Dissertation

The influence of prematurity and early-onset sepsis on typical and atypical bile acids in neonates

submitted by

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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 guidelines of 'Good Scientific Practice'

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ABBREVIATIONS

aBA	Atypical BA
ALT	Alanine transaminase
AMCA	Alphamuricholic acid
ASBT	Apical sodium-dependent BA transporter
AST	Aspartate transaminase
BA	Bile acids
BM	Breast milk
BMCA	Betamuricholic acid
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CRP	C-reactive protein
CYP27A1	Sterol 27-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
DCA	Deoxycholic acid
EDTA	Ethylenediaminetetraacetic acid
EHC	Enterohepatic circulation
EOS	Early-onset sepsis

FGF-19	Fibroblast growth factor-19
FM	Formula milk
FT	Term
FXR	Farnesoid X receptor (FXR)
G	Glycine
GBS	Group B streptococcus infection
GGT	Gamma-glutamyl transpeptidase
GMCA = HCA	Gammamuricholic acid = hyocholic acid
HCA = GMCA	Hyocholic acid = gammamuricholic acid
HDCA	Hyodeoxycholic acid
HPLC	High performance liquid chromatography
HPLC-HRMS	High performance liquid chromatography high resolution mass spectrometry
HSD3B7	3β-hydroxy-Δ5-C27-steroid oxidoreductase
I-BABP	Intestinal BA binding protein
IL-6	Interleukin 6
IQR	Interquartile range
LCA	Lithocholic acid
MCA	Muricholic acid
MDR	Multidrug resistance associated protein
MRP	Multidrug resistance associated proteins

MS	Mass spectrometry
OATP	Organic anion transporters
OMCA	Omegamuricholic acid
OSTα-OSTβ	Organic solute transporter alpha-beta
PCT	Procalcitonin
PT	Preterm
SHP	Small heterodimer partner
T	Taurine
tBA	Typical BA
TGR5	G-protein-coupled BA receptor 1
UDCA	Ursodeoxycholic acid
WHO	World Health Organization

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ABSTRACT

Background: Bile acids (BA) are essential for intestinal lipid absorption. Although BA mainly circulate between liver and gut, extraportal serum BA concentrations correlate with total BA quantity. Of note is that total serum BA levels and the composition of the BA pool (the "BA profile") differ in healthy adults and neonates. In adults, the typical BA, cholic and chenodeoxycholic acids, are the predominant BA species. In both term and preterm neonates, these typical BA predominate as well; however, "atypical" BA - otherwise mainly found in rodents – such as alpha-, beta-, gamma-, or omega-muricholic acid also are synthesized. This study initially aimed to determine standard value ranges of total BA levels including both typical and atypical BA in term and preterm neonates. Secondly, because serum BA concentrations rise in septic adults, BA values were determined in early-onset neonatal sepsis (EOS), a common and serious disease in neonates, to learn if monitoring serum BA values in neonates could identify sepsis at its onset.

Methods: The total BA profile – serum levels and composition of typical and atypical BA - was determined in 102 neonates using high-performance liquid chromatography – high-resolution mass spectrometry: 67 term neonates (47 healthy and 20 with EOS) and 35 preterm neonates (22 healthy and 13 with EOS) were included.

Results: In healthy term neonates, the median reference value of total BA was 8.7 µmol/L (interquartile range [IQR]: 5.0 – 13.5), higher than in healthy adults (0.3 – 6.5 µmol/L). In contrast to healthy term neonates, term neonates with EOS had significantly lower median BA values (6.2 µmol/L, IQR: 3.8 – 8.6; $p<0.01$). In healthy preterm neonates, the median reference value of total BA was 11.2 µmol/L (IQR: 5.6 – 16.9). Preterm neonates with EOS did not have significantly altered BA values (7.4 µmol/L, IQR: 4.0 – 10.0). Independently of gestational age and state of health of the neonate, the BA pool consisted primarily of taurine-conjugated BA: the most abundant BA was a typical BA, tauro-chenodeoxycholic acid. The most common atypical BA in healthy term and preterm neonates were tauro-gamma-muricholic acid and tauro-alpha-muricholic acid, respectively. In EOS (equal term and preterm), tauro-omega-

muricholic acid (TOMCA) was the most predominant atypical BA species, present in quantities significantly higher than those in healthy subjects ($p<0.01$).

Conclusion: This is the first study to determine standard value ranges of total BA levels in neonates including both typical and atypical BA. We propose that "atypical" BA are common in early infancy. Additionally, in contrast to adults with sepsis, total BA values in term neonates suffering from EOS are significantly lower than in healthy controls. Hence, we suggest serum BA in general – and TOMCA specifically - as an EOS biomarker with potential clinical value.

ZUSAMMENFASSUNG

***Hintergrund*:** Gallensäuren (GS) sind die Endprodukte des Cholesterinstoffwechsels und ein wichtiger Bestandteil der Galle. GS sind für eine optimale Fettverdauung unabdinglich und ihre Konzentrationen können ohne invasiven Eingriff indirekt über das Serum gemessen werden. Von Interesse ist, dass sich die Serum-Gesamt-GS und die Zusammensetzung des GS-Pools im Serum (ergeben zusammen das „GS-Profil“) zwischen gesunden Erwachsenen und Neugeborenen unterscheiden. Zwar sind Cholsäure und Chenodeoxycholsäure typische Vertreter der menschlichen GS, sowohl im Erwachsenen- als auch im Kindesalter, jedoch gelten C6-hydroxylierte GS wie Alpha-, Beta-, Gamma-, und Omega-Muricholsäure bei Erwachsenen als “atypisch”, aber wurden in höheren Konzentrationen bei frühen und reifen Neugeborenen nachgewiesen. Diese Studie diente zu allererst dazu, aufgrund der fehlenden Datenlage Normwerte von Gesamt-GS (typische und atypische GS) im Serum bei Früh- und Reifgeborenen festzulegen. Des Weiteren sollte der Einfluss von Sepsis auf die Serum-GS-Werte bei frühseptischen Neugeborenen analysiert werden, da die Frühsepsis als eine ernsthafte Komplikation bei Neugeborenen gilt und bekannt ist, dass bei septischen Erwachsenen aufgrund einer entzündungs-bedingten Cholestase Serum-GS signifikant erhöht sind.

Methodik: Untersucht wurden Gesamt-GS (typische und atypische GS) von gesunden Reifgeborenen (RG; n=47), gesunden Frühgeborenen (n=22) und Neugeborenen mit Frühsepsis (Frühgeborene: n=20; Reifgeborene: n=13) mittels Hochleistungschromatographie gekoppelt mit Massenspektrometrie.

Ergebnisse: Referenzwerte von Gesamt-GS wurden bei gesunden Reifgeborenen mit einem Median von 8,7 µmol/L, der in einem Interquartilbereich zwischen 5,0 und 13,5 µmol/L lag, festgelegt, sowie bei gesunden Frühchen mit 11,2 µmol/L (5,6 – 16,9). Die Normwerte beider Gruppen (Reif- und Frühgeborene) lagen deutlich über den Normwerten gesunder Erwachsener (0,3 – 6,5). Neugeborene mit Frühsepsis zeigten erniedrigte Gesamt-GS Werte: signifikant niedriger in Reifgeborenen (6,2 µmol/L, IQR: 3,8 – 8,6; $p<0,01$) und niedriger in Frühgeborenen (7,4 µmol/L, IQR: 4,0 – 10,0). GS in Taurin-konjugierter Form waren – unabhängig vom der Schwangerschaftswoche und des Gesundheitszustandes des Kindes - die

häufigste Spezies. Taurochenodeoxycholsäure, eine typische GS, war in allen Gruppen als konzentrierteste vorzufinden. Die atypische GS waren unterschiedlich häufig vorzufinden: Taurogammamuricholsäure dominierte bei Reifgeborenen, wohingegen Tauroalphamuricholsäure bei Frühgeborenen als einzige atypische GS detektiert wurde. Tauroomegamuricholsäure (TOMCS) war, unabhängig vom Reifegrad der Kinder, bei Frühsepsis signifikant erhöht ($p<0,01$).

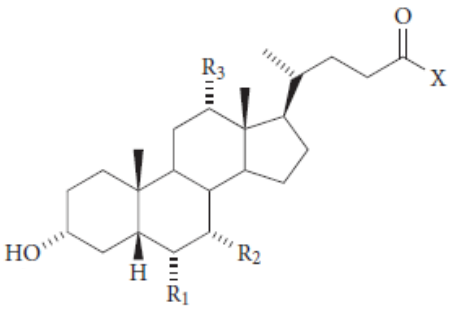
Schlussfolgerung: Dies war die erste Studie zu typischen und atypischen GS im Serum von Früh- und Reifgeborenen und deren Beeinflussung durch Frühsepsis. Erstens wurde gezeigt, dass „atypische“ GS bei Neugeborenen, unabhängig vom Reifegrad, als typisch gelten. Die physiologische und pathophysiologische Rolle muss jedoch in Zukunft erst geklärt werden. Zweitens zeigten die Neugeborenen - im Gegensatz zu septischen Erwachsenen - signifikant erniedrigte GS-Werte im Vergleich zu gesunden Probanden des gleichen Schwangerschaftsalters. Basierend auf diesen Ergebnissen könnten GS ganz allgemein – oder TOMCS im Spezifischen - zukünftig als zusätzlich diagnostischer Marker bei der Erkennung von Frühsepsis eingesetzt werden.

1. INTRODUCTION

1.1. BILE ACIDS (BA)

1.1.1. FORMATION

BA are formed from cholesterol in hepatocytes. BA synthesis is a complex, multi-enzymatic process in which an insoluble cholesterol molecule is converted into an amphipathic, membrane-dissolving and water-soluble emulsifier. BA have a 19 carbon backbone (4 ring sterol structure), 1-3 α orientated hydroxyl groups at C3, C7 or C12, 2 methyl groups at C18 and C19, and a saturated 5 carbon side chain that terminates in a carboxyl acid. This carboxyl acid can be further esterified with the amino acids glycine (G) or taurine (T), converting a weak acid to a strong acid, before BA are excreted from the hepatocyte. Consequently, conjugated BA are fully ionized at the pH range present in the small intestine (1). In bile, BA are almost completely conjugated, because the conjugation of BA in the hepatocyte is a highly efficient process. In adults, most BA are conjugated with G, the minority with T (2). All common BA are illustrated in Figure 1.



	R_1	R_2	R_3
CA	H	OH	OH
CDCA	H	OH	H
DCA	H	H	OH
LCA	H	H	H
MCA	OH	OH	H
UDCA	H	OH	H

Free BA: X = OH
Glyco-conjugated BA: X = $NHCH_2CO_2H$
Tauro-conjugated BA: X = $NHCH_2CH_2SO_3H$

Figure 1: Chemical structures of the most common BA: cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary BA, and deoxycholic acid (DCA) and lithocholic acid (LCA) are the secondary BA. Muricholic acid (MCA) and ursodeoxycholic acid (UDCA) are primary BA predominantly produced in rodents and bears, respectively (3).

1.1.1.1. PRIMARY BA FORMATION

Synthesis of BA requires multiple individual enzymes and occurs in multiple intracellular compartments that include the cytosol, endoplasmic reticulum, mitochondria, and peroxisomes (4).

The major pathway for the synthesis of BA is referred to as the 'classic', or 'neutral', pathway and is initiated via hydroxylation of cholesterol at the C-7 position via the action of CYP7A1. CYP7A1 is one of the cytochrome P450 metabolic enzymes. The classical pathway accounts for at least 75% of the total bile-acid pool. The 'alternative' pathway, or 'acidic' pathway, involves hydroxylation of cholesterol at the C-27 position via sterol 27-hydroxylase (CYP27A1) (5). CYP27A1 generates BA-intermediates that are subsequently hydroxylated on the C-7 position by oxysterol 7α-hydroxylase (CYP7B1) (3). Although the acidic pathway is not the major route for human BA synthesis in human adults, it is important for neonates as demonstrated by neonates harboring a mutation in the CYP7B1 gene. These infants present with severe cholestasis leading to cirrhosis and liver dysfunction (6). Sterol ring modification is achieved by 3β-hydroxy-Δ5-C27-steroid oxidoreductase (HSD3B7). This reaction is critical and children with mutations in the HSD3B7 gene develop progressive liver disease with cholestatic jaundice (7). HSD3B7 products can proceed via two pathways whose end products are the primary BA CA and CDCA. The distribution of these two BA is determined by the activity of sterol 12α-hydroxylase (CYP8B1). The intermediates of the HSD3B7 reaction that are further converted by CYP8B1 become CA and the others will become CDCA. Therefore, CYP8B1 eventually determines the ratio of CA to CDCA.

1.1.1.2. SECONDARY BA FORMATION

Anaerobic bacteria in the colon also attack the hydroxyl group at C-7. Bacterial dehydratases remove the hydroxyl group to form 7-deoxy BA (the term deoxy means that an oxygen-containing group has been lost). By this process, cholic acid (with hydroxy groups at C-3, C-7, and C-12) is converted to DCA; a dihydroxy bile acid with hydroxyl groups at C-3 and C-12. Similarly, 7-dehydroxylation of CDCA results in the formation of a monohydroxy bile acid with a hydroxyl group at only C-3. This BA is called lithocholic acid (LCA) because it was first isolated from a gallstone taken from a calf. DCA and LCA are called secondary BA because they are formed from primary BA. Both are absorbed to some extent from the colon and returned to the hepatocyte.

In the hepatocyte, secondary BA undergo differing fates. DCA further circulates with the primary BA. In most adults, DCA constitutes about 20% of the biliary BA, the others being CA and CDCA in roughly equal proportions (1). LCA is conjugated, sulfated at the C-3 position and excreted into bile. However, in contrast to the conjugates of primary BA and of DCA, LCA is not efficiently absorbed from the small intestine but promptly eliminated from the body, and never constitutes more than 5% of the BA pool. This elimination is essential since LCA is highly hepatotoxic in experimental animals (8).

Another BA, ursodeoxycholic acid (UDCA), is also present in trace amounts. It is an epimer of CDCA: the hydroxyl group at C-7 of UDCA has a β configuration instead of an α configuration in CDCA. It was first isolated from the bile of the polar bear (9).

1.1.2. FUNCTION

BA act in different physiological areas. The most important physiological function of BA is essential to lipid absorption: BA emulsify lipids and fat-soluble vitamins as micelles. In the small intestine, BA promote dietary lipid absorption by emulsifying dietary lipids and fat-soluble vitamins (A, D, E, and K1) as mixed micelles accelerating diffusion through the unstirred layer of chyme that adjoins enterocytes. In the biliary canaliculus, BA solubilize biliary phospholipids and cholesterol in mixed micelles. Such micelle formation promotes cholesterol elimination via the fecal route. Inborn defects in the conversion of cholesterol to BA can cause severe hepatic or systemic disease. Further functions of BA include the stimulation of bile flow and stimulation of biliary phospholipid secretion. BA are actively transported into the biliary canaliculi between hepatocytes and induce bile flow by their osmotic properties. BA promote the transfer of phospholipids from the canalicular membrane into bile. The presence of phospholipids in bile results in a greater fraction of BA existing in the form of mixed micelles that lessen BA-caused damage to bile duct epithelium (2).

Besides their well-established roles in dietary lipid absorption and cholesterol homeostasis, BA also act as metabolically active signaling molecules that coordinate hepatic triglyceride, glucose and energy homeostasis. They are ligands for TGR5, the G-protein-coupled BA receptor 1, and activate nuclear receptors such as farnesoid X receptor (FXR). Signal transduction pathways downstream of BA-mediated FXR activation are also involved in the regulation of hepatic gluconeogenesis, glycogen synthesis and insulin sensitivity. Dysregulation of BA transport and impaired BA receptor signaling may contribute to the pathogenesis of non-alcoholic fatty liver disease (10).

1.1.3. ENTEROHEPATIC CIRCULATION OF BA

Under physiological conditions BA undergo enterohepatic circulation (EHC) between liver and intestine. BA are secreted into bile and stored in the gallbladder, reabsorbed in the terminal ileum, and finally taken up again by the liver. Only a small fraction of BA passes into the colon, where BA are passively reabsorbed after a series of modifications in the human large intestine including deconjugation and oxidation of hydroxyl groups (11). BA are spilled over into sinusoid blood when concentrations increase in the hepatocytes. BA in the non-portal circulation that enter urine via ultrafiltration at the glomerulus are reabsorbed into plasma by renal tubular epithelium and are returned to the liver via the systemic circulation. A small amount of protonated, uncharged BA undergoes so called "cholehepatic shunting". This pathway includes passive biliary absorption followed by transfer of BA back to hepatocytes for re-secretion into bile (12).

Absorption of BA from the distal intestine permits return of BA to the liver. The body's BA are termed the BA pool. This is about 3 g in size in adults. The BA pool cycles several times with each meal. High efficiency in EHC of BA minimizes BA losses. Only 5% of BA are eliminated via the feces (2). These are replaced by BA synthesized de-novo in the liver (0.2 to 0.6 g/d). BA de-novo synthesis from cholesterol is controlled via negative feedback. The concentration of BA in the hepatocyte acts as a signal: when high, BA synthesis is low; when low, BA synthesis is increased. Because BA are synthesized from cholesterol, cholesterol synthesis undergoes a concomitant increase (13).

1.1.4. INTESTINAL BA TRANSPORTERS AND REGULATORY NUCLEAR RECEPTORS

BA uptake into the enterocyte occurs principally in the terminal ileum via the apical sodium-dependent BA transporter (ASBT) (Figure 4). Na+-independent uptake of BA via organic anion transporters (OATP isoforms) is possible in the small-bowel enterocyte (14,15), whereas entry into colonic enterocytes seems to occur exclusively by passive diffusion (2). The intestinal BA binding protein I-BABP is believed to be required for transcellular / intracellular transport in enterocytes of both terminal ileum and colon. Several exporter proteins at the basolateral as well as at the apical membrane actively move BA into the blood or back into the intestinal lumen. The basolateral efflux pumps are multidrug resistance associated protein (MRP3) and the organic solute transporter alpha-beta, OSTα-OSTβ (16). Enterocytes also express multidrug resistance associated proteins 2 and 4 (MRP2 and MRP4) (17), which constitute two apical BA export systems. In the kidney, bile acids undergo glomerular filtration, but are reabsorbed in the proximal renal tubule by ASBT (18).

The nuclear receptor for BA, FXR, plays a key role in the regulation of transporter expression in the EHC. FXR is expressed in large amounts in tissues exposed to high BA concentrations (mainly liver and intestine). FXR is activated by BA, its natural ligands (19). FXR activation by BA leads to downregulation of BA uptake systems in the liver and intestine and to upregulation of BA efflux pumps (*i.e.*, OSTα-OSTβ) in both tissues as well as to repression of the rate-limiting step in endogenous BA synthesis, that catalyzed by cholesterol 7α-hydroxylase (CYP7A1). While positive feedback regulation is mediated via direct binding of FXR to response elements in gene promoter regions of OSTα-OSTβ and MRP2, small heterodimer

partner (SHP), a further nuclear orphan receptor, is a central mediator of negative feedback regulation of CYP7A1 and ASBT. However, CYP7A1, pivotal for BA synthesis, also responds to FXR-SHP independent feed-forward and feedback mechanisms (12). In addition, intestinal FXR activation induces the expression of fibroblast growth factor-19 (FGF-19), a growth factor secreted in the ileum that is important for gut-liver signaling. FGF-19 is involved in BA synthesis through repressing hepatic CYP7A1 expression (20), refilling of the gallbladder and in regulation of beta-oxidation (21). Preliminary evidence suggests that ileal FGF-19 may be involved in the control of ASBT expression (Ben Shneider, personal communication).

1.1.5. BA AND CYTOTOXICITY

BA are cytotoxic; in excess they can cause major liver damage (22). BA cytotoxicity is strongly affected by the number, position and orientation of hydroxyl groups: the lower the hydrophilicity, the greater the cytotoxicity. Hydrophobicity is defined by the extent to which BA bind to hydrophobic surfaces (23). The natural BA CDCA and DCA, harboring two hydroxyl groups, are highly hydrophobic, and hence, highly cytotoxic. CA is "in between", being non-cytotoxic at low concentrations, but cytotoxic at very high concentrations (24). UDCA, although a di-hydroxy BA, is a hydrophilic BA due to the planar orientation of the hydroxyl group at the C-7 position, and is devoid of cytotoxic properties in most model systems. UDCA is now used as a therapeutic agent in hepatobiliary disorders including primary biliary cirrhosis, drug- and parenteral nutrition-induced cholestasis, pregnancy-induced intrahepatic cholestasis, cystic fibrosis and progressive familial intrahepatic cholestasis (25).

1.1.6. BA AND NUTRITION

In the fasting (pre-prandial) state, BA are found in low concentrations in the peripheral blood (serum BA levels). These represent a small 'leakage' of BA from the EHC. Serum BA levels are a measure of this spillover. After the ingestion of a (high fat) meal, the upper gastrointestinal system releases cholecystokinin, a hormone that mediates gall bladder contraction. Subsequently, the release of BA from enterocytes into the portal vein increases, and this elevation is reflected as an increase in serum BA levels in the post-prandial state. LaRusso *et al.* showed peak concentrations of serum BA levels 90 min after eating (26). Serum BA can also indicate liver disease. In this setting, the ability of hepatocytes to recapture BA may be decreased, resulting in high serum BA levels.

Ingestion of food may not only influence BA levels but also BA composition. G-conjugated BA are the predominant BA species in human adults, whereas T-conjugates are the predominant BA-species in neonates. This phenomenon may be nutrition-determined: T is the most abundant free amino acid in breast milk (27). The importance of T in the diet of preterm and term neonates is not fully understood yet. However, studies suggest that T may be a 'conditionally essential' free amino acid in neonates (28,29). Serum values dropped in neonates fed with T-free formula and urine values also fall, maybe resulting from an attempt of the kidney to conserve T (30)(31,32). T has an important role in fat absorption and in preterm (and possibly in term) neonates, since T-conjugated BA are better fat emulsifiers than are G-conjugates (30). In preterm neonates, T insufficiency results in impairment of fat absorption, BA secretion, renal function and neurodevelopment (27). All these consequences can be reversed by T supplementation (33). It may also play a role in the prevention of granulation of the retina (34). In cats, T is an essential amino acid: deficiency leads to retinal degeneration and eventual

blindness (35,36). Additionally, T is an important regulator of cell volume in the brain and the renal medulla (37).

In summary, T is essential in the diet of preterm neonates and may also be beneficial in term neonates. Based on this, T is now a standard supplement in formula milk.

1.1.7. BA IN PEDIATRICS

1.1.7.1. BA IN THE FETUS AND NEWBORN

What we know about the ontogeny of human BA synthesis and metabolism is incomplete and is mostly based on studies in animals (38). In rats, the production of BA starts very early in development, with peak serum BA values during the first days of life (39). In humans, biliary BA levels are low in the fetus and the neonate and increase concurrently with the maturation of BA synthesis pathways and BA transport capacity (40). A study of fetal bile reveled low total BA levels before 17 weeks of pregnancy which increased 20-fold between the 16 and 20th week (41), but at birth biliary BA concentrations were still lower than in older children and adults (42). This might be due to an immature fetal and neonatal gallbladder which is not so capable of concentrating BA as is the adult gallbladder.

In the fetus, the predominant BA species is CDCA conjugated to T (41). During the first trimester of pregnancy, BA levels are low and correlate with a small BA pool that increases with increasing weeks of gestation. In addition, studies in amniotic fluid measured higher

proportions of CDCA than CA and low BA concentrations (43). In preterm neonates of 32 weeks gestation, the size of the BA pool is approximately one-sixth that of adults and increases within one month postnatally (44).

The development of the EHC is still a largely unexplored field. The efficiency of hepatic transport was investigated using serum BA concentrations. In the mature liver, uptake of BA is highly efficient and more than 90% of conjugated primary BA are absorbed. In fetal life, serum BA are determined by spillover into the systemic circulation of BA that reach the liver from the intestine. BA values are lower in umbilical artery blood than in umbilical vein blood, indicating that fetal serum BA levels are kept low by net transport across the placenta to the mother. Specific transport mechanisms allow bidirectional BA transfer between the fetal and maternal circulations (45). In neonates, conjugated primary serum BA increase rapidly. Their concentrations are significantly higher than those seen in older children, adolescents and healthy adults (46,47); indeed, they are similar to those in patients with cholestatic liver disease. Hence, this elevation of BA in the serum of neonates has been referred to as 'physiological cholestasis' (47). The high levels of serum BA in early infancy are remarkable considering the small size of the BA pool and the immature mechanisms for intestinal BA reabsorption.

1.1.7.1.1. ATYPICAL BA (ABA)

As mentions before, the BA pool in neonates differs from that in adults. Of particular note is the appearance of aBA. During the 1970s and 1980s, studies first investigated the appearance of unusual BA besides usual BA. Indeed, studies reported the existence of tri-hydroxylated BA in umbilical cord blood and amniotic fluid from neonates (43,48). These aBA are typical for

the development of the liver. Hydroxylation is a hepato-protective mechanism: by enhancing the polarity of BA, elimination via the kidneys is facilitated and cytotoxic potential is lowered.

In subsequent studies various C-6-α and 6-β hydroxylated BA have been isolated in substantial amounts from biological materials excreted by patients with hepatobiliary diseases and by neonates and fetuses. Hyocholic acid (HCA, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid), also known as γ-muricholic acid (γ-MCA), isolated from pig bile by Haselwood, was one of the earliest 6-α-hydroxylated BA known. The C-6 epimers of HCA, 3α,6β,7α-, 3α,6β,7β- and 3α,6α,7β-trihydroxy-5β-cholanoic acids (α-, β-, and ω-MCA, respectively) (48) also are excreted by humans (43,49). In addition, C-6-α hydroxylation of DCA to hyodeoxycholic acid (HDCA; 3α,6α-dihydroxy-5β-cholanoic acid) has been identified in humans (50,51,52).

MCA are a group of BA mainly found in mice. Human primarily synthesize CA and CDCA, whereas mice synthesize CA, α-MCA (AMCA) and β-MCA (BMCA). In mice, CYP8B1 is required for CA synthesis, whereas the enzyme that catalyzes the formation of BMCA is not yet characterized. A fourth MCA, ω-MCA (OMCA), is an epimer of BMCA and is produced by the normal microbiota (53). Earlier studies suggested HDCA as another possible substrate for OMCA synthesis (54). In conventional mice, the primary BA AMCA and BMCA and the secondary BA γ-MCA (GMCA), OMCA and HDCA, with various sulphated forms, are found. MDCA, murideoxycholic acid (3α,6β-dihydroxy-5β-cholanoic acid), is also found in conventional mice (55); however, this BA was not included in the present studies. Germ-free mouse studies reported an alteration in the BA pool, since the gut microbiota has a profound systemic effect on BA metabolism. It was shown that the microbiome can suppress biosynthetic genes in the liver, which is consistent with increased FXR activation in the ileum due to reduced

levels of T-MCA. As in humans, MCA is further conjugated to G (predominant in human adults) and T forms (predominant in mice and neonates) (111). T-MCA-conjugates were shown to be potent antagonists of the BA receptor FXR (112).

Interestingly, the composition of the BA pool in the fetus and the neonate reflects the same situation present in patients with cholestatic liver disease, indicating a switch of liver physiology towards early metabolic pathways (50,56). Therefore, it is speculated that there are common altered metabolic pathways in fetal liver and cholestatic liver; however, the exact pathways are still unclear. In summary, aBA have been investigated in healthy and cholestatic adult subjects and neonates; however, standard values in pediatrics are lacking.

1.1.7.2. SERUM BA

In pediatrics the interest in BA as serum markers for inborn and acquired hepatobiliary disorders like biliary atresia, different types of progressive familial intrahepatic cholestasis, inborn errors of BA synthesis, or sclerosing cholangitis is increasing (57,58). Serum BA are an indirect marker of the EHC. By measuring serum BA the concentration and composition of BAs in the EHC are calculable.

1.1.7.2.1. NORMAL BA VALUES IN CHILDREN AND ADOLESCENTS

Until now, very few accurate data are available on serum BA levels during childhood. The few and small studies characterizing the BA profile in children have yielded inconsistent results (59) (60). Serum BA studies are often not comparable due to different measurement methods. In addition, the ontogeny of BA handling and EHC maturation is a largely un-explored field.

The expansion of BA synthesis and EHC activity as placentomaternal support is withdrawn and enteral alimentation begins, with microbiologic colonization of the newborn organism and acquisition of the functional enterobiome, are not well understood, and the same holds for metabolic shifts with the onset of puberty. Until recently, no comprehensive study has existed using modern liquid chromatography coupled with tandem mass spectrometry (MS) methods to define serum BA levels in healthy neonates, children and adolescents.

Several studies have already reported considerable differences in BA metabolism in neonates, after infancy, and during adolescence until adulthood (47,59,61,62). Niijima *et al.* published normal BA values in sera of a healthy pediatric cohort in 1985, with high values in neonates that became continuously lower with progressing growth (61). High BA values in neonates were also shown by Polkowska *et al.* with a peak at an age of one month. Surprisingly, after one year of life values of total BA (tBA) reached adult standards (59). However, both these studies used methods with several limitations in sensitivity and selectivity.

In 2015 our group aimed to define normal range values of BA in the serum of children and adolescents for different age groups (46). Measurements were performed using high performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry (HPLC-HRMS). 194 children and adolescents without hepatobiliary disorders were included in this study. We noted a high serum tBA concentration in neonates until the 24th month, henceforward, becoming continually lower until constant values comparable with those in adults were reached after the age of 11 years (Table 1) (46).

Table 1: Normal values of BA in different age groups (46).

Age	N	BA [µmol/l]	Confidence interval (95%)
0-5 months	17	5.1 ± 2.2	3.9 - 6.3
6-24 months	13	8.0 ± 5.6	6.6 - 9.4
3-5 years	22	5.4 ± 2.7	4.3 - 6.4
6-11 years	44	4.4 ± 2.7	3.6 - 5.1
>11 years	98	3.6 ± 1.9	3.1 - 4.1

With respect to conjugation patterns, in neonates BA were primarily conjugated with T, but after 6 months G conjugates clearly predominated.

1.2. PREMATURITY

A preterm baby is defined as a baby born alive before the 37th week or 259th day of gestation. Prematurity is further categorized into three sub-groups: extremely preterm (<28 weeks), very preterm (28 to <32 weeks) and late preterm (32 to 36 weeks). The World Health Organization (WHO) reports that 15 million babies are born too early every year, making up more than 1 in 10 babies, and that preterm birth rates are increasing. Preterm birth rates have been reported to range from 5% to 7% of live births in some developed countries, but are estimated to be substantially higher in developing countries (63). In Austria 8% of all neonates are preterm babies (Statistics Austria, 2016).

Birth at extremely premature gestational ages is associated with a significant increase in morbidity and mortality (64). Worldwide, prematurity is the leading cause of death in children under the age of 5. Although survival of extremely premature infants has improved, morbidity remains high, with intraventricular hemorrhage, retinopathy of prematurity, necrotizing enterocolitis, and chronic lung disease (64,65). In addition, complications increase the risk of long-term neurologic, cognitive, and behavioral sequelae (66).

Events leading to preterm birth include a variety of reasons that are still not completely understood. Most preterm births happen spontaneously, but some are due to early induction of labour or caesarean birth. Common causes of preterm birth include medical conditions of the mother (chronic conditions such as diabetes and high blood pressure) or fetus, genetic influences, environmental exposure and multiple pregnancies; however, often no cause is identified (67). Obviously, risk factors vary depending on the region. In developed countries, the increasing age of women giving birth leads to more maternal complications and caesarean sections, which may contribute to the rising numbers of cases. Increasing rates of multiple pregnancies due to increasing infertility treatments may be another explanation. In undeveloped countries, on the other hand, high levels of preterm birth are likely due to intrauterine infection or lack of proper treatment (67).

1.3. EARLY-ONSET SEPSIS

Based on the timing of infection, neonatal sepsis has been classified into early-onset sepsis (EOS) and late-onset sepsis (68) with EOS occurring at ≤72 h, versus <7 days in late-onset

sepsis. EOS is a severe illness with a mortality rate ranging from 2-3% in term infants to 20-30% in preterm infants (64). Although advances in neonatal care have improved survival and reduced complications in preterm infants, sepsis still contributes significantly to mortality and morbidity, especially among very-low-birth-weight (<1500 g) infants (69). Mortality rates for EOS have been stabilized, since screening procedures for maternal group B streptococcus infection (GBS) are widely used intrapartum (70). However, GBS remains the leading cause of EOS, while *Escherichia coli* (E. coli) is responsible for the majority of deaths (71).

1.3.1. PATHOPHYSIOLOGY

Neonates may acquire pathogens either *in utero* (intra-amniotic infection prior to the onset of labor) or *intrapartum* (72). EOS may originate from both mother and child. Maternal risks can arise before labor; dietary intake of contaminated foods with *Listeria monocytogenes* being the most common example. In addition, invasive procedures during pregnancy, which disrupt the amniotic cavity, including cervical cerclage and amniocentesis, may also increase the rates of intra-amniotic infection and EOS. During birth, prolonged rupture of membranes, fever, or vaginal colonization with GBS may favor EOS (73).

EOS risk-factors associated with the child are mostly prematurity and low birth weight, inborn anomalies and low APGAR scores (score of ≤6 at 5 min) (74,75). In addition to the immaturity of the immune system in premature infants, the barrier function of the skin and mucous membranes is not fully developed and might be more affected by invasive procedures, including intravenous access or intubation. Ethnic and social factors also are associated with EOS including poor prenatal care, low socioeconomic status of the mother, poor maternal nutrition and maternal substance abuse (76).

1.3.2. EOS IN PRETERM NEONATES

The degree of prematurity correlates with the risk for mortality from EOS and associated morbidities. Especially very-low-birth-weight infants are at the greatest risk of infection since neither the innate nor the adaptive immune system is functioning at optimal levels (77). A study by Lim *et al.* showed that the mortality rate was higher in preterm neonates with EOS than in preterm neonates with late-onset sepsis (40% vs. 5%, $p<0.01$) (78). Gram-negative organisms have been reported as causing the highest rates of death by EOS within the first 72 h (Pisani 2012). Other studies including all causes of EOS in the preterm population have reported mortality rates of 26 to 37% (79). Overall, diagnosis of EOS is associated with higher all-cause mortality in the first 120 days (77). These studies emphasize the very serious nature of EOS in preterm neonates.

1.3.3. EOS IN TERM NEONATES

Mortality rates are significantly lower in term than in preterm neonates; however, EOS still is a serious complication. Term neonates are at higher risk of infection if they have comorbidities such as impaired immune function, meconium aspiration or pulmonary abnormalities (80). In the term neonate population, the organism contributing most to mortality is *E. coli*, despite fewer total infections than GBS.

1.3.4. DIAGNOSTICS

The signs and symptoms of EOS are nonspecific, including fever, respiratory distress such as cyanosis and apnea, feeding difficulties, hepatomegaly, unexplained jaundice, or very simply

"just not looking right" (81,82). Infants with hypoxia-associated respiratory acidosis may gasp *in utero* which could lead to pneumonia and meconium aspiration. Diagnosis requires obtaining a complete white blood cell count with a single blood culture. Increased levels of various cytokines, either pro- or anti-inflammatory, have been tested as laboratory biomarkers, with C-reactive protein (CRP) and procalcitonin (PCT) the two most commonly studied acute-phase reactants. CRP remains the most available and most frequently used laboratory test for the diagnosis of EOS (83,84). However, CRP shows a physiological 3-day increase after birth, resulting in low sensitivity for detection of sepsis at an early stage. EOS initiates the release of cytokine interleukin 6 (IL-6), which stimulates an increase in CRP concentrations. Studies report a CRP sensitivity range of 41 to 96% and a specificity range of 72 to 100% for EOS (85). Most neonatal care units use a cutoff value of 10 mg/liter.

Monocytes and hepatocytes produce a propeptide of calcitonin (PCT). Concentrations of PCT significantly elevated during infections in neonates, children, and adults (86). The normal level for neonates >72 h of age is <0.5 ng/ml. In general, PCT is more sensitive for earlier detection of EOS than CRP. However, a physiologic increase in the PCT concentration occurs within the first 24 h of birth, and elevated levels in serum can occur under noninfectious conditions (*e.g.,* respiratory distress syndrome, hemodynamic instability, and maternal diabetes). In addition, PCT levels are more likely to be elevated during bacterial infections than during viral ones (85).

Cytokines, including IL-6, interleukin, gamma interferon, and tumor necrosis factor alpha, and cell surface antigens, have also all been studied as biomarkers for EOS, but specificities are low for all (68,85).

Hyperbilirubinemia and jaundice are also well-known complications of sepsis but may also occur in non-bacterial infections (87).

1.3.1. TREATMENT

In EOS, the current approach to the treatment of GBS and *E. coli* infection is a combination of ampicillin and aminoglycoside (*e.g.,* gentamicin) (114). Alternatively, third-generation cephalosporins (*e.g.,* cefotaxime) are available. Cephalosporins are attractive in the treatment of meningitis because of their lack of dose-related toxicity and their excellent cerebrospinal fluid (CSF) penetration; however, several studies have reported rapid development of resistance when cefotaxime has been used routinely for the treatment of EOS (88). Ceftriaxone is contraindicated in neonates with hyperbilirubinemia since ceftriaxone competes with bilirubin for binding to human serum albumin, replacing bilirubin and thereby posing a risk for kernicterus (89). In general, antimicrobial treatment may be necessary for 7-10 days and discontinuation of drugs may be indicated when culture results remain negative for 48-72 hours.

Antimicrobial therapy in neonates with negative blood cultures merits discussion: women receive prophylactic antimicrobial agents during birth to prevent GBS infections or when intra-amniotic infection is suspected (74). This may lead to sterile postnatal blood cultures and false negative results. In such cases, the duration of therapy should depend on the clinical course of the neonate. Furthermore, the risks associated with longer courses of antimicrobial treatment should be considered: studies suggest an association between prolonged administration of antimicrobial agents (>5 days) in neonates with suspected EOS (and negative blood cultures) and death as well as necrotizing enterocolitis (90,91). Finally, antimicrobial resistance is increasing worldwide not only in the general population but also in neonatal intensive-care units due to extensively multidrug resistant bacteria that pose a significant treatment dilemma.

In summary, more reliable, quickly measurable biomarkers for EOS would be of clinical value to avoid not only underdiagnosis but also unnecessary antimicrobial treatment.

1.4. AIM OF THE STUDY

I sought to prospectively investigate total BA levels (tBA and aBA) and the BA profile in:

1) healthy term neonates
    (a) in fasting or fed condition
    (b) fed with breast milk or formula diet
2) healthy preterm neonates
3) term neonates with EOS
4) preterm neonates with EOS

within the first 3 days of life.

Ad 1) In the first step I aimed to determine reference values in healthy term neonates without disturbances of the hepatobiliary tract as a control group since there were no accurate and reliable data available for this study population. I hypothesized that BA levels are higher in neonates than in adults. Additionally, I aimed to document the nutritional status of neonates - if possible - in order to investigate possible influences on serum BA levels. I hypothesized that BA levels are higher in the fed state than in fasting condition, and that there are differences in the BA pool between breast milk and formula diet fed neonates.

Ad 2) In a second step healthy preterm neonates without disturbances of the hepatobiliary tract were to be included, on the one hand, as a second control group, and on the other hand, to investigate differences between preterm and term neonates. I hypothesized that BA levels in preterm neonates are higher than in adults but also than in term born neonates.

Ad 3) and 4) In a last step the influence of EOS on BA metabolism was sought in both preterm and term neonates. I hypothesize that BA levels are high in neonates, as in adults with sepsis-induced cholestasis. Work in neonates with sepsis, then, whose illness is relatively early in its course (within three days) and whose organisms are relatively "naïve", may offer the opportunity to analyze perturbations of BA handling in sepsis at a fundamental level and to derive insights that may be applicable to patients of all ages with respect both to disease mechanisms and to treatment by manipulation of BA pools and BA signaling.

2. PATIENTS AND METHODS

2.1. STUDY DESIGN AND PATIENT CHARACTERISTICS

Patients without disorders of EHC were studied prospectively at the Department of Pediatrics and Adolescent Medicine, Medical University Graz, from March 2014 until May 2015. The clinical study was approved by the Medical University Graz ethics committee (24-549 ex 11/12 and 26-215 ex 13/14). Parental consent was obtained for each subject. Neonates of less than 37 weeks' gestational age were considered preterm. All neonates (preterm and term) born at the Medical University of Graz aged 1 to 3 days were evaluated for inclusion. Exclusion criteria comprised elevated serum transaminase activity levels, primary hepatic diseases, asphyxia, or death within 1 month after birth. In controls EOS was excluded.

2.1.1. CONTROL GROUP

Fasting blood sample collection in neonates is difficult, since neonates are fed at least every 4 hours. However, feeding status (fed/fasting and breast milk/formula milk) was identified retrospectively when possible. Patients not fed within 2 hours before blood sampling were considered "fasted".

2.1.2. EOS GROUP

In EOS studies, only neonates with proven EOS were included. Diagnosis was confirmed by successful culture of microorganisms from blood, cerebrospinal fluid, or tracheal aspirate. Concentrations of CRP in serum, of PCT and IL-6 in cord blood, and of liver parameters

bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), and gamma-glutamyl transpeptidase (GGT) in serum were measured by standard laboratory methods.

2.1.3. OVERVIEW OF GROUPS

The study included overall 236 neonates (111 females, 125 males) aged one to 3 days. An overview of groups and numbers of overall studied neonates is given in Figure 7. In contrast to tBA, which were measured in all participants (n=236), aBA and henceforward total BA levels could only be determined in a subgroup of participants (n= 102; 43 females, 59 males) due to a shortage of sample volume. Neonates were divided into a reference study group and an EOS study group and were further divided into term and preterm.

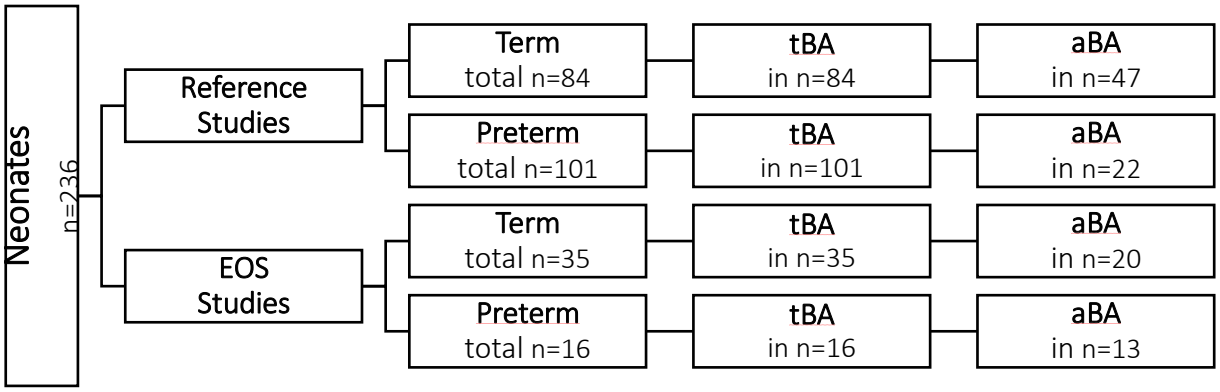


Figure 2: Overview of groups and numbers of all neonates (healthy and EOS, term and preterm). Atypical BA (aBA) – in contrast to typical BA (tBA) – were measured only in a subgroup of participants.

2.2. SAMPLE COLLECTION

Obtaining blood solely to define reference values of serum BA in neonates is ethically moot. Hence, blood sampling in healthy term neonates was performed during routine screening for

phenylketonuria. In EOS, blood was taken within 24 hours after first clinical signs of EOS and before first antibiotic administration. Venous blood samples were collected in EDTA (ethylenediaminetetraacetic acid) tubes and centrifuged for 10 min at 3000 U/min. All plasma samples were stored at -80°C until batch analysis.

2.3. BA ANALYSIS

Unconjugated, tauro- (T) and glyco- (G) conjugated BA were determined using HPLC-HRMS including typical BA (tBA) CA, CDCA, LCA, DCA, UDCA and their G- and T-conjugates in addition to aBA AMCA, BMCA, GMCA, OMCA and HDCA and their G- and T-conjugates (Table 2).

Table 2: List of all BA measured by HPLC-HR-MS

Unconjugated BA	**G-conjugated BA**	**T-conjugated BA**
CA	GCA	TCA
CDCA	GCDCA	TCDCA
DCA	GDCA	TDCA
LCA	GLCA	TLCA
UDCA	GUDCA	TUDCA
AMCA	GAMCA	TAMCA
BMCA	GBMCA	TBMCA
GMCA	GGMCA	TGMCA
OMCA	GOMCA	TOMCA
HDCA	GHDCA	THDCA

2.3.1. SAMPLE PREPARATION AND BA STANDARDS

Unconjugated CA, CDCA, DCA, LCA, and UDCA, AMCA, BMCA, GMCA, OMCA and HDCA, as well as their respective T- and G-conjugates, and the internal standards d_4-DCA, d_4-LCA, d_4-GLCA and d_4-GCDCA (all Sigma Aldrich, Taufkirchen, Germany) were used for identification and quantification in MS analysis and as bases for synthesis of other standards not commercially available. All aBA used as external standards (AMCA, BMCA, GMCA and OMCA, as well as their T- and G-conjugates) were synthesized by Benno Amplatz as part of his master's-degree thesis at the Clinical Institute of Medical and Chemical Laboratory Diagnostics of the Medical University of Graz in 2014 (Amplatz *et al*., submitted).

Plasma samples were prepared after the protocol of Humbert *et al.* (92). After addition of internal standards d_4-DCA, d_4-LCA, d_4-GLCA, d_4-GCDCA and d_4-TDCA, 0.2 nmol each, plasma samples (10µl) were vortexed for one minute. 400 µl of acetonitrile (80% v/v; Sigma Aldrich, Taufkirchen, Germany) were added for deproteination and - after vortexing - the precipitate was removed by centrifugation at 3200 g for 12 minutes. The supernatant was dried under a stream of nitrogen. The samples were redissolved in 100 µl of mobile phase B (methanol with 1.2% v/v formic acid and 0.38% w/v ammonium acetate) and transferred to autosampler vials.

2.3.2. CHROMATOGRAPHY

Samples (10 µl), stored in a cooled stack (Thermo Fisher Scientific), were introduced into the chromatographic system by an autosampler (Accela Open AS, Thermo Fisher Scientific). A nucleoshell C18 reversed phase column (2.7 µm, 50 x 2.0 mm) (Macherey-Nagel, Düren,

Germany) mounted in an oven with column switching unit (Mistraswitch, Maylab, Vienna, Austria) set to 25°C was used for HPLC of tBA samples. The HPLC-pump was a 1250 Accela (Thermo Fisher Scientific). For aBA a kinetex pentafluorophenyl column (2.6 µm, 100 x 6.0 mm, Phenomenex, Aschaffenburg, Germany) was used. 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 for separation and elution. HPLC gradient is indicated in Table 3. Flow rate was 500 µl/min. The lower limit of detection was at 0.012 nmol/mL.

Table 3: HPLC gradient used for elution of unconjugated, G- and T- conjugated BA

Time [min]	**Eluent A [%]**	**Eluent B [%]**
0	60	40
1	60	40
9	30	70
11	35	65
12	0	100
15	0	100
19 (reequilibration start)	60	40
23 (reequilibration end)	60	40

2.3.3. MS

A Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) was used with a heated electrospray ionization (ESI) ion source. The settings used for ionization are indicated in Table 4. Full scan was set between m/z = 370 to m/z = 570 in negative ion mode; resolution was 70,000. Because formic acid was used as an ionization modifier, formic acid adducts of unconjugated BA (addition of m/z = 46.0058 to $[M-H]^-$) were observed in different amounts. Therefore the sum of abundances of m/z values of corresponding ions (unconjugated $[M-H]^-$ plus adducts) was added in qualitative and quantitative experiments.

Table 4: ESI settings used for analysis of unconjugated and G- and T- conjugated BA

Sheath gas [ml/min]	40
Aux gas flow rate	10
Sweep gas flow rate	0
Spray voltage [kV]	3.00
Capillary temperature [°C]	350
S-lens RF level	50
Aux gas heater temp [°C]	300

2.3.4. CALIBRATION

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

2.4. STATISTICAL ANALYSIS

Patient characteristics and biochemical variables are presented as median and interquartile range (IQR). Non parametric tests (Mann-Whitney U-test) were performed when data were not normal-distributed. Correlations between BA and liver parameters were assessed using Spearman's correlation analysis. Multivariate regression analysis was used on combined results for total BA and inflammation parameters to identify variables independently associated with EOS. All statistical tests were two-tailed and p values of <0.05 were considered statistically significant. The SPSS Statistics package 23.0.0 (IBM SPSS, Armonk, NY) was used for all analyses.

3. RESULTS

3.1 BA IN HEALTHY TERM NEONATES

3.1.1 TOTAL BA PROFILE IN HEALTHY TERM NEONATES

3.1.1.1 TOTAL BA LEVELS

Reference values for total BA in healthy term neonates were defined in a subgroup of 47 participants for whom feeding status also was defined. The median BA levels were 8.7 µmol/L (IQR: 5.0 – 13.5). BA values differed significantly between the two feeding statuses: Levels were significantly higher in the fed group (n=30; 11.0 µmol/L; IQR: 6.4 – 17.1) than in the fasting group (n=17; 6.0 µmol/L; IQR: 4.4 – 10.7; $p<0.05$; Figure 8). No differences between breast milk (n=37; 7.9 µmol/L [IQR: 4.8 – 13.5]) and formula milk (n=10; 7.3 µmol/L [IQR: 4.5 – 11.2]) fed neonates were observable.

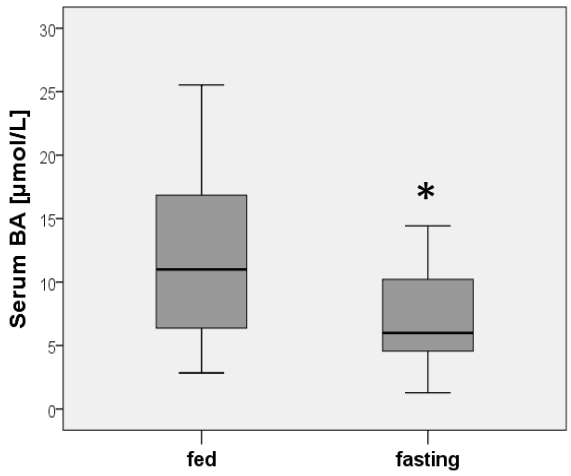
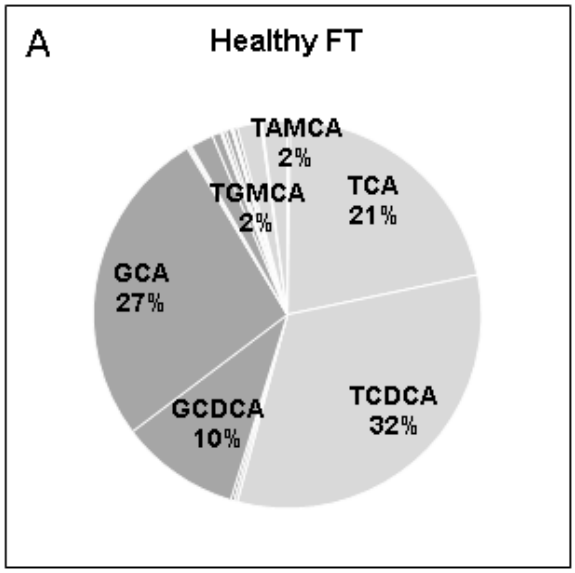


Figure 3: Influence of fasting condition on total BA levels (tBA + aBA). Total BA are significantly lower in the fasting group than in the fed group (*$p<0.05$).

3.1.1.2 TOTAL BA PROFILE

TDCA, GCA, TCA, and GCDCA were not only the most abundant tBA in healthy term neonates, but also the most abundant BA in total (Figure 9, A). aBA accounted for 5% and 8% of total BA in the fed and fasting condition, respectively. TAMCA and TGMCA were the most abundant aBA. Also investigated was if nutrition status has an effect on BA profile: within the two groups (fed and fasted) T-conjugates predominated (56% and 57%, respectively) over G-conjugates (40% and 34%, respectively). Unconjugated BA were in both groups <10%. Minor differences in BA profile were observed between both fed and fasting condition and breast milk and formula milk: GCA was slightly higher in the fed than in the fasting condition, and again in breast milk fed neonates than in formula milk fed neonates, respectively. In contrast, CA was slightly higher in the fasting than in the fed condition, and again in formula milk fed neonates than in breast milk fed neonates (Figure 9, B-E).



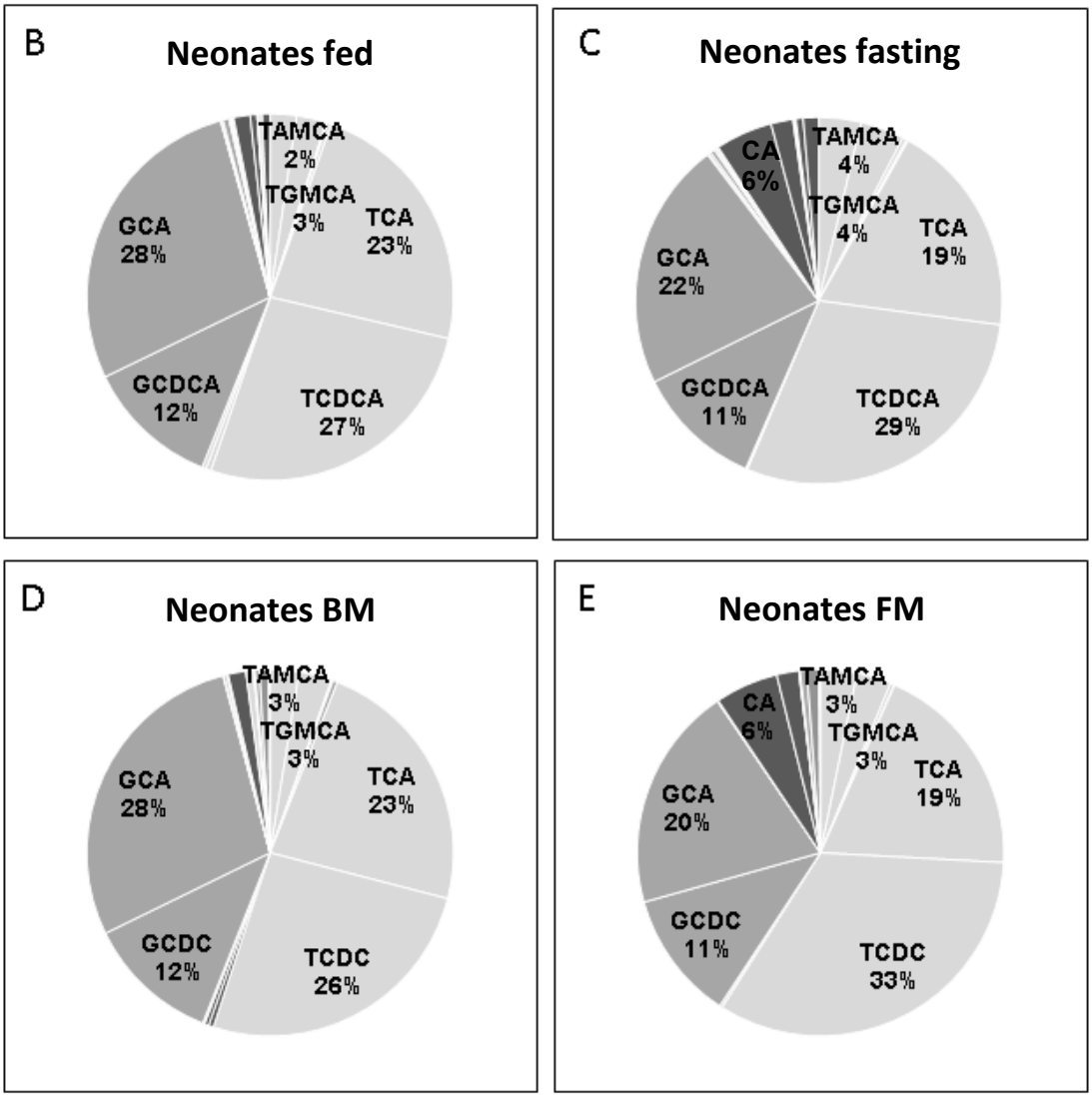


Figure 4: BA pool composition of total BA (A) is similar in healthy term (FT) neonates in the B) fed and C) fasting condition and when fed with D) breast milk (BM) and formula milk (FM). In all conditions TCDCA and GCA were the most abundant species and the presence of unconjugated and secondary BA was <10%.

3.1.2 TBA IN HEALTHY TERM NEONATES

3.1.2.1 TBA LEVELS

Reference values for tBA in healthy term neonates were defined in a group of 84 participants. The median BA levels were 8.0 µmol/L (IQR: 4.6 – 12.9). Influence of fasting condition was determined in 47 healthy term neonates (median BA levels 7.8 µmol/L; IQR: 5.0 – 13.0); in 37 cases the feeding status was unclear. Interestingly, BA values differed significantly between

the two feeding statuses: levels were significantly higher in the fed group (n=30; 10.1 µmol/L; IQR: 6.2 – 15.5) than in the fasting group (n=17; 5.8 µmol/L; IQR: 4.3 – 7.9; $p<0.01$). No differences between breast milk (n=37; 7.9 µmol/L [IQR: 4.8 – 13.5]) and formula milk (n=10; 7.3 µmol/L [IQR: 4.5 – 11.2]) fed neonates were observable.

3.1.2.2 TBA PROFILE

TCA, TCDCA, GCA, and GCDCA were the most abundant BA, whereas secondary BA did not exceed 0.1 µmol/l. Within the two groups (fed and fasted) T-conjugates predominated (55% and 54%, respectively) over G-conjugates (42% and 37%, respectively). However, no differences in BA profile were observed between fed and fasting conditions (Figure 10, A-B). The influence of natural or synthetic diet also was investigated; however, no differences were identified between breast-milk or formula-milk fed neonates (Figure 10; C-D).

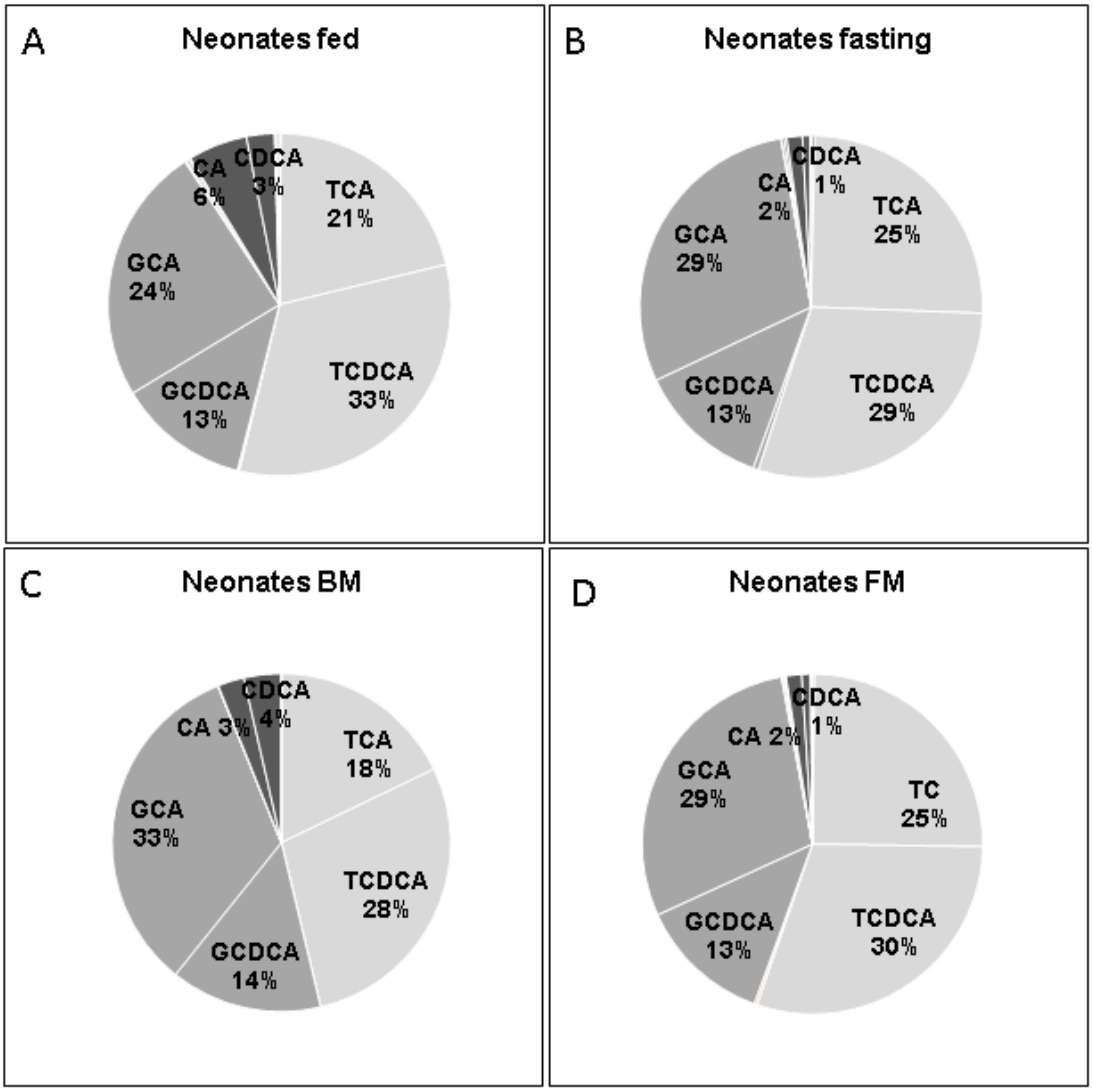


Figure 5: BA pool composition of tBA is similar in healthy term neonates in A) fed and B) fasting condition. No differences were observed between C) breast milk (BM) and (D) formula milk (FM) fed neonates.

3.1.3 aBA IN HEALTHY TERM NEONATES

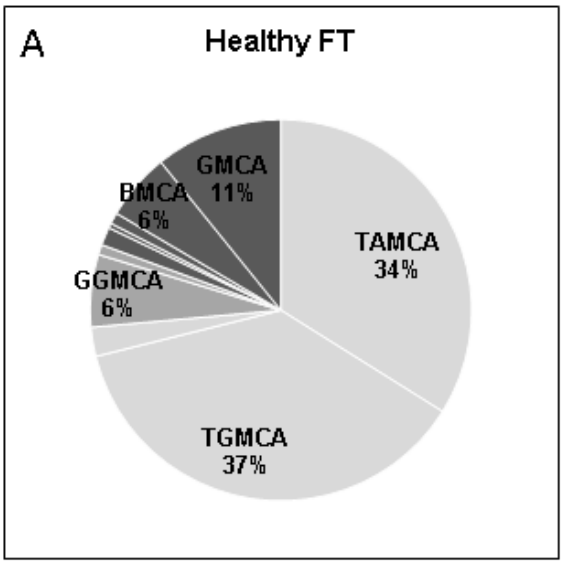
3.1.3.1 aBA LEVELS

Reference values for aBA in healthy term neonates were defined in the subgroup of 47 participants for whom feeding status also was defined. The median BA levels were 0.5 µmol/L (IQR: 0.3 – 1.3). Levels were slightly higher in the fed group (n=30; 0.6 µmol/L; IQR: 0.4 –

1.3) than in the fasting group (n=17; 0.5 µmol/L; IQR: 0.2 – 1.2;). Again, no differences between breast milk (n=37; 0.5 µmol/L [IQR: 0.3 – 1.2]) and formula milk (n=10; 0.5 µmol/L [IQR: 0.2 – 1.4]) fed neonates were observable.

3.1.3.2 aBA PROFILE

TGMCA and TAMCA were the predominant aBA in healthy term neonates; GMCA, BMCA and GGMCA were also present (Figure 11 A). HDCA and its conjugates were below the detection limit. Also investigated was if nutrition status has an effect on aBA profile: in both groups (fed and fasted) T-conjugates predominated (73% and 75%, respectively) over G-conjugates (10% and 6%, respectively), and unconjugated BA predominated (17% and 20%, respectively) over G-conjugates. However, no differences in BA profile were observed between fed and fasting conditions and breast and formula milk fed neonates (Figure 11, B-E).



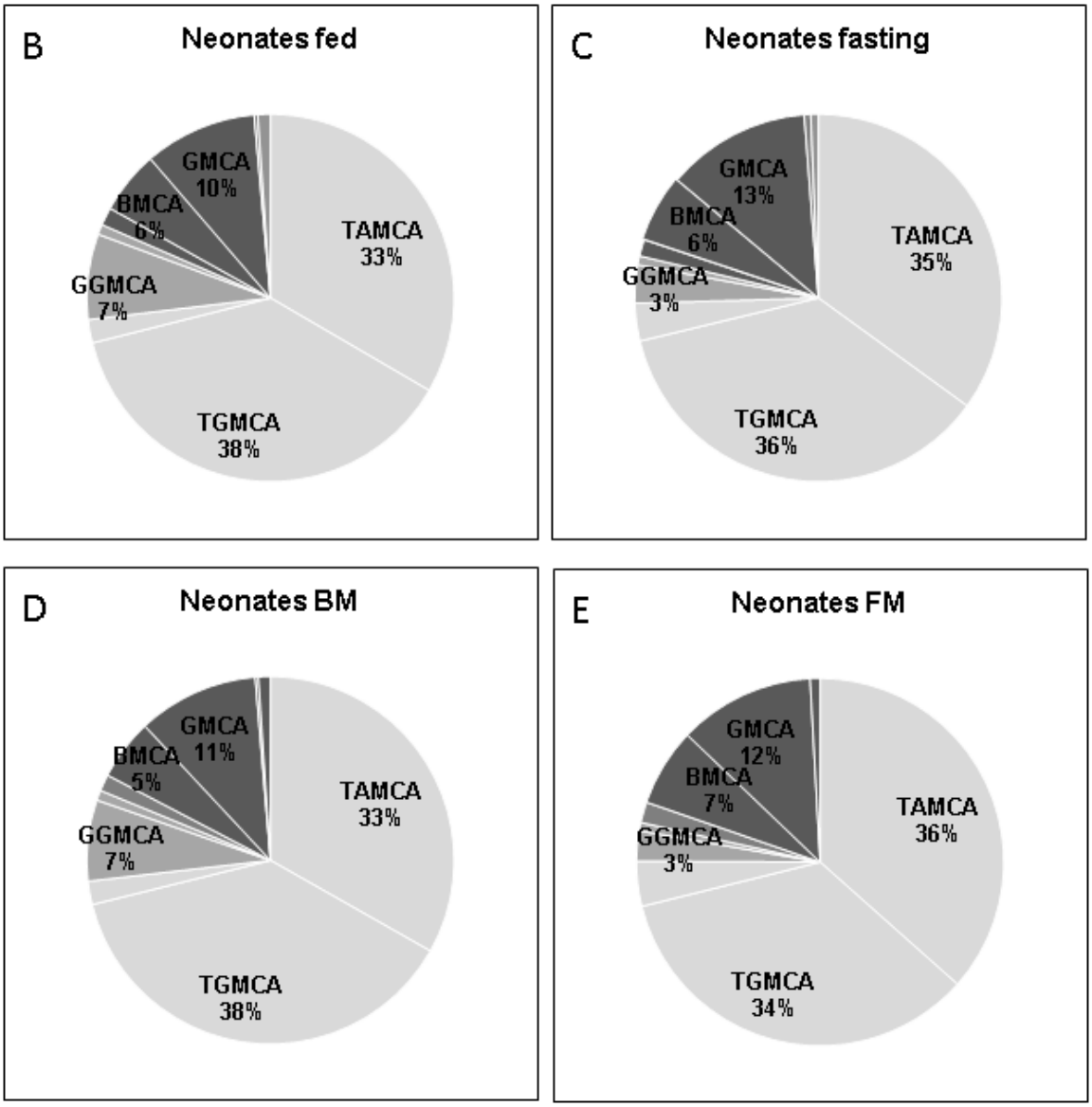


Figure 6: BA pool composition of aBA is similar in all healthy term (FT) neonates (A). The aBA profile is similar in B) fed and C) fasting condition. There were no differences between C) breast milk (BM) and (D) formula milk (FM) fed neonates. In all conditions, TGMCA and TAMCA were the most abundant species.

3.2 BA IN HEALTHY PRETERM NEONATES

3.2.1 TOTAL BA PROFILE IN HEALTHY PRETERM NEONATES

3.2.1.1 TOTAL BA LEVELS

Reference values for total BA in healthy preterm neonates were defined in the subgroup of 22 participants for whom aBA also could be determined. Feeding status in all preterm neonates was defined as "fed" since they were fed regularly. The median total BA levels were 11.2 µmol/L (IQR: 5.6 – 16.9; Table 5) and therefore similar to term neonates in the fed state with 11.0 µmol/L (IQR: 6.4 – 17.1).

Table 5: Total BA (tBA + aBA) levels of heathy preterm neonates and in FT controls

	n		**Total BA**	
	all	**f / m**	**Median**	**IQR**
FT controls	47	27 / 20	8.7	5.0 – 13.5
FT controls (fed)	30	13 / 17	11.0	6.4 – 17.1
FT controls (fasting)	17	13 / 4	6.0	4.4 – 10.7
PT controls	22	8 / 14	11.2	5.6 – 16.9

3.2.1.2 TOTAL BA PROFILE

In healthy preterm TCDCA, GCA, TCA, and GCDCA were not only the most abundant tBA neonates, but also the most abundant BA in total (Figure 12). Additionally, TCDCA is significantly higher in preterm than in term neonates ($p<0.05$). TAMCA is the only detectable aBA in healthy preterm neonates; however, it accounts for <1% of total BA.

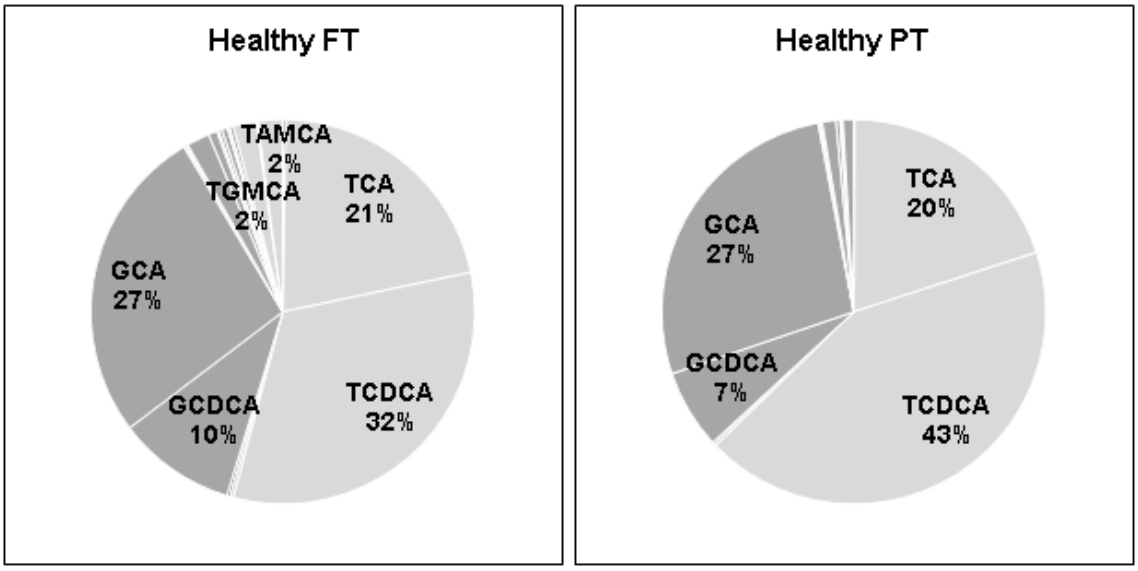


Figure 7: Total BA profiles of healthy term (FT) and preterm (PT) neonates. T-conjugates outweighed G-conjugates in both healthy term and preterm neonates. aBA are higher in healthy term neonates than in preterm neonates.

3.2.2 TBA IN HEALTHY PRETERM NEONATES

3.2.2.1 TBA LEVELS

In a second step, I determined if prematurity influences BA levels. Reference values for tBA in healthy preterm neonates were defined in a group of 101 participants and compared to those in healthy term neonates (Table 6). Feeding status in all preterm neonates was defined as "fed" since they were fed regularly. BA levels in preterm neonates were similar to fed term controls (10.1 µmol/L; IQR: 5.7 – 15.7 vs. 10.1 µmol/L; IQR: 6.2 – 15.5, respectively).

Table 6: tBA levels of heathy preterm neonates and in FT controls

	n		**tBA**	
	all	**f / m**	**Median**	**IQR**
FT controls	47	27 / 20	5.6	0.3 – 1.3
FT controls (fed)	30	13 / 17	10.1	6.2 – 15.5
FT controls (fasting)	17	13 / 4	5.8	4.3 – 7.9
PT controls	101	47 / 54	10.1	5.7 – 15.7

3.2.2.2 TBA PROFILE

In preterm neonates primary BA TCA, TCDCA, GCA, and GCDCA were the most abundant BA, whereas secondary BA did not exceed 0.1 µmol/l (Figure 13). In both preterm and term neonates T-conjugates predominated (55% and 53%, respectively) over G-conjugates (42% and 44%, respectively).

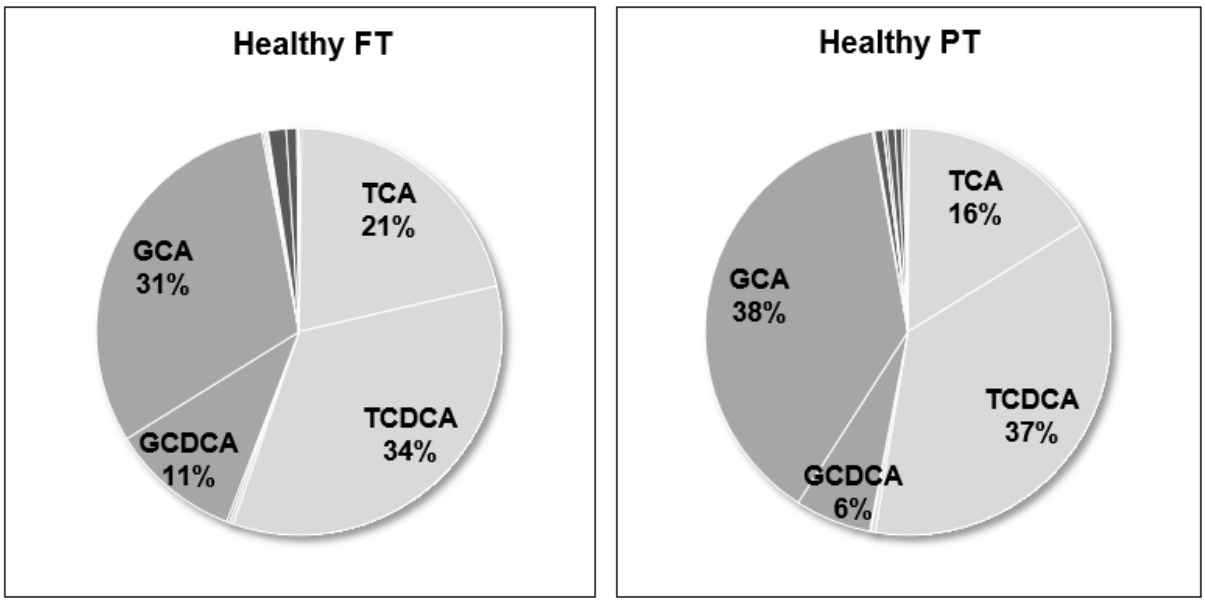


Figure 8: tBA profiles of healthy term (FT) and preterm (PT) neonates. T-conjugates outweighed G-conjugates in healthy term and preterm neonates.

3.2.3 ABA IN HEALTHY PRETERM NEONATES

3.2.3.1 ABA LEVELS

Reference values for aBA in healthy preterm neonates were defined in a group of 22 participants and compared to those in healthy term neonates. Feeding status in all preterm neonates was defined as "fed" since they were fed regularly. aBA levels in preterm neonates were below the detection limit except from TAMCA, whose median concentration was 0 µmol/L, but with an

IQR of 0 – 0.2, though (Table 7). In total, aBA were significantly higher in healthy term neonates than in healthy preterm neonates ($p<0.01$).

Table 7: aBA levels of heathy preterm neonates and in FT controls

	n		aBA	
	all	**f / m**	**Median**	**IQR**
FT controls*	47	27 / 20	0.5	0.3 – 1.3
FT controls* (fed)	30	13 / 17	0.6	0.4 – 1.3
FT controls* (fasting)	17	13 / 4	0.5	0.2 – 1.2
PT controls	22	8 / 14	0.0	0.0 – 0.2

3.2.3.2 aBA PROFILE

TAMCA was the only aBA found in healthy preterm neonates. Compared to healthy term neonates, GMCA, TGMCA, TOMCA, GGMCA and BMCA were significantly lower in preterm neonates (Figure 14).

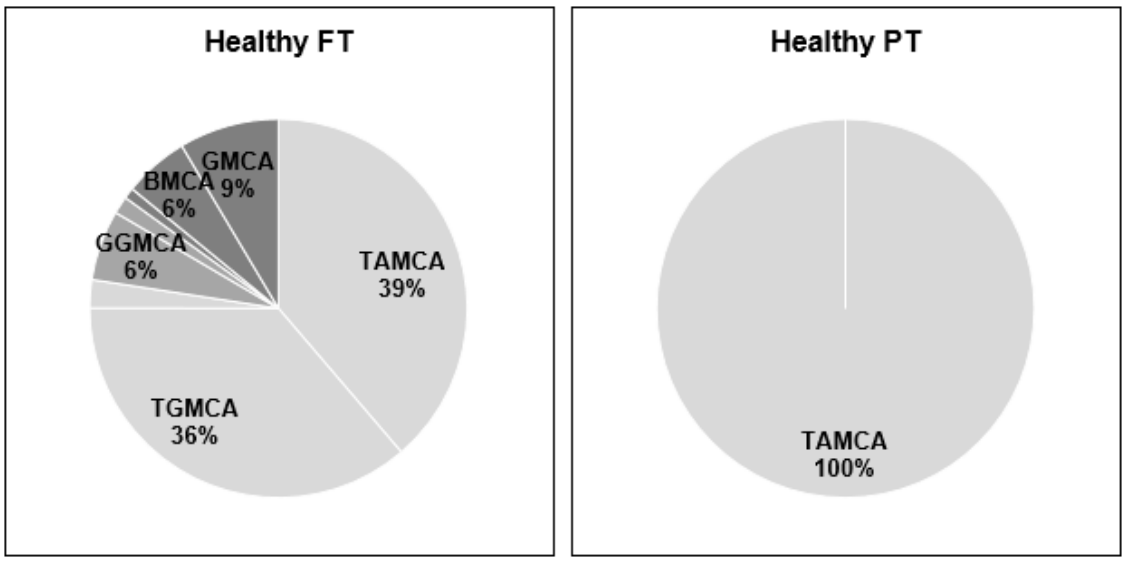


Figure 9: aBA profiles of healthy term (FT) and preterm (PT) neonates. TAMCA is the only aBA over the detection limit in preterm neonates.

3.3 BA IN EOS

3.3.1 BA IN EOS TERM NEONATES

3.3.1.1 LABORATORY PARAMETERS IN EOS TERM NEONATES

Alterations of BA levels in septic neonates were studied, since BA levels are known to be elevated in septic adults. Twenty term neonates with EOS presented with inflammation marker concentrations above normal ranges of CRP, IL-6, but not PCT levels. Median levels of total serum bilirubin - a marker of cholestasis - were within the normal range at 4.5 µmol/L but 20 percent were above the reference range (IQR: 1.9 – 8.9). Serum activities of liver parameters ALT, AST and GGT were within the normal range. All laboratory parameters and reference ranges are listed in Table 8.

Table 8: Mean laboratory in EOS term (FT) neonates during first 72 h of life. Expected ranges: CRP, <5 mg/l; IL-6, <7 pg/ml; PCT, <0.5 ng/ml; total bilirubin, 1.4-5.3 mg/dl; ALT, <67 U/L; AST, <77 U/L and GGT, <216 U/L.

	FT EOS (n=20)
CRP, mg/L	12.6 (9.6 – 20.5)
PCT, ng/mL	0.4 (0.3 – 0.6)
IL-6, pg/mL	204 (117 – 1090)
Bilirubin, µmol/L	4.5 (3.3 – 8.5)
ALT, U/L	17.0 (11.0 – 35.0)
AST, U/L	56.5 (28.0 – 69.0)
GGT, U/L	107.0 (90.5 – 197.5)

3.3.1.2 TOTAL BA IN EOS TERM NEONATES

3.3.1.2.1 TOTAL BA LEVELS

Total BA in EOS term neonates were defined in the subgroup of 20 participants for whom aBA also could be determined. The median total BA levels were 6.2 µmol/L (IQR: 3.8 – 8.6) and significantly lower than in term controls with 8.7 µmol/L (IQR: 5.0 – 13.5) ($p<0.05$).

3.3.1.2.2 TOTAL BA PROFILE

BA profiles revealed different results for healthy and EOS term neonates. In EOS term neonates the three tBA TCDCA, TCA, and GCA made up the largest part of the BA pool, but with GCA significantly lower in EOS than in controls ($p<0.05$). Interestingly, the aBA TOMCA took the fourth place with 15% of total BA and was significantly higher in EOS than in term controls ($p<0.01$; Figure 15).

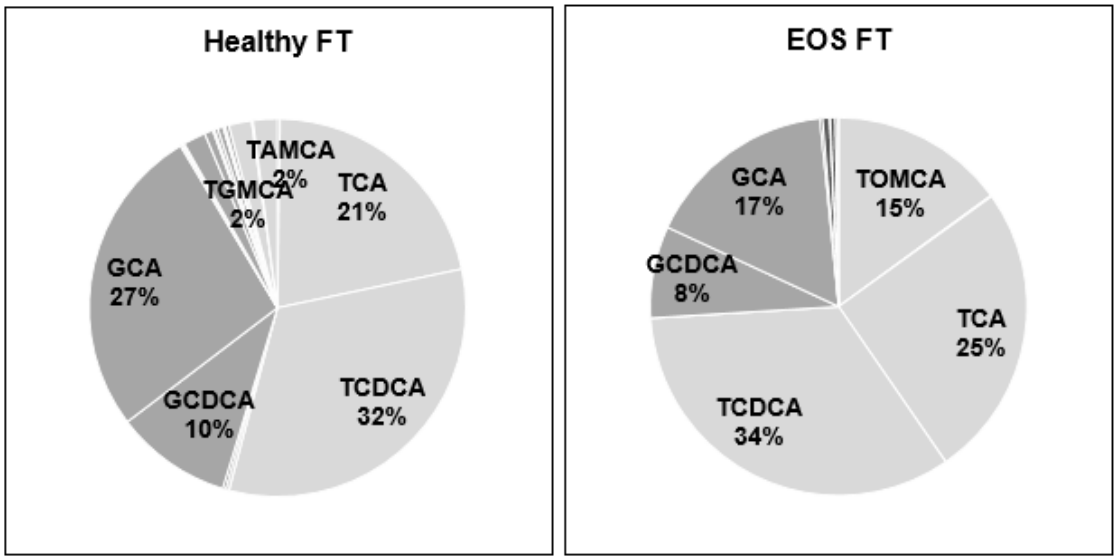


Figure 10: Total BA profiles of healthy and EOS term (FT) neonates. TCDCA, TCA, and GCA were the most abundant BA in both groups, but with tBA GCA significantly lower and TOMCA significantly higher in EOS neonates than in controls.

3.3.1.3 TBA IN EOS TERM NEONATES

3.3.1.3.1 TBA LEVELS

BA levels were investigated in 35 term neonates with EOS. Serum BA levels were significantly less at 4.7 µmol/l (2.3 – 7.5 µmol/l) than in controls at 8.0 µmol/l (4.8 – 12.9 µmol/l; $p<0.05$).

3.3.1.3.2 TBA PROFILE

In term neonates T-conjugates predominated in both EOS patients and term controls (69% and 56%, respectively) over G-conjugates (30% and 42% respectively). In summary, T-conjugates outweighed G-conjugates in the septic state, with a significant decline ($p<0.05$) in G-conjugated BA (GCA) in term neonates with EOS. Secondary BA accounted for less than 10% of total BA levels (Figure 16).

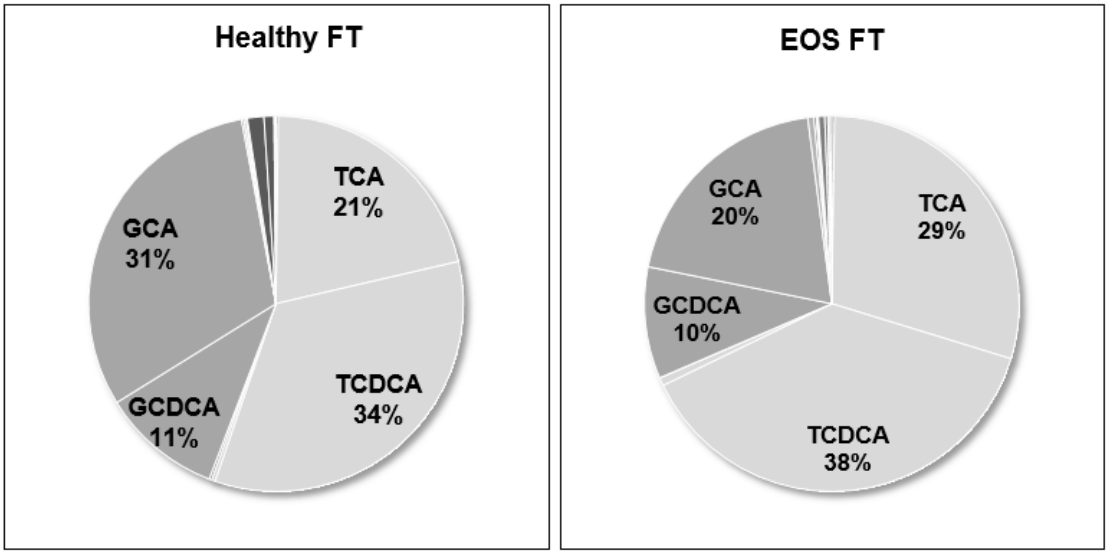


Figure 21: tBA profile in EOS term (FT) neonates. BA profile is similar in term controls as in neonates with EOS: T-conjugates outweigh G-conjugates. In both conditions the presence of unconjugated and secondary BA was <10%.

3.3.1.4 aBA in EOS term neonates

3.3.1.4.1 aBA levels

ABA levels were determined in 20 term neonates with diagnosed EOS. Interestingly, aBA levels were comparable in term neonates with EOS and in term controls with 0.5 µmol/L (IQR: 0.3 – 1.3) and 0.6 µmol/L (IQR: 0.1 – 1.6), respectively.

3.3.1.4.2 aBA profile

The aBA profile in EOS term neonates differed from that in healthy term neonates: TOMCA was significantly higher in EOS ($p<0.01$) and accounted for 95% of all aBA (Figure 17). TAMCA was significantly lower in EOS ($p<0.01$). TGMCA, GMCA, BMCA and GGMCA, which were - in addition to TAMCA - the most abundant aBA in healthy term neonates, were below the detection limit in EOS.

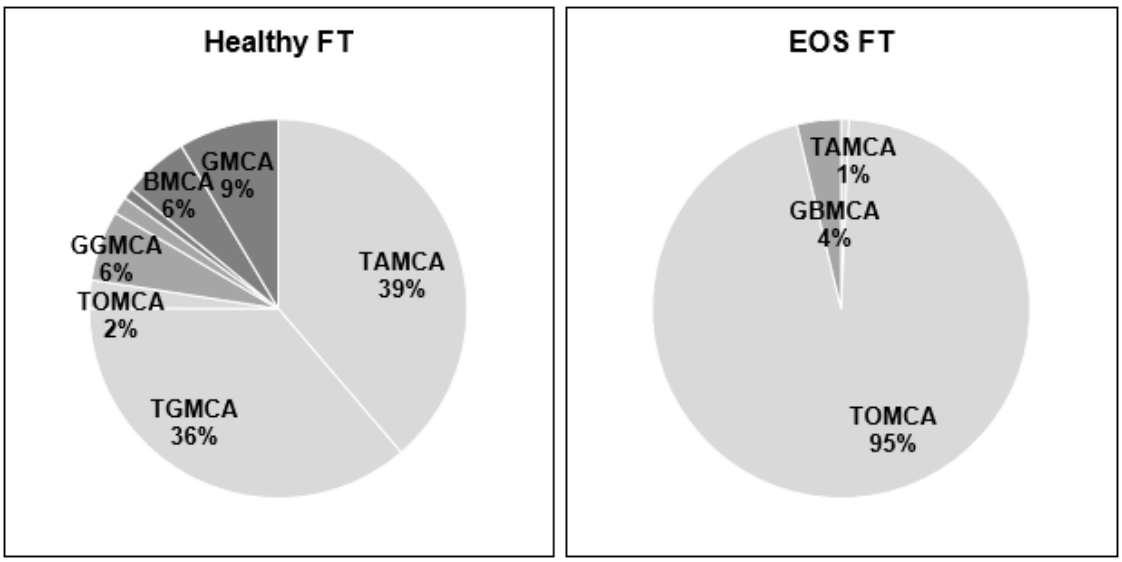


Figure 32: aBA profile in healthy and EOS term (FT) neonates. aBA profile in term controls is more diverse than in EOS. TOMCA is significantly higher in EOS and makes up the largest part of aBA.

3.3.2 BA IN EOS PRETERM NEONATES

3.3.2.1 LABORATORY PARAMETERS IN EOS PRETERM NEONATES

Twenty-two preterm neonates with EOS showed inflammation marker concentrations above normal ranges of CRP, IL-6 and bilirubin, but not PCT levels. Median levels of liver parameters ALT, AST and GGT were within the normal range. All laboratory parameters and reference ranges are listed in Table 9.

Table 9: Mean laboratory in EOS preterm (PT) neonates during first 72 h of life. Expected ranges: CRP, <5 mg/l; IL-6, <7 pg/ml; PCT, <0.5 ng/ml; total bilirubin, 1.4-5.3 mg/dl; ALT, <67 U/L; AST, <77 U/L and GGT, <216 U/L.

	PT EOS (n=22)
CRP, mg/L	52.0 (17.3 – 86.8)
PCT, ng/mL	0.3 (0.2 – 22.2)
IL-6, pg/mL	44 (9 - 1400)
Bilirubin, µmol/L	5.8 (1.7 – 10.0)
ALT, U/L	9.5 (1.0 – 18.0)
AST, U/L	24.0 (17.0 – 31.0)
GGT, U/L	44.0 (27.0 – 61.0)

3.3.2.2 TOTAL BA IN EOS PRETERM NEONATES

3.3.2.2.1 TOTAL BA LEVELS

Total BA in EOS preterm neonates were defined in the subgroup of 22 participants for whom aBA also could be determined. The median total BA levels were 7.4 µmol/L (IQR: 4.0 – 10.0) and therefore lower than in preterm controls (11.2 µmol/L [IQR: 5.6 – 16.9]), although not significantly so.

3.3.2.2.2 TOTAL BA PROFILE

The BA profile including tBA and aBA differed between EOS preterm neonates and controls: T-conjugates were significantly decreased in EOS ($p<0.05$). In healthy preterm neonates tBA TCDCA, GCA, TCA and GCA were the most abundant BA species and aBA – other than TAMCA with <1% of total BA – were below the detection limit. In contrast, in EOS preterm neonates TCDCA was significantly lower ($p<0.05$) and TOMCA significantly higher than in controls ($p<0.01$) making making TOMCA the fourth most important BA (Figure 18).

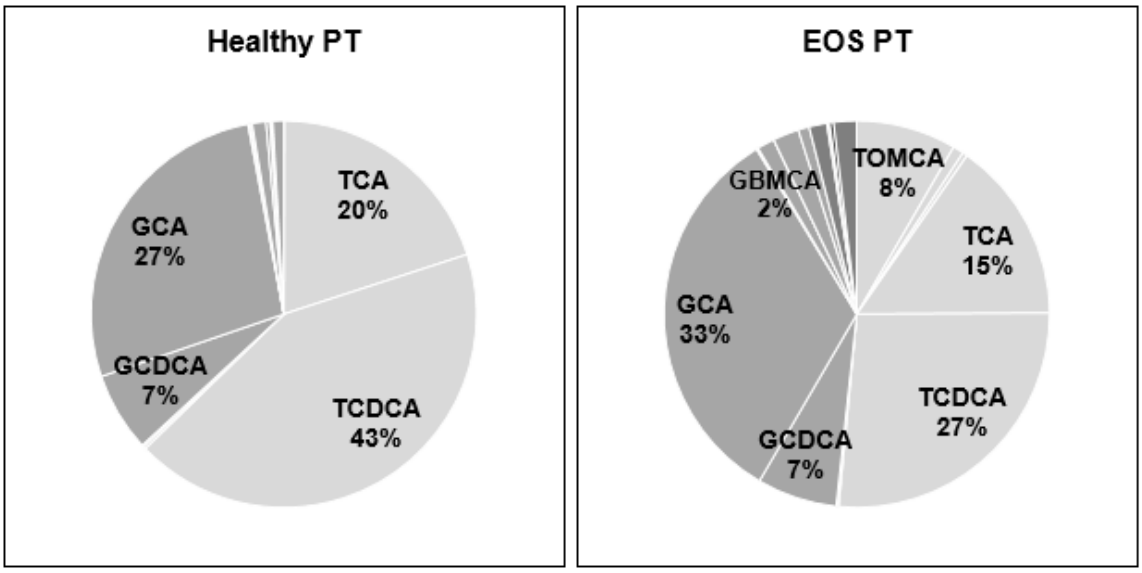


Figure 43: Total BA profiles of healthy and EOS preterm (PT) neonates. T-conjugates outweighed G-conjugates in both healthy and EOS term neonates, but with a decline in T-conjugates in EOS. aBA levels were higher in EOS preterm neonates than in those with EOS. TCDCA was the most abundant tBA in both healthy and EOS preterm neonates, TAMCA the only aBA in healthy preterm neonates (<1%) and TOMCA the most abundant aBA in EOS.

3.3.2.3 TBA IN EOS PRETERM NEONATES

3.3.2.3.1 TBA LEVELS

I investigated if and to which extent EOS in combination with prematurity affects BA levels. tBA levels were determined in 16 preterm neonates with diagnosed EOS. Interestingly, tBA levels in preterm neonates with EOS were significantly lower than in preterm controls (6.4 µmol/L [IQR: 3.5 – 8.4] vs. 10.1 µmol/L [IQR: 5.7 – 15.7]; $p<0.01$).

3.3.2.3.2 TBA PROFILE

In preterm neonates profiles of controls and those with EOS (Figure 19) and were similar: T-conjugates in controls (56% and 53%, respectively) predominated over G-conjugates (42% and 45%, respectively). TCDCA and GCA were the two most abundant BA, with 37% vs. 39% and 38% vs. 34% (preterm controls vs. EOS, respectively). Secondary BA accounted for less than 10% of total BA levels.

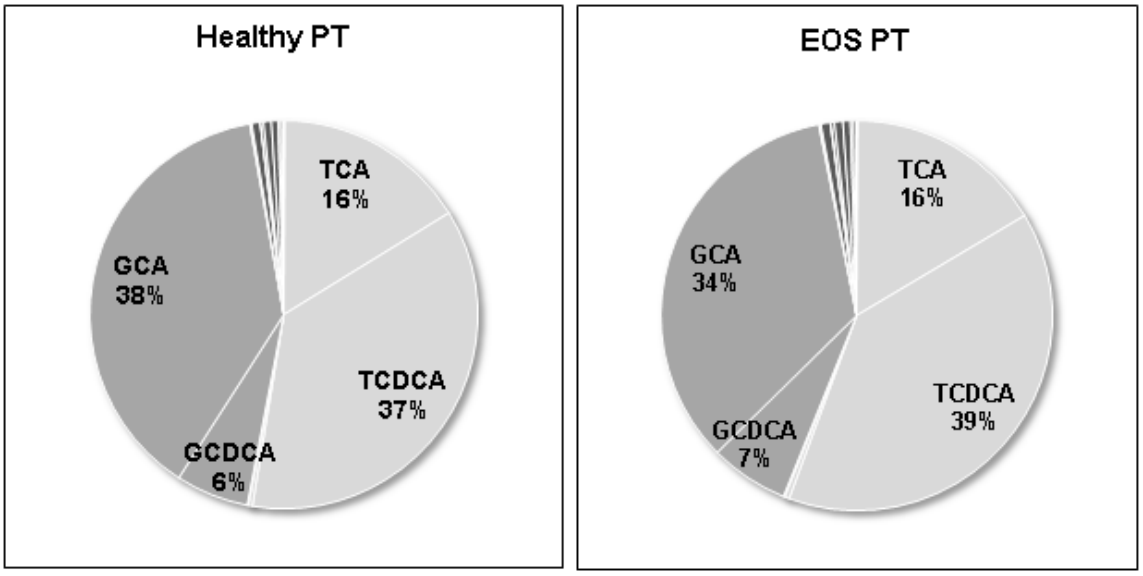


Figure 54: tBA profile in EOS preterm (PT) neonates. BA profiles were similar in PT controls (A) and in neonates with EOS (B): T-conjugates outweighed G-conjugates. In both conditions the presence of unconjugated and secondary BA was <10%.

3.3.2.4 ABA IN EOS PRETERM NEONATES

3.3.2.4.1 ABA LEVELS

ABA levels were determined in 13 preterm neonates with diagnosed EOS. ABA levels in preterm neonates with EOS were significantly higher than in preterm controls (0.6 µmol/L [IQR: 0.2 – 1.5] vs. 0 µmol/L [IQR: 0.0 – 0.2]; $p<0.01$).

3.3.2.4.2 ABA PROFILE

The aBA profile in EOS preterm neonates differed from that in healthy preterm neonates: TOMCA was significantly higher in EOS ($p<0.01$) and accounted for 72% of all aBA, followed by GBMCA (17%), TAMCA (8%) and BMCA (3%; Figure 20). TAMCA – the only aBA in healthy preterm neonates - was significantly lower in EOS ($p<0.01$).

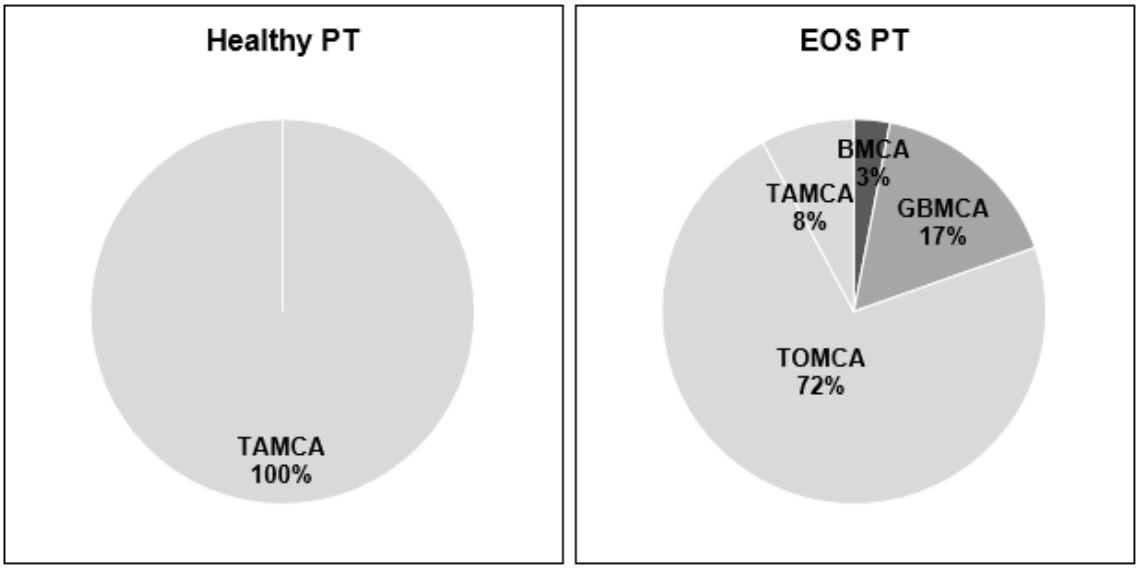


Figure 15: aBA profile in healthy and EOS preterm (PT) neonates. In preterm controls, TAMCA was the only aBA, whereas in EOS preterm neonates the aBA were varied. TOMCA was significantly higher in EOS preterm neonates than in controls making up the largest part of aBA in EOS.

3.3.3 THE ROLE OF BA AS BIOMARKERS IN EOS

In a last step, the association between laboratory parameters (liver parameters, inflammatory parameters and serum BA levels) were analyzed in order to investigate the potential role of serum BA as a biomarker for EOS. Statistical analyses in both term and preterm neonates with EOS (n=33) revealed that serum BA levels are independently associated with EOS. No correlation was found between BA and liver parameters ALT (ρ=-0.22; p=0.56), AST (ρ=-0.13; p = 0.73) and GGT (ρ=0.07; p=0.87). Moreover, multivariate regression analysis of BA, CRP, PCT, IL-6 and bilirubin revealed that serum BA levels are an independent factor associated with EOS (r=0.97, p=0.10); BA are not to be predicted by CRP (p=0.10), PCT (p=0.56), IL-6 (p=0.12) and bilirubin (p=0.13). Besides, neither prematurity nor individual BA (tBA and aBA) influenced correlation and/or regression results.

4. DISCUSSION

In the work presented in this thesis, I determined reference ranges of total BA values (tBA and aBA) for healthy term and preterm neonates using highly sensitive HPLC-HRMS. I found that, in general, total BA levels are higher in neonates (both term and preterm) than in adults and specifically are higher in preterm than in term neonates. In addition, I sought to investigate whether sepsis affects BA levels in neonates, given that serum BA concentrations rise in septic adults. Total BA levels were found to be lower in both term and preterm neonates suffering from EOS than in healthy age-matched controls.

My first aim was to establish total (tBA and aBA) BA reference values for healthy term neonates. It has been clearly shown that total BA levels are higher in term neonates than in healthy adults (5.0 – 13.5 vs. 0.28 – 6.50 μmol/L) (97). High levels of serum BA in term neonates have been reported by Polkowska *et al*., with peak values of 22.2 ± 5.1 μmol/L at the age of 1 month measured by enzymatic colorimetry, and by Niijima *et al*., who measured serum BA levels between the ages of 0 and 4 weeks (11.0 ± 8.7 μmol/L) by HPLC (59,61). In 2015, our group aimed to define normal range values of BA in the serum of children and adolescents for different age groups using HPLC-HRMS, a state-of-the-art method that has numerous advantages, including substantially higher sensitivity, selectivity, and reproducibility resulting in better comparison between laboratories, and higher validity of our standard values (46). Serum concentrations of tBA in infants until the 24th month were higher (3.9 – 6.3 μmol/L) than in adults, which accords with our present results. In my work, results of total BA were comparable to levels found by Niijima *et al.*, perhaps due to similarities in age and method. Enzymatic colorimetry, used by Polkowska *et al*., is a simple way to determine total BA; however, its sensitivity is poor. Today,

HPLC-MS is the method of choice to produce sensitive and reliable values for BA concentrations, enabling the generation of an individual BA profile.

A closer examination of total BA level results in neonates showed that postprandial BA levels of both tBA and aBA were significantly higher than fasting levels (>2 hours fasting). This accord with the work of LaRusso *et al.* in adults, who found peak levels of BA and conjugates 90 min after a meal (26). Suchy *et al.* reported high BA levels in term neonates; they postulated that frequent food intake induces *de novo* BA synthesis and stimulates EHC (47), which is in line with higher levels in neonates than in adults, independent of feeding status. Of further note are the high concentrations of T-conjugates in healthy neonates. This profile differs substantially from those in healthy adults, where G-conjugates are the predominant species (97). T is the most abundant free amino acid in breast milk. Since the presence of T has been noted as essential in preterm neonates and also may be beneficial in term neonates, it has become a standard supplement in formula milk (27). Apart from its role as an osmolyte in the brain and renal medulla (37), T is very important for fat absorption: T-conjugates have been found to be better fat emulsifiers that G-conjugates, which might be essential for the immature preterm neonate. Interestingly, I found no major differences in BA profiles between neonates fed breast milk and neonates fed formula. Minor differences between those two diets (GCA was higher in breast milk, whereas CA was higher in formula milk) were probably not due to a different diet but due to the fed and fasting condition of the neonate. In summary, whether the neonate is fed by breast or with formula milk causes no difference in the BA pool and hence, formula milk can be used in this regard without hesitation.

The composition of individual BA differs from that in neonates with that found in adults. It has been suggested that the main pathway of BA biosynthesis in the neonate is the alternative pathway (98) wherein large amounts of CDCA are produced, the precursor of GMCA and AMCA. This is in accordance to my finding. In healthy term neonates TCDCA, followed by TCA, was the most abundant tBA and TGMCA, followed by TAMCA, was the most abundant aBA. The presence of typical secondary BA is low in neonates, rise together with increasing bacterial actions and are firstly signaled by the presence of DCA in bile. In this study, secondary tBA DCA, LCA and UDCA were present in low concentrations in all study groups. My findings accord with results obtained by Barbara *et al.* and Poley *et al.*, who reported high primary BA levels in serum of fetuses and of neonates (62,99). Low serum concentrations of typical secondary BA in infancy are explained by immaturity of the colonic microbiome, which develops within the first year (100). Since the microbiome is essential for the generation of secondary BA and hence the whole BA pool diet, antibiotic therapy, and disease states may have a major effect on BA homeostasis. In contrast to tBA, GMCA, a secondary aBA, was the most abundant aBA in healthy term neonates. Studies reported from high amounts of GMCA in the meconium (95), suggesting that the fetal pathway persists throughout early infancy. However, the purpose of existing aBA in early infancy has still not been defined.

Secondly, I aimed to investigate the BA profiles of preterm neonates in order to gain insight into early BA metabolism. My work demonstrates that serum BA levels are comparable in term and preterm neonates. Since preterm neonates are fed regularly, their levels were compared to BA levels of fed term neonates. In summary, BA levels are similar in neonates born at term and preterm in the fed state, but are higher than reference values in adults (97). Despite lower BA synthesis rate and decreased BA pool size, serum BA levels are increased in healthy neonates,

both term and preterm, and may reach values as high as those found in adults with clinical cholestasis (47). The elevated serum BA levels during this period are termed 'physiological cholestasis' and are ascribed to poor hepatic extraction of bile salts from the portal circulation. An improvement in the hepatic uptake of BA occurs over the first years of life and tracks a decrease in peripheral serum BA levels during childhood (46).

Although BA levels are similar in term and preterm neonates, the two BA profiles differ. In preterm neonates, TCDCA was significantly lower than in term neonates. Additionally, all aBA were below the detection limit, except the primary BA TAMCA, which was only found in small amounts. These findings lead to the hypothesis that in the preterm child the 'classical' pathway of BA synthesis, where CA is the main product, may be the predominant pathway. Also possible is that in the preterm neonate aBA other than di- or trihydroxylated BA are more concentrated. For instance, Strandvik *et al.* measured high amounts of tetrahydroxylated and ketonic BA in the urine of healthy term and preterm infants (101). Unfortunately, it was not possible to measure these BA species in our laboratory.

My third aim was to investigate the influence of EOS on BA metabolism. Demand is great for sepsis biomarkers that will permit an early and reliable diagnosis of EOS. CRP plays a central role in the humoral response to bacterial invasion. However, its delayed synthesis during the inflammatory response accounts for its low sensitivity during the early phase. Nonetheless, only CRP levels are used in the routine for diagnosis mainly due to costs and technical reasons. Il-6 is released after the invasion of microorganisms as an acute inflammatory reaction and is responsible for the production of acute-phase proteins in the liver, including CRP (84). In general, cytokines as sepsis biomarkers might be of limited value, since numerous noninfectious

diseases also can induce their synthesis. Additionally, hyperbilirubinemia is frequently observed in patients with sepsis (87). All these - CRP, IL-6 and bilirubin levels - were above normal ranges in our EOS participants independent of gestational age. PCT, also an acute-phase reactant, has the advantage of increasing rapidly after infection with levels rising after 4 h (102). Changes in PCT can support the diagnosis of bacterial infection and PCT thus is an improvement on CRP. However, based on current evidence, PCT lacks the necessary accuracy to be used without clinical judgment, since nonbacterial systemic inflammation such as that of birth stress in neonates has also been reported to increase PCT levels (102). In this study, median PCT levels were within the normal range in both term and preterm neonates, indicating that PCT was an unreliable biomarker for EOS.

BA have attracted interest as potential biomarkers not only of liver injury, but also of intestinal or infectious disorders (103,104). That BA levels rise in septic adults due to a cholestatic effect of cytokines is well known (105). We aimed to examine the influence of sepsis, in our case EOS, and of an excess amount of cytokines on BA metabolism in the very young infant. Total BA levels and tBA levels in term neonates suffering from EOS were significantly lower than in age-matched controls. aBA concentrations did not differ between these two groups. Given that the liver plays a major role in host defense against bacterial infection and sepsis this might be caused by impaired hepatic BA synthesis, conjugation, or secretion (106). Interestingly, liver and biliary parameters were within the normal range in all patients with EOS and no correlation was observed between BA and liver parameters arguing against liver damage as underlying cause for lower BA levels. Furthermore, when evaluated in multivariate regression analysis with other inflammation parameters, serum BA levels were identified as an independent factor associated with EOS which emphasizes the role of serum

BA measurements in this patient group. Also of interest is that our study results are not in agreement with the behavior of BA levels in septic adults; these increase (sepsis-induced cholestasis) (107). BA levels in our patients were measured early in sepsis, whereas BA levels in septic adults may be measured only when sepsis is advanced, with frank icterus among its manifestations. Lipopolysaccharide, a substance found in the walls of bacteria, can affect BA metabolism: Cholesterol-7-alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in BA synthesis, is repressed in C57BL/6 mice and Wistar rats at the transcriptional level by lipopolysaccharide treatment (108). Under normal conditions, hydrophobic BA repress CYP7A1 transcription via binding to the farnesoid X receptor (FXR) and interaction with the BA response element II in the CYP7A1 promoter. Bacteria-induced hepatobiliary alterations during sepsis, thus may share pathways that mediate negative feedback regulation of CYP7A1 by BA, including those involving FXR (108-110). I hypothesize that this is the predominant mechanism at an early stage of sepsis, resulting in lower BA levels as seen in neonatal EOS. However, with progressing inflammation a severe cholestasis might shift this balance to increased BA levels, as reported in adult sepsis. This hypothesis needs to be tested in a longitudinal study, but positive results may highlight the importance of BA as an early biomarker for sepsis preventing cholestasis and liver injury.

Lastly, tBA, but not total BA levels, were significantly lower in preterm neonates with EOS than in preterm controls. The cause is found within the BA profile. In EOS term neonates, aBA levels are constant; however, the BA profile has changed. TOMCA was significantly increased, whereas all other aBA decreased below the detection limit, except GBMCA and TAMCA, which were found only in small amounts. The same held true in preterm neonates. TAMCA, the only aBA in healthy preterm neonates, significantly decreased resulting in a more

miscellaneous BA profile with TOMCA as the clearly predominating aBA species. Hence, TOMCA may represent an important BA in EOS. In the total BA profile, we see clearly that in both term and preterm neonates TOMCA is one of the major BA; it is fourth most abundant (replacing GCDCA). This results in an overall shift towards a higher fraction of aBA, while total BA remain constant. Our results let us speculate that EOS may alter BA homeostasis: during fetal development, the synthesis of aBA is high, decreasing as maturation progresses and reaching low levels in healthy adults. Studies in rats showed that OMCA might be a result of the 6-isomerase conversion of BMCA, a primary BA, and that OMCA might therefore be a secondary BA (54). Other authors suggest that rat hepatocytes contain a 7-β-hydroxylase that can convert HDCA, a bacterial metabolite and hence a secondary BA, into OMCA, which might then be termed a 'tertiary' BA (54). Our results let us speculate that EOS may enhance the conversion of either BMCA or HDCA into TOMCA. However, these assumptions await further investigation in future studies.

As a last point, I want to discuss the limitations of this study. Firstly, it should be noted that total BA levels of our study cohort were compared with total BA levels measured in healthy adults by Tagliacozzi *et al.* (97). Tagliacozzi *et al.* did not include aBA in their results; but since aBA are not present in healthy adults, this limitation might be trivial. Secondly, feeding status could be determined in only a subgroup of participants. Since there are visible differences in BA levels, but not in the BA profile, the feeding status of the neonates must be taken into account when interpreting BA levels. Thirdly, aBA and in turn total BA could also be determined in only a subgroup of participants due to sample restriction. Hence, sample volume should be calculated generously. Fourthly, methods limitations did not allow detection of aBA at concentrations below 0.012 μmol/L. This was a problem especially in preterm neonates,

where TAMCA was the only measurable aBA. However, BA species present in concentrations below 0.012 µmol/L may not be clinically important. Fifthly, only tri-hydroxylated BA were measured in this study, but others' work has also revealed high levels of tetra-hydroxylated and ketonic BA in neonates. Unfortunately, tetra-hydroxylated and ketonic BA could not be determined by the technology employed in this study. As a final limitation I must emphasize that until now there is no clear evidence that BA may act as an early marker for EOS with potential clinical value. However, future studies may allow defining an exact cut-off value for BA levels in EOS using receiver operating characteristic (ROC) curve analysis for medical diagnostic test evaluation.

5. CONCLUSION

This is the first study to determine standard value ranges of tBA and aBA in neonates. Firstly, we propose that “atypical” BA are not atypical in early infancy, and that to find them may not indicate metabolic disease. The physiological and pathological importance of these BA awaits elucidation. Secondly, in contrast to adults with sepsis, neonates suffering from EOS exhibit lower total BA values than do controls of the same gestational age. Additionally TOMCA was found to be significantly higher in neonates with EOS. Hence, we suggest serum BA in general – and TOMCA in specific - as a candidate for an EOS biomarker, which needs to be further evaluated in future studies.

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7. APPENDIX

Overview of all median BA levels: total BA, tBA and aBA of all studied groups [term (FT) controls – fed and fasting, preterm (PT) controls, EOS FT and EOS PT].

	n		Total BA [µmol/L]	
	all	**f / m**	**Median**	**IQR**
FT controls	47	27 / 20	8.7	5.0 – 13.5
FT controls (fed)	30	13 / 17	11.0	6.4 – 17.1
FT controls (fasting)	17	13 / 4	6.0	4.4 – 10.7
FT controls (breast milk)	37	18 / 19	9.1	5.3 – 14.2
FT controls (formula milk)	10	6 / 4	8.3	4.6 – 12.2
PT controls	22	8 / 14	11.2	5.6 – 16.9
EOS FT	20	5 / 15	6.2	3.8 – 8.6
EOS PT	13	3 / 10	7.4	4.0 – 10.0

	n		tBA [µmol/L]	
	all	**f / m**	**Median**	**IQR**
FT controls	84	47 / 37	8.0	4.6 – 12.9
FT controls	47	27 / 20	7.8	5.0 – 13.0
FT controls (fed)	30	13 / 17	10.1	6.2 – 15.5
FT controls (fasting)	17	13 / 4	5.8	4.3 – 7.9
FT controls (breast milk)	37	18 / 19	7.9	4.8 – 13.5
FT controls (formula milk)	10	6 / 4	7.3	4.5 – 11.2
PT controls	101	47 / 54	10.1	5.7 – 15.7
EOS FT	35	14 / 21	4.7*	2.7 – 7.6
EOS PT	16	3 / 13	6.4	3.5 – 8.4

	n		aBA [µmol/L]	
	all	**f / m**	**Median**	**IQR**
FT controls	47	27 / 20	0.5	0.3 – 1.3
FT controls (fed)	30	13 / 17	0.6	0.4 – 1.3
FT controls (fasting)	17	13 / 4	0.5	0.2 – 1.2
FT controls (breast milk)	37	18 / 19	0.5	0.3 – 1.2
FT controls (formula milk)	10	6 / 4	0.5	0.2 – 1.4
PT controls	22	8 / 14	0.0	0.0 – 0.2
EOS FT	20	5 / 15	0.6	0.1 – 1.6
EOS PT	13	3 / 10	0.6	0.2 – 1.5

DATA SHEET FOR BASELINE CHARACTERISTICS IN HEALTHY TERM NEONATES

Datum: **Uhrzeit**:

1. **Patientennummer**: _____
2. **Alter:** _____ Tage
3. **Ernährung**: □ Stillen □ Formula
   (_____)
4. **Letzte Mahlzeit**: _____
5. **Schwangerschaftsdiabetes**: □ ja □ nein
6. **Schwangerschaftscholestase**: □ ja □ nein
7. **HELLP** □ ja □ nein (_____)

8. **Geburt (aus MKP)**:
   a. Schwangerschaftswoche _____ Woche
   b. Geburtsart □ Sectio □ spontan
   c. Geburtseinleitung □ ja □ nein (_____)
   d. APGAR _____
   e. Geburtsgewicht _____
   f. Geburtsgröße _____
   g. ph-Wert _____
   h. Medikamente: UrsoFalk □ ja □ nein (_____)

PARENTAL CONSENT FOR BLOOD SAMPLING IN HEALTHY TERM NEONATES DURING ROUTINE SCREENING FOR PHENYLKETONURIA

UNIVERSITÄTSKLINIK FÜR KINDER-UND JUGENDHEILKUNDE – GRAZ
Vorstand: Univ.-Prof. Ch. Urban
Klinische Abteilung für Allgemeine Pädiatrie, Leiter: Univ.-Prof. Dr. W. Muntean
Gastroenterologie, Hepatologie und Ernährung; Oberarzt Dr. J. Jahnel
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Bestimmung von „Normwerten" der Gallensäuren im Serum bei Neugeborenen:
Elterninformation und Einverständniserklärung
zur Teilnahme an der klinischen Studie

Sehr geehrte Eltern!

Bei Ihrem Neugeborenen ist in den ersten Lebenstagen ein Stoffwechselscreening (die sog. „PKU-Untersuchung") vorgeschrieben. Dabei werden Bluttropfen aus der Ferse Ihres Neugeborenen entnommen. Am Ende der Blutentnahme erfolgt eine übliche Blutstillung durch Watte. Wir möchten Sie nun fragen, ob wir die Blutstillung mit Hilfe einer Kapillare bzw. eines Filterpapiers durchführen dürfen und die minimale Blutmenge, welches sich in der Kapillare bzw. im Filterpaper befindet, für die Bestimmung von „Normwerten" von Gallensäuren bei Neugeborenen verwenden dürfen?

Ihr Kind wird keinen Nutzen aus der Studie ziehen, aber es gibt auch kein Risiko; Sie helfen uns damit in Zukunft bestimmte Laborwerte besser beurteilen zu können. Die **Teilnahme an dieser Studie erfolgt freiwillig und Ablehnung der Teilnahme hat keine nachteiligen Folgen für die weitere medizinische Betreuung Ihres Kindes.** Beim Umgang mit den Daten werden die Bestimmungen des Datenschutzgesetzes beachtet und alle PatientInnen-Daten werden anonymisiert.

Verantwortliche Ansprechperson für diese Studie: **Oberarzt Dr. J. Jahnel** (0316-385-80039)

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(Datum und Unterschrift der Mutter bzw. Vater des Neugeborenen)

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(Datum, Name und Unterschrift der/des verantwortlichen Ärztin/Arztes)

PROTOCOL FOR BA DETERMINATION BY HPLC-HRMS

Laufmittel:

A: 1000 ml A.dest von Fluka Nr.3953-1L-R BLAU
+ 120 µl Ameisensre
+ 0,38 g Ammoniumacetat
5 Min ultraschallen

LMA: in 1000 ml A.dest. 38 g Ammoniumacetat + 12 ml Ameisensäure lösen=
Stocklösung LMA, davon 10 ml in 1000 ml Aqua dest. geben = Arbeitslösung LMA

B: 1000 ml Methanol (MeOH) von Sigma-Aldrich NR.34885-1L-R GELB
+ 120 µl Ameisensre
+ 0,38 g Ammoniumacetat
5 Min ultraschallen

LMB: in 1000 ml MeOH. 38 g Ammoniumacetat + 12 ml Ameisensäure lösen=
Stocklösung LMB, davon 10 ml in 1000 ml LMB geben = Arbeitslösung LMB

Ansatz:

10 µl Serum
100 µl Standard 1 : 10 verdünnt
vortexen
400 µl Acetonitril
Vortexen
Zentrifugieren 12 Min 3200 U/Min
Überstand abheben und abblasen
In 100 µl Laufmittel B aufnehmen

Säule: ZORBAX Extend-C18 P.N. 735700-902 Fa Agilent,2,1 x 50 mm 3-5-Micron

Vor dem Starten der Proben:

- Unter Instr.Method klicken – File open –Accela –Accela 1250 Pump - open File – Direct Control – Fenster rechts unten geht auf – *Take pump under control* anklicken – Flow:1000 µl A 60 und B 40
- Flügelschraube öffnen Start mit Dreieck aktivieren, einige Minuten purgen, dann Stop mit Quadratzeichen und Fenster schließen. SCHRAUBE ZUDREHEN!

Zum Starten der Proben:

- Xcalibur öffnen –Fenster mit 6 Symbolen erscheint – Sequence Setup aufmachen: File – open – Local DiskD **Pfad**: **Data Fauler\Gallensäuren\data\data\EK** - open. .
- Die letzte leere Zeile anklicken, unter **File Name** Leerwert (LW) 1 hineinschreiben, mit ENTER öffnet sich das nächste Feld ,in den einzelnen Feldern kann man mit **F2** Änderungen vornehmen.
- Einen 2. Lw starten.
- Oder: **Anzahl** der Proben, die ich starten möchte in der Tabelle anklicken, mit strg C und strg V kopieren. Probennummern und Vialpositionen überschreiben.
- Als 3.Position eine beliebige KO
- Anschließend die Nummern der Proben, und zwar die lange Nummer (Anforderungsnummer) eintragen.
- **Spülen der Säule nach 10 Proben**.
- **Instr.Method: D/DataFauler/Gallensäuren\Methods/GS_FSneg_optimierte Methode(_Maus)**
- **Proc.Method: D/DataFauler/Gallensäuren\Methods/GS**
- **Sample Type:** Unknown für Proben, Blank für LW
- **Injection Vol**:10 µl
- **Path:**D:\Data Fauler\Gallensäuren\data\EK_xx_xx_xxxx (Datum) mit dem Datum abspeichern
- Zum Schluss wieder 2 LW eingeben.
- Die Sequence! (EK_xx_xx_xxxx save)
- auf der Taskleiste **RUN SEQUENCE** anklicken um die Proben zu starten.

Während der Messung:

- Auf der linken Seite unter *Aquisition Queue* sieht man die zu messenden Proben
- Unter Status kann man den Status des Autosamplers (Accela open AS),
- der Pumpe (Accela 1250 Pump),
- der Orbitrap (Q-Exactive – Orbitrap MS),
- und des Säulenofens (Mag lab Mistra Switch) verfolgen.
- Lauf startet, wenn alle 4 ready sind.
- Bei Real Time Plot View kann man die Laufzeit anschauen.

Auswertung:

- In Sequence Setup Sequence, in der die Proben gelaufen sind, öffnen.
- Alle zu quantifizierenden Proben markieren Edit copy – oder strg C – EK_xx_xx_xxxxquan öffnen.
- Letzte Zeile in der EK_xx_xx_xxxxquan anklicken, mit – Edit paste - oder strg V die Proben in diese EK hineinspeichern.
- **Speichern** mit dem jeweiligem Datum.
- Nur diese übertragenen Proben markieren und mit Summenzeichen (auf der Taskleiste) rechnen lassen.
- **Warten bis Rechenvorgang beendet ist, Symbol mit Rechenblättern verschwindet.**
- Im Quan Browser richtigen File z.B. EK_11_11_2013quan als sld-File öffnen.
- **ALL** anklicken.
- Zuerst die 4 internen Standards anschauen, ob 1. die Zeit stimmt u. 2. der Peak richtig integriert ist, dazu zuerst den 1.Eichpunkt (EK1_01) anklicken, Zeit merken.

- Anschließend alle Proben kontrollieren.
- Speichern als xqn – File unter dem jeweiligem Datum.
- Während der Auswertung immer wieder speichern
- SAVE ALL
- SAVE AS
- Nach der Auswertung: File - Export data to Excel – Eport short Excel report.
- Excel Report - save as – Desktop – save

Eichkurve:

Von jeder Gallensäure eine 2 mmol/L Lösung herstellen

Gallensäure	Signatur	Molekular-gewicht [g/mol]	mg/10 ml MeOH
Cholsäure	Sigma 084KO784 C1129	408 6	8,17
Glycholsäure	Sigma 41K5304 G-7132	487,2	9,74
Taurocholsäure	Sigma 102H5011 T-4009	536,1	10, 72
Deoxycholsäure	Sigma 074K0062 D2510	391,6	7,83
Glycodeoxycholsäure	Steraloids Batch:7595 Ref.:131011	471,6	9,41
Taurodeoxycholsäure	Aldrich Lot. 05811KB 287245	520,3	10,41
Chenodeoxycholsäure	Sigma Lot:094KO975 C9377	392,2	7,84
Glycochenodeoxycholsäure	Sigma: Lot: 121K5317 G-0759	471,2	9,42
Taurochenodeoxycholsäure	Sigma Lot: 110K5009	521,3	10,43
Lithocholsäure	Sigma: Lot: 053K1682 L6250	376,2	7,52
Glycolithocholsäure	Steroloids Batch. G801 Ref.:131011	455,2	9,1
Taurolithocholsäure	Steraloids. Batch H255 Ref.:131011	505,07	10,11
Ursodeoxycholsäure	Sigma Lot: 074K1080 U5127	392,2	7,84
Glycoursodeoxycholsäure	Steraloids Batch: H894 Ref.: 131835	449,6	8,99
Tauroursodeoxycholsäure	Steraloids Batch: L1655 Ref.:131011	521,3	10,42

Die Konzentration der Standards beträgt 2 mmol/l = Stammlösung

Verdünnung für die Eichkurve:

Je 50 µl der Stammlösung + 250 µl MeOH entspricht dem 1. Eichkurvenkonzentration =
EK1= 100 nmol/ml

In weiteren 12 Röhrchen 500 µl MeOH vorlegen,

EK2: 500 µl MeOH + 500 µl EK1 = 50 nmol/ml
EK3: 500 µl MeOH + 500 µl EK2 = 25 nmol/ml
EK4: 500 µl MeOH + 500 µl EK3 = 12,5 nmol/ml
EK5: 500 µl MeOH + 500 µl EK4 = 6,25 nmol/ml
EK6: 500 µl MeOH + 500 µl EK5 = 3,125 nmol/ml
EK7: 500 µl MeOH + 500 µl EK6 = 1,5625 nmol/ml
EK8: 500 µl MeOH + 500 µl EK7 = 0,7812 nmol/ml
EK9: 500 µl MeOH + 500 µl EK8 = 0,3906 nmol/ml
EK10: 500 µl MeOH + 500 µl EK9 = 0,1953 nmol/ml
EK11: 500 µl MeOH + 500 µl EK10 = 0,0976 nmol/ml
EK12: 500 µl MeOH + 500 µl EK11 = 0,0488 nmol/ml
EK13: 500 µl MeOH + 500 µl EK12 = 0,0244 nmol/ml

Ansatz - Eichkurve:

10 µl 3 % BSA +
10 µl Standard (EK1 bis EK13) +
100 µ Interner Standard 0,2 nmol/100µl
Vortexen +
400 ml Acetonotril
Vortexen
10 Min bei 3000 U/Min (Zentrifuge: Thermo Megafuge 16R)
Überstand abheben und unter Stickstoff abblasen
Resuspendieren mit 100 ml Laufmittel B und in Vials überführen,
falls die Lösung nicht klar ist, Vials in einem Eppendorfhütchen bei 13000 U/Min
3 Min in der Eppendorfzentrifuge 5415 D zentrifugieren, Überstand muß klar sein!!

Interne Standards:

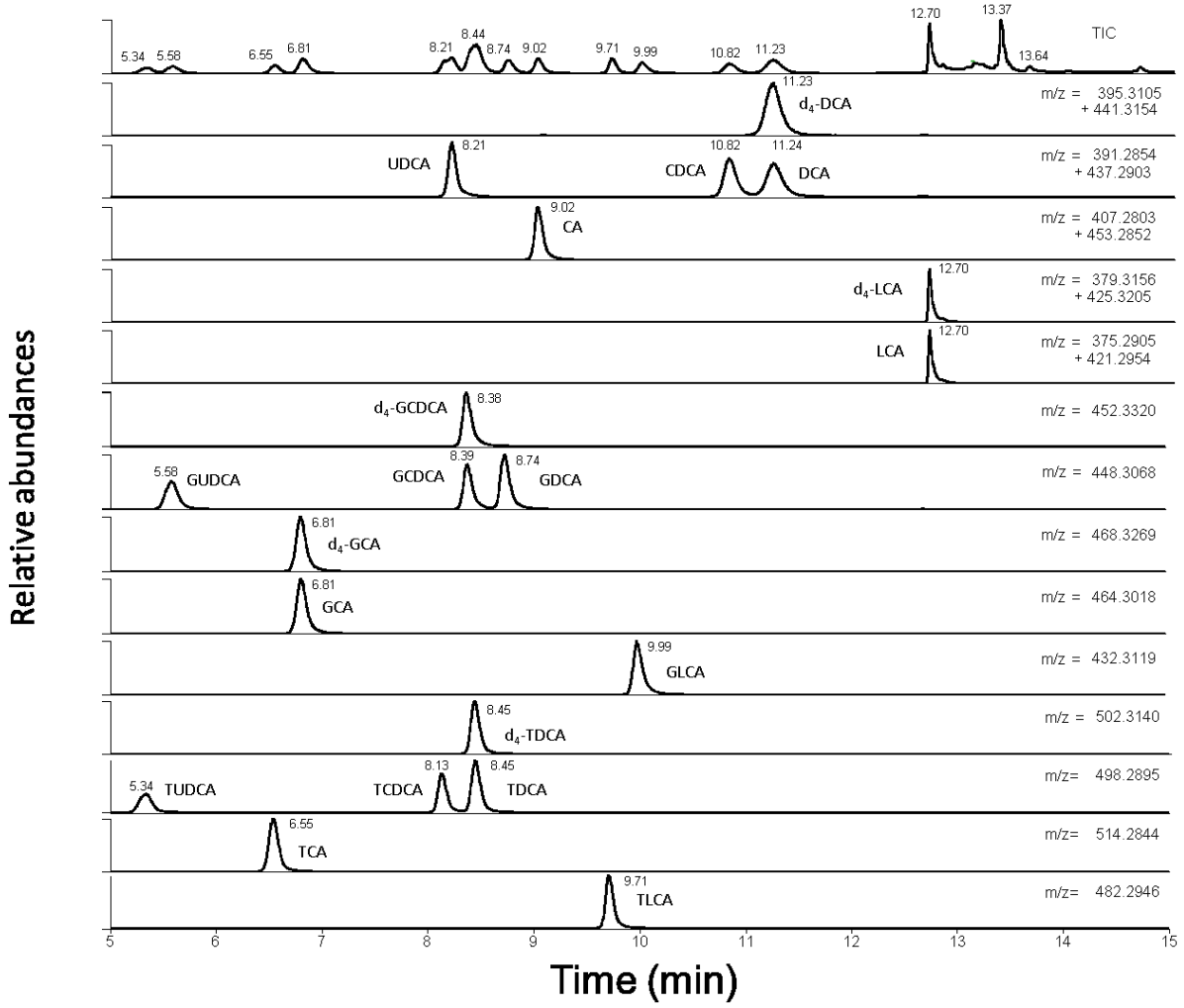
1 bis 4: Stammlösung ist 2 mmol/l in MeOH
5 (Taurodeoxycholsre D4): 412 nmol/ ml

1) Glycochenodeoxycholsäure-d4	CDN Isotopes	Lot: G317P3 D-5673	9,07 mg/10 ml MeOH
2) Glycocholsäure-d4	CDN Isotopes	Lot: U500BP3 D-3878	9,39 mg/10ml MeOH
3) Deoxycholsäure-d4	CDN Isotopes	Lot: J204P20 D-2941	7,93 mg/10ml MeOH
4) Lithocholsäure-d4	CDN Isotopes	Lot:U501P12 D-3742	7,61 mg/10 ml MeOH
5) Taurodeoxycholsäure-d4	hausintern synthetisiert		

Von den ersten 4 Internen Standards je **100** µl, von der Taurodeoxycholsäure-d4 **485** µl auf 10 ml MeOH verdünnen = 2 nmol/100µl → diese Verdünnung 1:10 mit Methanol weiterverdünnen =
GEBRAUCHSLÖSUNG für Orbitrap = 0,2 nmol / 100µl

Auswertung: Chromatogramm – typische und atypische Gallensäuren

1) Chromatogramm von typischen Gallensäuren



2) Chromatogramm von atypischen Gallensäuren