjugated BA levels of pruritus ASC patients are interesting as previous studies already described differing ratios of G- versus T-conjugates in PSC versus PBC patients (232) and may indicate a varying autoimmune component.

Interestingly, the increase in tMCA was more distinct than that of tBA in pediatric PFIC and ASC patients compared to controls. TMCA levels were significantly higher in pruritic ASC patients compared to non-pruritic ASC patients. Moreover, the MCA profile was distinctly different with less heterogenicity in pruritic ASC patients than in non-pruritic patients. No correlation has been found between tMCA levels and PVAS values (1).

Various studies showed that the BA pool in neonates differs from that in adults (70). The existence of “usual” BA, also called “atypical” BA or MCA, has been described earlier in literature in amniotic fluid and umbilical cord blood (65). These tri-hydroxylated BA are typically present during liver development, as hydroxylation is hepato-protective (13,38). Significantly higher MCA levels could have been demonstrated in full-term- compared to preterm neonates (70). Besides neonates, the existence of MCA have been described in cholestatic patients. Those findings lead to the suggestion of an existing altered pathways in BA metabolism in both: fetal and cholestatic liver (13).

In PFIC patients under rifampicin-therapy, we observed the highest levels auf tMCA. Rifampicin is a strong PXR-agonist which is frequently used in pediatric and adult CCLD patients with pruritus and is known to induce C6-hydroxylation. (213,234). PXR-activation inhibits BA-synthesis by suppression of CYP7A1 and induces hepatic transport proteins such as OATP2 und MRP2 (235,236). Furthermore, activation of PXR increases the activity of the microsomal enzyme CYP3A, which induces detoxification through urinary BA-excretion and C6-hydroxylation (212). For that reason, similar mechanisms of action are assumed for rifampicin (213,234). Therefore, the increased MCA levels in our pediatric pruritic ASC patients may be a sign of an enhanced liver clearance. However, these suggestions need further investigation in a larger study population.

It is known that single MCA have influence on FXR: Antagonistic effects on FXR are known for AMCA and its conjugates (46). This is in accordance with our findings as TAMCA was prevailing in pruritic ASC patients. GAMCA, however, predominated in non-pruritic ASC patients. If TAMCA and GAMCA have a different antagonistic potential of FXR between has not been described so far in literature. Significantly increased TOMCA levels could have been

shown in preterm infants with early-onset sepsis (70). Therefore, a diagnostic potential was assumed for TOMCA (70). In our patients, TOMCA levels were comparably high in all ASC patients independently of the existence of pruritus. Based on our findings, the potential of TOMCA and MCA as biomarkers seems to vary in different pathophysiologies.

We observed significantly increased ATX antigen levels compared to healthy age-matched controls n PFIC patients, howver, not in ASC patients. Furthermore, ATX antigen levels did not differ significantly pruritic and non-pruritic ASC patients (1).

Through its lysophospholipase D activity, ATX is generating LPA through cleaving choline from LPC (177,237). We determined ATX antigen, as a role of LPA in cholestatic pruritus has been described in literature. LPA is considered as strong neuronal activator and is furthermore, considered as potential pruritogen (237). An elevated ATX activity in serum in ICP and PBC patients with pruritus has been described by Kremer et al. Furthermore, ATX activity was described to correlate with the intensity of pruritus (177,198). An interruption of EHC by nasobiliary drainage led to a decreased ATX activity in serum and pruritus sensation of their CCLD patient population. However, ATX protein could not be determined in bile directly leading to the suggestion that ATX expression and/or activity is increased by a substance contained in bile (198).

Also, in children with CCLD with pruritus such as ALGS, extrahepatic biliary atresia and PFIC, significantly increased ATX levels has been described compared to pediatric patients with BA synthesis defects, in which tBA are low are pruritus is usually not observed (199). In our patient cohort, ATX levels were correlating with tBA-, however, not with tMCA levels in our pruritic ASC patients. Different to other CCLD (e.g., ALGS or PFIC), ATX antigen levels seem to be of little diagnostic or prognostic potential in ASC patients. Based on our findings, not only an elevated ATX activity alome seems not to be itching in pediatric ASC patients. However, one possible explanation concerning the different findings may be that Kremer *et al.* determined ATX activity and not ATX antigen levels. Furthermore, due to the small patient count in which a PVAS value was available in our study, our results might be underpowered.

As already mentioned, neither tBA nor tMCA did correlate significantly with pruritus assessed by a PVAS in our study group of pediatric ASC patients. Also, ATX antigen levels did not correlate with PVAS values in pediatric pruritic and non-pruritic ASC patients (1).

Currently, there is no epidemiological study concerning pruritus in children and adolescents. As itch is a perception, its evaluation is difficult. In our pediatric patients older than 5 years of age, pruritus was assessed using a PVAS. As the application of the PVAS is not validated in children under 5 years of age, we stratified younger children to the pruritus study group if itching, displayed by scratching behavior, was present. Besides pruritus, visual analogue scales (VAS) are also used to assess other perceptions such as pain (238). However, the application of VAS comprises several potential sources of error: Firstly, VAS-Scores generally tend to cluster at the midpoint and the extremes. Secondly, interpatient variability in indicating the scales is evident. Thirdly, factors like age, ability to think abstractly or mental organization are known to contribute to respondent error (239). Whether a PVAS displays a sufficient and reliable index in terms of evaluating the severity pruritus in cholestasis especially in younger children is uncertain (181,215,238,240).

As scratching is a behavioral consequence of itching, Bringhurst *et al.* used wrist accelerometers to objectivate scratch behavior in adults and children >2 years of age during night, which seemed to reflect scratch severity and sleep disturbances. However, most of included children were diagnosed with atopic dermatitis thus generalizability is questionable (151,241). Moreover, sleep disturbances in younger children are common and may not reflect pruritus severity (151). Additionally, specific challenges exist for very young infants as motor skills development does not allow children less than six months to sufficiently scratch themselves. This may lead to underestimation of pruritus-related discomfort (150,151).

4.2 Results of ICP Patients

In ICP patients tBA were significantly higher compared to pregnant controls. However, tBA levels were significantly higher in both, pediatric ASC- and PFIC patients, than in ICP patients. BA profiles showed almost no significant differences in ICP patients compared to healthy pregnant controls, although unconjugated BA tended to be lower. Only CA and its G- and T-conjugates were found to be significantly higher compared to pregnant controls. Moreover, TDCA was significantly higher in ICP-patients. In ICP patients, the U:T ratio tended to be lower compared to pregnant controls, however, without a significant difference.

Increased serum tBA levels are the key laboratory finding which is present in >90% of ICP patients (124). In literature, there are deviations concerning the cut-off values due to different methods of measurement, fasting state, or gestational age. The most used cut-off value of tBA

concentrations is 10µmol/L for diagnosing ICP (129). Under physiological conditions, fetal BA are eliminated via the placenta into the maternal circulation (29). However, this mechanism is often reversed in ICP: maternal BA can accumulate in the fetus as well as in amniotic fluid by crossing the placenta (242). In that cases, there is a higher risk for intrauterine demise, preterm delivery, or meconium-stained amniotic fluid (126).

The risk of fetal demise is associated with higher tBA levels in serum, especially >100µmol/L (131). The reason underlying fetal death in ICP is poorly understood. However, high tBA levels may induce vasospasm of the placental chorionic surface vessels (243). In rats, increased TCA levels have been shown to be linked to fetal demise through inhibition of the synchronous beating of fetal cardiac myocytes, however, concentrations of used TCA in this experiments were far higher than determined in affected women (244). Based on a study by Puljic *et al.* some centers deliver women with ICP before term at 36 weeks of gestation to reduce the risk of fetal or infant death (245). Furthermore, an increased risk for neonatal respiratory distress syndrome isnsits which is supposed to be BA-induced (246).

Especially, increased levels of the primary BA CA und CDCA and the secondary BA DCA have been reported in literature in ICP (129,130). Our findings are in accordance with those of Tribe *et al.*, who also found CA and its conjugates as most abundant BA in ICP patients. They also determined a general predominance of T- and G-conjugated BA in serum of ICP patients with particularly increased TCA levels (247). In older studies, Brites *et al.* described a predominance of T-conjugated BA whereas Bacq *et al.* suggested an equivalence of T- and G-conjugates (248,249). Also in our study, conjugated BA predominated with almost equally distributed G- and T- conjugates. In accordance with Tribe *et al.*, TCA was significantly elevated in our ICP study group either.

In ICP patients, UDCA is the first-line therapy as most other therapeutic options are prohibited in pregnancy. It has been shown that ICP patients treated with UDCA had lower tBA levels compared to untreated pregnant women (250). Those findings indicate that UDCA either directly reduces tBA or prevents a further rise of BA levels in ICP patients (247). As T is the most common free amino acid in breast milk and important for brain- and normal growth development and retinal photoreceptor activity our findings of predominating T-conjugated BA in our ICP patients seems to be conclusive (65,67). T intake of pregnant women during late gestational period has led to more body weight and a greater length of the newborn compared to children of pregnant women with low T intake (69). However, a specifically reduction of CA,

TCA, TCDCA and TDCA levels in serum of ICP patients in association with UDCA treatment has been described whereas G-conjugated BA were almost not affected (247).

A preserved FXR signaling is important to limit pathological BA overload in cholestatic states. However, various FXR single nucleotide polymorphisms (SNPs) are known to predispose to ICP (251). Most proven FXR variants in ICP are heterogenous (2,251). Therefore, it is suggested that one copy of the wild-type allele is not enough to prevent cholestatic states during pregnancy when the liver is generally challenged (251). Admittedly, rodent studies could further demonstrate reduced FXR expression of the liver in pregnant mice (252). Consistent with these findings, stimulation of FXR-signaling has been shown to reduce BA synthesis and restore bile flow (253,254).

Literature concerning MCA in women with ICP is lacking. In our ICP patients, tMCA levels tended to be slightly higher compared to pregnant controls, however, without statistical significance. In ICP patients, MCA were always measurable in trace amounts. Furthermore, MCA profiles showed almost no significant differences in ICP patients compared to healthy pregnant controls.

ATX antigen levels tended to be higher in ICP patients compared to pregnant controls, however, without statistically significance. In ICP patients as well as in pregnant controls. ATX antigen levels were significantly increased compared to all our pediatric study groups.

Physiologically serum ATX levels have already been determined to be higher in females than in males and to be highest in pregnant women (185,197). Furthermore, a positive correlation between increased ATX- and LPA levels with gestational age have been reported in pregnant women and rapidly return to pre-pregnancy levels after birth (204,255). In vitro, LPA signaling has been shown to contribute to the production of chemokines from human trophoblast cells. Those chemokines have been shown to regulate migration, proliferation, and angiogenesis of human endometrial epithelial cells (256). Furthermore, ATX is known to be essential for angiogenesis and neuronal development of the fetus (201). It is assumed that fatty tissue and liver may contribute to the increased ATX levels (187). Besides, it has been postulated that placental trophoblasts and synctiotrophoblasts are sources of enhanced ATX synthesis (202,203).

In the past, significantly enhanced ATX activity has been reported in ICP patients compared to healthy pregnant controls, which correlated with pruritus intensity (177). In a more recent study, ATX has also been found to be increased compared to pregnant control, though the differences were not statistically significant (205). Those findings are in accordance with our findings. Moreover, no relation between ATX levels and pruritus severity or preterm birth could have been found in ICP patients (205). It is also interesting that ATX antigen levels in our ICP patients were significantly higher compared to our pediatric CCLD patients with pruritus. One hypothesis for these findings is that the main source of ATX in pregnant woman is the placental trophoblasts and synctiotrophoblasts whereas in CCLD, ATX is mainly built by the liver. Cholestatic states, in which the liver represents an additional ATX source (e.g., ICP), might lead to pruritus sensation. In ICP, tBA might not correlate with ATX activity as ATX is still overwhelmingly source of the placenta in these states.

Controversially, tBA and tMCA levels did neither correlate in ICP patients nor in pregnant controls with ATX antigen levels.

4.3 Strengths and Limitations

Firstly, the small size of the included study groups may limit the representativeness of our results. However, collecting data in the pediatric population is difficult due to ethical reasons. Moreover, finding the appropriate balance of risk and benefit is especially important as children are a vulnerable population.

Secondly, the cellular source of ATX and the influence of BA on ATX secretion should be investigated by ongoing in-vitro studies for exclusion of confounding factors. Nevertheless, we believe in the clinical impact of our results as they provide new insights in the pathophysiology of pruritic and non-pruritic ASC patients. Furthermore, our results point out that the extent of ATX activity seems to be not a trigger of pruritus alone in pediatric ASC patients.

Thirdly, for an optimal determination of tBA/tMCA levels and BA/MCA profiles, fasting serum sampling is the gold standard as the feeding status is an essential component in BA/MCA physiology. The serum sampling of our patients was performed in a nonfasting state. Blood sampling in a fasting state was particularly impossible due to due to ethical reasons in pediatric patients. Therefore, we cannot exclude alterations compared to fasting states completely in our pruritic/non-pruritic ASC/PFIC patients. Same accounts for ICP patients and pregnant controls. However, in pruritic ASC patients, the differences we found in MCA and T-conjugated BA are substantially larger than variations we usually observe in fasting and postprandial samples.

Fourthly, we acknowledge that our pediatric pruritic ASC patients had higher baseline total bilirubin, ALT- and AST levels compared to non-pruritic ASC patients. However, these parameters do not account as sensitive or specific markers of pruritus in cholestasis. Moreover, there were no significant differences in ALP, GGT and LDH. Therefore, we think, that the severity of cholestasis in our pediatric patients pruritic/non-pruritic ASC seems largely comparable and it is therefore unlikely that severity of cholestasis has biased our serum BA/MCA results.

5 CONCLUSION

To date, data concerning pruritus in cholestasis in children an adolescents but also in adult patients are sparse. With this study we are representing for the first time a completely different BA- and MCA profile with significantly elevated tMCA levels in pruritic ASC patients compared to non-pruritic ASC patients. This ASC-specific surge of MCA is a novel observation associated with itching in cholestasis which may allow a more detailed classification of ASC in the future (1). In PFIC patients, ATX antigen levels were significantly increased. In ASC patients, however, ATX antigen levels were not significantly different compared to control subjects. Furthermore, ATX antigen levels did not differ significantly between pruritic and non-pruritic ASC patients which indicates that ATX is not the sole source of pruritus genesis in ASC patients in contrast to other cholestatic states (e.g., PFIC or ALGS). Moreover, tBA/tMCA as well as ATX antigen levels did not correlate significantly with PVAS values and therefore pruritus severity (1).

Similarly, in ICP patients, we found significantly increased tBA levels compared to pregnant controls. Different to pediatric CCLD patients with pruritus, ICP patients tended to have higher tMCA level, however, without a statistical significance compared to pregnant controls. ATX antigen level were significantly elevated in ICP patients and pregnant controls than in all pediatric study groups but did not correlate with tBA/tMCA level. The significant elevation of ATX antigen levels in ICP patients and healthy pregnant controls despite significantly lower tBA/tMCA level compared to all pediatric CCLD patients argues for additional factors besides BA/MCA inducing a surge in ATX activity. This, however, needs to be further evaluated in future studies. Once again, our findings are highlighting the multimodality and complexity of pruritus genesis in different CCLD and the need for further investigations in this field.

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