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CHOLESTASIS IN INFANTS AT RISK

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CHOLESTASIS IN INFANTS AT RISK

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Jonas Teng

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POPULAR SCIENCE SUMMARY OF THE THESIS

BACKGROUND

Cholestasis is a condition when bile from the liver is not adequately excreted to the gut. Bile is crucial for the digestion and uptake of dietary fats and for excretion of waste products, such as bilirubin. Bilirubin is the yellow pigment produced when red blood cells are degraded and replaced by new ones. At first, an unconjugated variant of bilirubin is formed, which can harm the brain. The liver detoxifies it, forming the atoxic conjugated bilirubin.
In cholestasis, bile is retained in the liver and leaks back to the blood, causing a yellow discoloration in the skin and whites of the eyes called jaundice. The conjugated bilirubin is not harmful, but the bile acids, the detergents of bile that are meant to dissolve the dietary fats, are toxic. During cholestasis, they accumulate and damage the liver, but also affect other organs. If the cholestasis is severe, it may be fatal unless a liver transplantation is performed. Cholestasis is rare in term infants, affecting about 1 in 2500. There are multiple, sometimes genetic, causes for cholestasis in these infants.

In preterm infants or neonates with other underlying conditions such as for example hemolytic disease of the fetus and newborn (HDFN) cholestasis is far more common. In preterms, several risk factors for cholestasis have been identified. Low gestational age or birth weight, intestinal malformations or complications and bacterial infections are such risk factors. The role of viruses such as cytomegalovirus (CMV) is less studied. The most important risk factor is prolonged need for intravenous infusion of nutrients, so called parenteral nutrition. The exact mechanism for how parenteral nutrition contributes to cholestasis is not known, but much research suggests that the source of fat used is important.

In HDFN, pregnant mothers have developed antibodies against the red blood cells of the fetus. The antibodies pass across the placenta to the fetus and cause destruction of red blood cells, a process called hemolysis. The fetus may need intrauterine blood transfusions to survive. It takes months for the antibodies to be degraded, and the process continues after birth. Many neonates with HDFN rapidly develop jaundice with high levels of toxic unconjugated bilirubin that need treatment to avoid brain damage. It has been known for a long time from case reports, that some of these infants also develop cholestasis with high levels of conjugated bilirubin, but actual research studies are very few.

AIMS

This thesis focuses on cholestasis in these infants at risk, the preterm infants and infants with HDFN. We aimed to investigate the incidence of cholestasis and to establish the most important risk factors. In preterms, we also aimed to evaluate the role of two different parenteral fat sources and the role of CMV in the development of cholestasis. We also aimed at investigating the long-term liver outcome in preterm infants and in those with HDFN.

METHODS AND RESULTS

In paper I and II, we retrospectively investigated the entire population of very preterm infants born before 30 weeks of gestation in the Stockholm region during two two-year periods for cholestasis. A soy oil-based parenteral fat was used in the first era and a predominantly olive oil-based in the second. We compared the cholestatic infants to non-cholestatic controls. As many as 1 out of 7 preterm infants developed cholestasis. This figure did not differ greatly between the 2 different eras, but patients in the second era seemed to be at a slight advantage. The incidence was highest among the most premature infants.
Prolonged parenteral nutrition and the intestinal complication of necrotizing enterocolitis were the strongest risk factors. Cholestatic infants had worse outcomes; they had more retinopathy of prematurity and bronchopulmonary dysplasia even after adjusting for other risk factors. Mortality was 14% in cholestatic infants, five times higher than among the non-cholestatic. Those with severe cholestasis had a particularly high mortality. The survivors all recovered from cholestasis, and none had significant liver disease at ten years of age.

In paper III, cholestatic preterm infants born before 37 weeks of gestation were tested for CMV DNA in white blood cells, blood plasma and urine and compared to non-cholestatic controls tested at the same age. CMV DNA was detected in 69% of cholestatic infants versus 13% of non-cholestatic, and the difference was not explained by the risk factors mentioned above. In cholestatic infants, mortality was higher if they also were CMV DNA positive.

All infants with HDFN in the region of Stockholm during a twelve-year period were examined for cholestasis in paper IV. 7% (11/149) were cholestatic and compared to the ones who were non-cholestatic. Having received intrauterine blood transfusions and presence of multiple maternal antibodies including at least one of those known to be associated with a more severe HDFN, the D-, c- or K-antibodies, were the most important risk factors. Onset of cholestasis was very early and often present at birth. Cholestasis was severe in the majority of cases, but resolved in all. No liver disease was evident at two years of age.

CONCLUSIONS

Cholestasis is more than 300 times more common in very preterm infants, and almost 200 times more common in infants with HDFN than in term infants in general. Preterms at extra high risk for cholestasis are the most premature ones, those developing necrotizing enterocolitis and those who need prolonged parenteral nutrition.
CMV may play a role in development of cholestasis in preterms, and further investigation is needed on this subject.
Cholestasis can be severe in both preterms and infants with HDFN. It may be associated with worse outcome and increased mortality in preterms. If surviving, the liver prognosis is good in both preterms and infants with HDFN, and other underlying liver disease in these infants presenting as transient cholestasis as a neonate is improbable.
We recommend routines for screening for cholestasis in both preterms on parenteral nutrition and in infants with HDFN for early detection to ensure correct management.

POPULÄRVETENSKAPLIG SAMMANFATTNING

BAKGRUND

Kolestas (gallstas) är ett tillstånd med otillräcklig utsöndring av galla från levern till tarmen. Gallan behövs för upptaget av dietärt fett samt för utsöndring av restprodukter, som bilirubin. Bilirubin är det gula pigmentämne som bildas när röda blodkroppar bryts ner. Först bildas en okonjugerad variant av bilirubin, som kan skada hjärnan. Levern omvandlar det okonjugerade bilirubinet till konjugerat bilirubin som är atoxiskt.
Vid kolestas ansamlas gallan i levern och läcker även tillbaka till blodet. Bilirubinet orsakar då en gul missfärgning i huden och ögonvitorna som kallas gulsot. Det konjugerade bilirubinet är inte skadligt, men gallsyrorna, gallans fettlösningsmedel, är toxiska. Vid kolestas ansamlas gallsyrorna i levern och orsakar skada, men påverkar också andra organ. Om kolestasen är mycket allvarlig kan den leda till dödsfall om inte levertransplantation utförs. Kolestas är sällsynt hos fullgångna nyfödda och drabbar cirka 1 av 2500. Det finns ett flertal, ibland genetiska, orsaker till kolestas hos dessa barn.

Hos för tidigt födda barn, eller nyfödda med andra underliggande tillstånd som till exempel hemolytisk sjukdom till följd av graviditetsimmunisering (hemolytic disease of the fetus and newborn, HDFN) är kolestas betydligt vanligare. För prematurer har flera riskfaktorer för kolestas identifierats. Låg gestationsålder eller födelsevikt, tarmmissbildningar eller tarmkomplikationer och bakterieinfektioner är sådana riskfaktorer. Betydelsen av virus, som till exempel cytomegalovirus (CMV), är mindre studerad. Den viktigaste riskfaktorn är dock ett långvarigt behov av intravenös näringstillförsel, så kallad parenteral nutrition. Den exakta mekanismen för hur parenteral nutrition bidrar till kolestas är inte känd, men mycket forskning tyder på att typen fett som används i näringsinfusionen har betydelse.

Vid HDFN har gravida mödrar utvecklat antikroppar mot fostrets röda blodkroppar. Antikropparna passerar över moderkakan och via navelsträngen till fostret och orsakar ökad nedbrytning av röda blodkroppar, vilket kallas hemolys. Fostret kan då behöva intrauterina blodtransfusioner för att överleva. Det tar månader innan antikropparna bryts ned, och processen fortsätter därför efter födseln. Många nyfödda med HDFN utvecklar snabbt gulsot med höga halter av giftigt okonjugerat bilirubin och behöver behandling för att undvika hjärnskador. Från fallrapporter har det har varit känt länge att vissa också utvecklar kolestas med höga nivåer av konjugerat bilirubin, men antalet forskningsstudier är mycket få.

SYFTE

Denna avhandling fokuserar på kolestas hos dessa barn med förhöjd risk, för tidigt födda barn och nyfödda med HDFN. Vi ville undersöka förekomsten av kolestas och fastställa de viktigaste riskfaktorerna i dessa grupper. Vi ämnade också utvärdera betydelsen av CMV, samt två olika parenterala fettsorter för utvecklingen av kolestas hos prematurer. Ytterligare ett mål var att undersöka det långsiktiga leverutfallet hos dessa barn som haft kolestas som nyfödda.

METOD OCH RESULTAT

I studie I och II undersökte vi retrospektivt under två tvåårsperioder alla barn födda före graviditetsvecka 30 för kolestas. En sojabaserad parenteral fettlösning användes på neonatalavdelningarna under den första tidsperioden och en huvudsakligen olivoljebaserad under den andra. Vi jämförde kolestatiska prematurer med icke-kolestatiska kontroller. 1 av 7 utvecklade kolestas. Det var ingen tydlig skillnad i kolestasförekomst mellan tidsperioderna, möjligen var risken något lägre då olivoljebaserat fett användes. Förekomsten var högst hos de mest för tidigt födda. Långvarig parenteral nutrition och tarmkomplikationen nekrotiserande enterokolit var de viktigaste riskfaktorerna.
Kolestatiska barn hade sämre utfall. De hade mer prematuritetsretinopati och bronkopulmonell dysplasi, även efter justering för andra riskfaktorer. Dödligheten var 14 % hos kolestatiska prematurer, fem gånger högre än hos icke-kolestatiska. Särskilt hög mortalitet sågs hos de med svår kolestas. De överlevande återhämtade sig från sin kolestas, och ingen betydande leversjukdom sågs vid tio års ålder.

I studie III testades kolestatiska prematurer födda före 37 graviditetsveckor för CMV DNA i vita blodkroppar, blodplasma och urin och jämfördes med icke-kolestatiska kontroller testade vid samma ålder. CMV DNA detekterades hos 69 % av de kolestatiska prematurerna och hos 13 % av de icke-kolestatiska. Skillnaden förklarades inte av de riskfaktorer som nämnts ovan. Hos kolestatiska spädbarn var dödligheten högre om de också var positiva för CMV DNA.

Alla nyfödda med HDFN i Stockholmsregionen under en tolvårsperiod undersöktes för kolestas i studie IV, och 7 % (11/149) hade drabbats. De kolestatiska jämfördes sedan med de icke-kolestatiska. Att ha fått intrauterina blodtransfusioner samt förekomst av flera maternella antikroppar, där minst en utgjordes av D-, c- eller K-antikroppar som är kända för att orsaka mer allvarlig HDFN, var de viktigaste riskfaktorerna. Kolestasen debuterade mycket tidigt och fanns ofta redan vid födseln. Majoriteten hade uttalad kolestas, men den var övergående hos alla. Ingen betydande leversjukdom kunde ses vid minst två års ålder.

SLUTSATSER

Kolestas är mer än 300 gånger så vanligt hos mycket för tidigt födda barn och nästan 200 gånger så vanligt hos barn med HDFN än hos nyfödda i allmänhet. Prematurer med extra hög risk för kolestas är de mest för tidigt födda, de som utvecklar nekrotiserande enterokolit och de som behöver långvarig parenteral nutrition. CMV kan ha betydelse för utvecklingen av kolestas hos prematurer, och ytterligare forskning behövs för att klarlägga detta samband.
Kolestasen kan vara allvarlig hos både de för tidigt födda och de med HDFN. Den kan vara förknippad med sämre utfall och ökad mortalitet hos prematurer. Hos de som överlever är leverprognosen bra hos både prematurer och barn med HDFN. Att andra underliggande leversjukdomar skulle finnas hos dessa spädbarn, som skulle debutera som en övergående episod av kolestas som nyfödd, är osannolikt.
Vi rekommenderar att rutiner för screening finns för att upptäcka kolestas både hos mycket för tidigt födda som får parenteral nutrition och nyfödda med HDFN för tidig upptäckt för att säkerställa rätt behandling.

ABSTRACT

Cholestasis is a condition when bile flow from the liver to the intestines is impaired. Unresolved, it may lead to liver transplantation or death. Overall, it affects 1 out of 2500 term infants, but is far more common in high-risk infants with other conditions such as prematurity and hemolytic disease of the fetus and newborn (HDFN). In preterms and other sick infants in the neonatal intensive care unit (NICU), cholestasis is associated with prolonged parenteral nutrition (PN), where the source of lipids used in PN has been implied in the pathogenesis. Bacterial infection is also a risk factor, but the role of hepatotropic viruses, such as cytomegalovirus (CMV), is less well studied. In infants with HDFN and cholestasis, very few studies are published. Reports on short- and long-term outcomes of cholestasis in specific infant groups at risk are scarce. The aim of this thesis was to study incidence, risk factors, course and outcomes of cholestasis in preterm infants in the NICU and in infants with HDFN.

In two population-based retrospective case-control studies, we included cholestatic very preterm infants (gestational age < 30 weeks) in the Stockholm region during two two-year periods in 2006-2008 and 2010-2011. In paper I, we found an incidence of cholestasis of 14.8% (37/250) in 2006-2008 when a soy-based lipid emulsion was used, and 12.7% (34/268) in 2010-2011 when an olive-based one was used (p=0.52). In a multivariable model we found that the olive-based lipid emulsion might carry a slightly reduced risk for cholestasis when adjusting for the risk factors necrotizing enterocolitis and PN duration.
In paper II, outcomes of cholestasis were evaluated in the cohort born 2006-2008. Cholestatic infants had higher rates of bronchopulmonary dysplasia (BPD) and retinopathy of prematurity (ROP) than controls, and mortality was 13.5% versus 2.7% (p=0.040). No significant long-term liver disease was found in those surviving the neonatal period.
In paper III, we prospectively analyzed CMV DNA in blood and urine samples from cholestatic preterm infants (<37 weeks) in two NICUs in 2008-2010. Non-cholestatic controls were sampled in 2015-2017 at a similar age. In 69 % (31/45) of cholestatic infants, CMV DNA was detected in at least one sample, versus 13% (3/24) in non-cholestatic controls (p<0.00001). Mortality was 26% among CMV positive cholestatic infants, whereas none of the CMV negative cholestatic infants died (p=0.044).
In paper IV, a retrospective population-based cohort study, we evaluated the incidence of cholestasis in infants with HDFN in the Stockholm region in 2004-2015. Cholestasis was found in 7% (11/149), with an early onset. Intrauterine blood transfusions and multiple maternal antibodies including D-, c- or K-antibodies were risk factors for cholestasis.

We conclude that cholestasis is common in very preterm infants and that switching from soy-based to olive-based parenteral lipid emulsion affects the incidence only marginally. CMV seems associated with cholestasis in preterms and further scientific attention should be encouraged. Cholestasis is common in infants with HDFN, and this warrants screening for early detection and correct management. Cholestasis may be associated with a more severe short-term outcome in preterms, but liver disease later in childhood is uncommon in both preterms and infants with HDFN.

LIST OF SCIENTIFIC PAPERS

I. **Teng J**, Arnell H, Bohlin K, Nemeth A, Fischler B. Impact of parenteral fat composition on cholestasis in preterm infants.
Final Peer-reviewed manuscript version.
The published article is available in *J Pediatr Gastroenterol Nutr.* 2015;60(6):702-7.

II. **Teng J**, Bohlin K, Nemeth A, Fischler B. Cholestasis after very preterm birth was associated with adverse neonatal outcomes but no significant long-term liver disease: A population-based study. *Acta Paediatr.* 2021;110(1):141-8.

III. **Teng J**, Elwin A*, Omarsdottir S*, Aquilano G, Vanpee M, Nemeth A, Rahbar A, Bohlin K, Fischler B[#], Söderberg-Nauclér C[#]. Cytomegalovirus and neonatal cholestasis in preterm infants. *Manuscript, submitted*.

IV. **Teng J**, Wickman L, Reilly M, Nemeth A, Fischler B, Bohlin K*, Tiblad E*. Population-based incidence and risk factors of cholestasis in hemolytic disease of the fetus and newborn. *Manuscript, submitted*.

*, # Shared contribution

CONTENTS

LIST OF ABBREVIATIONS

BPD	Bronchopulmonary dysplasia
BSEP	Bile salt export pump
BW	Birth weight
CB	Conjugated bilirubin
CI	Confidence interval
CMV	Cytomegalovirus
CMV-IE	Cytomegalovirus immediate early
CRP	C-reactive protein
CT	Cycle threshold
ELBW	Extremely low birth weight
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
EXPRESS	Extremely Preterm infants in Sweden Study
FXR	Farnesoid X receptor
GA	Gestational age
HbA	Adult hemoglobin
HbF	Fetal hemoglobin
HDFN	Hemolytic disease of the fetus and newborn
HDN	Hemolytic disease of the newborn
IBAT	Intestinal bile acid transporter
IFAC	Intestinal failure-associated cholestasis
IFALD	Intestinal failure-associated liver disease
IUT	Intrauterine erythrocyte transfusion
LBW	Low birth weight
MRP2	Multidrug resistance protein 2
n-3-PUFA	ω-3-polyunsaturated fatty acids
n-6-PUFA	ω-6-polyunsaturated fatty acids
NASPGHAN	North American Society for Pediatric Gastroenterology, Hepatology and Nutrition
NEC	Necrotizing enterocolitis

NICU	Neonatal intensive care unit
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PFIC	Progressive familial intrahepatic cholestasis
PN	Parenteral nutrition
PNAC	Parenteral nutrition-associated cholestasis
PNALD	Parenteral nutrition-associated liver disease
PUFA	Polyunsaturated fatty acids
Rh	Rhesus
ROP	Retinopathy of prematurity
SNQ	Swedish neonatal quality register
TB	Total bilirubin
TPN	Total parenteral nutrition
TPNAC	Total parenteral nutrition-associated cholestasis
UDCA	Ursodeoxycholic acid
UDP-glucuronyltransferase	Uridine 5'-diphospho-glucuronyltransferase
VLBW	Very low birth weight

1 INTRODUCTION

As a resident in pediatrics, when working in the neonatal intensive care unit, I noted that some of the preterm infants developed cholestasis. It was usually the sickest ones who were affected. Those who needed mechanical ventilation and parenteral nutrition for long periods of time due to recurrent sepsis episodes or due to the feared intestinal complication of necrotizing enterocolitis (NEC). Treatment options were few. The pediatric hepatologists were consulted, recommending ursodeoxycholic acid treatment if possible and vitamin K, and always suggesting tapering of parenteral nutrition, which was difficult due to the severe disease. A fatal outcome was not uncommon despite all measures of intensive care.

When discussing possible research projects with my future supervisors, I was intrigued to be able to start a small pilot project, investigating the course of cholestasis in ten extremely preterm infants in relationship to sepsis episodes (1). This was the starting point that led up to this thesis. In Figure 1 below, the course of cholestasis in one of these infants is illustrated. The conjugated bilirubin is slowly increasing during the initial episode of parenteral nutrition due to immaturity, feeding intolerance and sepsis, and then "explodes" after the onset of NEC and a second sepsis episode with the need for another long period of parenteral nutrition.

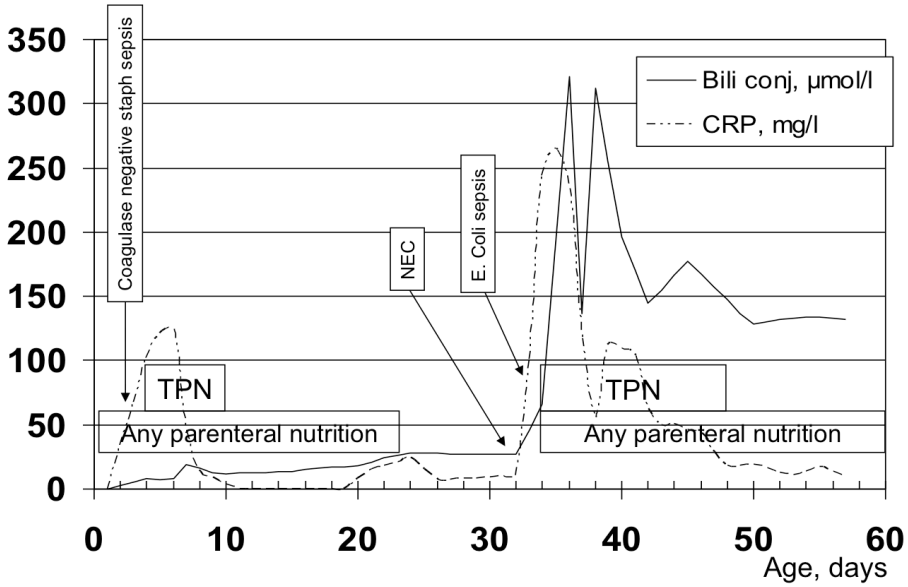

Figure 1. CRP and conjugated bilirubin levels in an extremely preterm infant. Abbreviations: CRP, C-reactive protein; TPN, total parenteral nutrition; NEC, necrotizing enterocolitis.

Another clinical episode that had an impact on me, was when I noted that in one infant we had been treating in the neonatal ward with exchange transfusion and intensive phototherapy due to maternal erythrocyte alloimmunization and hemolysis, the conjugated bilirubin level had increased in the past few days without us noticing. It now constituted almost all of the bilirubin. We stopped the phototherapy, and I was confused. I had never heard of cholestasis in these infants. I searched the web, but found nothing but single case reports.

Later, I was able to learn a lot more about this condition when we initiated the project on cholestasis in infants with hemolytic disease of the fetus and newborn (HDFN).

2 BACKGROUND

2.1 THE LIVER AND BILE FORMATION

The liver is an accessory organ of the intestinal tract that has multiple and crucial functions. It is situated in the upper right quadrant of the abdomen, and consists of two lobes that are subdivided into eight segments each, and each segment contains thousands of the functional units of the liver known as the liver lobules.

Oxygenated blood from the heart arrives in the hepatic artery branches to the liver lobule. The blood containing nutrients from the intestines connects through the portal vein branches and mixes with the arterial blood in the sinusoids where the hepatocytes are lined up. The blood is then returned through the central veins to the hepatic vein and the vena cava.

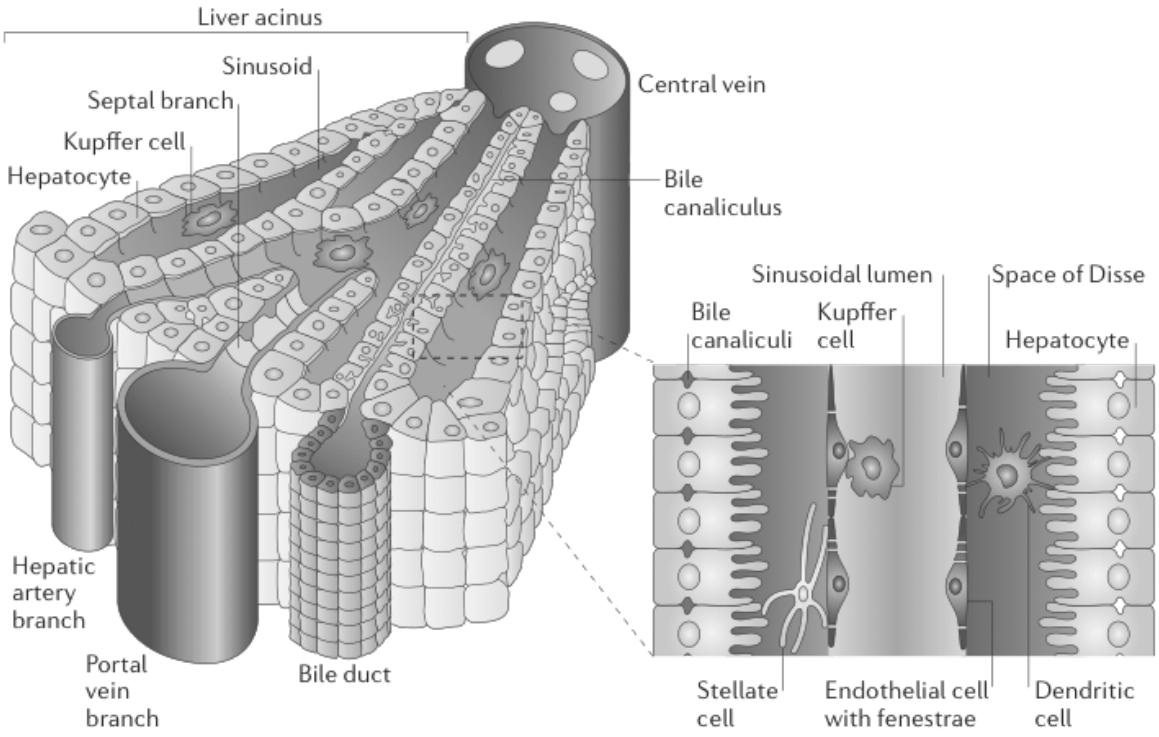

Figure 2. The structure of a liver lobule. From Adams et al, *Nature Reviews Immunology 2006* (2), with permission from Springer Nature.

The formation and excretion of bile is a unique and important function of the liver. Bile is crucial for digestion and excretion of waste products from the metabolism. Bile is formed in the liver cells (hepatocytes) and excreted to the adjacent smallest bile ducts, the bile canaliculi (Figure 2). The bile canaliculi join together to form larger and larger bile ducts like the tributaries of a river to eventually unite in the common bile duct that, via the reservoir of the gallbladder, empties into the duodenum.

The bile has a complex composition. Apart from water and electrolytes it is composed of bile acids, bilirubin, phospholipids and cholesterol (3, 4). Bile acids are important for digestion, while bilirubin and cholesterol are excess waste products to be excreted. The phospholipids in bile are synthesized by the hepatocytes to aid the bile acids to increase the water-solubility of the excreted cholesterol (5).

The principal bile acids cholic acid and chenodeoxycholic acid are synthesized by the hepatocytes from cholesterol and therefore referred to as the primary bile acids (6). The bile salt export pump (BSEP) is essential in transportation of bile acids over the canalicular membrane of the hepatocyte.

The bile acids act as a detergent through the emulsifying properties of the hydrophobic cholesterol-based skeleton and the hydrophilic side chain and are necessary for the adequate uptake of dietary fat and other fat-soluble nutrients such as fat-soluble vitamins (7). The primary bile acids are modified by the gut microbiome into several secondary bile acids (8), of which ursodeoxycholic acid (UDCA) is especially worth mentioning, since its choleretic properties are used therapeutically to increase the bile flow when impaired (9-11).

Reuptake of the bile acids occurs in the enterocytes, primarily in the terminal ileum, mainly through the ileal bile acid transporter (IBAT), and they are then transported through the portal vein back to the liver in what is referred to as the enterohepatic circulation. A common perception is that only about 5 % are excreted in the stools per day (12, 13). The bile acids circulate several times in the enterohepatic circulation before being excreted in the feces (14).

Bile acids also affect gene expression, for example through binding to the nuclear Farnesoid X receptor (FXR), which is present in many cells especially in the digestive organs. In the hepatocyte, stimulation of the FXR upregulates the BSEP on the canalicular membrane through increased gene transcription, and also affects the membrane pumps responsible for, among other compounds, bile acid uptake on the sinusoidal membrane.
Hence, feedback through the FXR affects the uptake and excretion of bile acids in the hepatocyte. This has opened possibilities for novel treatments, such as the FXR agonist obeticholic acid, which is used in adults with primary biliary cholangitis and currently evaluated for biliary atresia in infants (15, 16).

The IBAT in the ileum, the most important pump for reuptake of bile acids from the intestinal lumen, is also regulated through the FXR (17), and is also a target for development of new treatments, such as the IBAT-inhibitor odevixibat (18).

Mutations in the genes coding for important proteins in the bile acid metabolism and enterohepatic circulation can cause cholestatic diseases, as for example progressive familial intrahepatic cholestasis (PFIC), a group of recessively inherited cholestatic diseases with mutations affecting for example the transportation of bile acids over the canalicular membrane of the hepatocyte (19). Biallelic null mutations are associated with severe disease, and less severe mutations are associated with transient neonatal cholestasis or recurrent episodes of cholestasis with full recovery in between (20).

2.1.1 Fetal and neonatal bile acid metabolism

In the intrauterine environment, several functions of the fetal liver are immature and carried by the maternal liver, and the main function liver is erythropoiesis (21, 22). The fetal hepatic circulation differs fundamentally from the situation in extrauterine life. Nutrient and oxygen rich blood arrives to the fetal liver from the maternal circulation via the placenta and the umbilical vein, which obliterates after birth (23, 24).

The fetal bile flow to the intestines is low since nutrition occurs through the placental route and not the fetal intestines. However, the fetal liver does produce bile to some extent, bile acids and bilirubin has been found in fetal gallbladders, amniotic fluid and meconium (24-26). These bile acids and other cholephilic compounds such as bilirubin, however, mainly need to be eliminated from the fetal circulation via the placenta (27, 28).

The bile acid pool of the neonate i reduced in size as compared to adults, and reaches a similar size at almost 2 months of age (29). The serum bile acid levels increases in the first week after birth to higher levels than later in childhood or adulthood. Then, it decreases and reaches the normal levels of childhood and adulthood within the first 4-6 months of life, presumably due to lower reuptake from the hepatocytes in the small infant (30).

All infants, especially neonates, could therefore be perceived as having a condition of physiological cholestasis in infancy. This physiological cholestasis could be argued to make neonates, and perhaps more profoundly preterms, susceptible to developing cholestasis.

2.1.2 Bilirubin metabolism

The bile is an important route for the excretion of excess waste products such as cholesterol and bilirubin. Bilirubin is formed from degradation of heme within phagocytic cells in the spleen, liver (Kupffer cells) and bone marrow. The heme primarily originates from hemoglobin within the erythrocytes. Hemoglobin is the oxygen-carrying molecule of the erythrocytes (red blood cells), which consists of a protein part, the globin chains, and the iron-containing heme.

Within the phagocytic cells, the globin chains are separated from the heme and degraded to amino acids, and the heme is separated from the iron (Figure 3). The iron and amino acids from the globin can be reused. The heme is after removal of iron in several enzymatic steps converted to the yellow-colored unconjugated bilirubin, which is hydrophobic and transported in the serum by albumin to the liver. The unconjugated bilirubin can pass the blood-brain-barrier and is neurotoxic in high levels (31, 32).

The hepatocytes conjugate the bilirubin in two steps with the enzyme bilirubin uridine-5'-diphospho-glucuronyltransferase (UDP-glucuronyltransferase). First, bilirubin monoglucuronide is formed within the hepatocyte, and then bilirubin diglucuronide is produced, which in turn is excreted to the bile canaliculi.
The main transporter for this is the multidrug resistance protein 2 (MRP2). The bilirubin glucoronides, primarily the bilirubin diglucuronide, are referred to as conjugated bilirubin, which is atoxic and can be measured in serum. Mutations in the gene alleles of MRP2 can cause Dubin-Johnson syndrome with elevated levels of conjugated bilirubin in serum, usually without any other signs of liver disease, which demonstrates the lack of toxicity of conjugated bilirubin in itself (33).

In the intestines, the gut microbiome deconjugates the bilirubin and forms urobilinogen, which is then further metabolized to stercobilin and excreted in the feces. The stercobilin gives the feces their typical brown color. Some of the urobilinogen is reuptaken in the enterohepatic circulation, and can be transformed to urobilin and excreted to in the urine, or excreted in the bile once again (Figure 3).

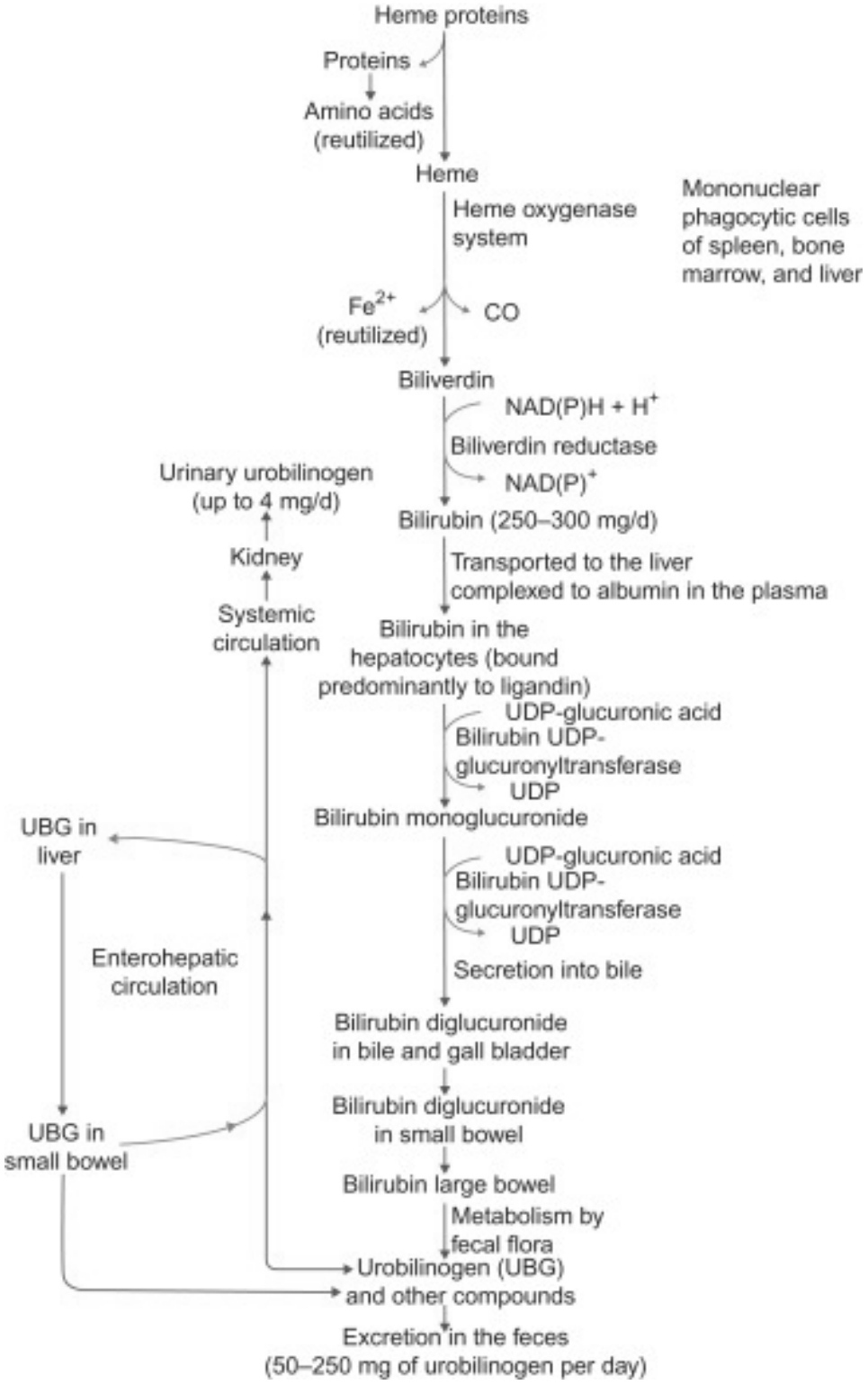

Figure 3. Heme degradation. From Bhagavan NV, Ha C-E. *Essentials of Medical Biochemistry (Second Edition): Elsevier; 2015* (34), with permission from Elsevier.

2.1.3 Jaundice and bilirubin fractions

When bilirubin levels rise in serum, jaundice, a yellowish tone in the skin and sclerae can be seen. Jaundice is a clinical sign that should always warrant further investigation throughout life.

Bilirubin and its fractions can be measured in serum, which is important for the evaluation of the jaundiced patient. Most clinical laboratories nowadays use methods to determine total and conjugated serum bilirubin levels. Earlier, and still in some parts of the world, the diazo method is used, measuring indirect (≈unconjugated) and direct bilirubin. The direct bilirubin includes both the free conjugated bilirubin, but also a covalently albumin bound fraction of bilirubin referred to as γ-bilirubin. This method is thus overestimating the true conjugated bilirubin level, especially if the total bilirubin levels are high (35).
Still, the terms direct bilirubin or direct hyperbilirubinemia are often used, even though conjugated bilirubin has been analyzed and the term conjugated hyperbilirubinemia would be more appropriate.

Bilirubin is measured in μmol/L using the SI-system, but the unit mg/dL is stilled used in many parts of the world. When converting, 17 μmol/L of bilirubin is approximately equal to 1 mg/dL.

2.1.4 Fetal and neonatal bilirubin metabolism

The hemoglobin content of fetal erythrocytes is to 70-90% made up by fetal hemoglobin (HbF), with higher affinity to oxygen than adult hemoglobin (HbA). This enables more efficient retrieval of oxygen from the maternal blood via the placenta (36). The fetal erythrocytes have a shorter life span than after the transition to extrauterine life.
The transition to primarily HbA-containing erythrocytes with HbF <1% is completed at about 6 months of postnatal age (37). From this age, erythrocytes have a life span of approximately 120 days. In the term neonate, the life span of an erythrocyte is about 60-90 days, and in preterm infants only 35-50 days (38, 39).
The level of hemoglobin is higher in the neonate than later in life. It increases from 140-150 g/L on average in extremely preterm infants (<28 weeks of gestational age) to about 180 g/L in the term neonate (40, 41). Shortly after birth, increasing production of HbA-containing erythrocytes begins but the rate of degradation of fetal erythrocytes is higher, and the total hemoglobin level starts to decrease.

The high hemoglobin levels in neonates, erythrocytes with a short life span, and the decrease in total hemoglobin after birth are factors that synergize towards a high neonatal production of bilirubin. Before birth, the maternal liver handles the bilirubin from the fetus, but after clamping of the umbilical cord, the immature neonatal liver with the bilirubin UDP-glucuronyltransferase enzyme system not yet in full use, suddenly has to handle this rapid increase of bilirubin.

The expression of the bilirubin UDP-glucuronyltransferase increases slowly in the fetus, from 0.1% of adult levels in gestational weeks 17-30, to about 1% from 30 weeks to term (42-44). Thus, newborn infants have a low conjugational capacity of bilirubin, especially if born preterm. The majority, between 60-90%, of newborns therefore develop jaundice with predominantly unconjugated hyperbilirubinemia (45).

In most cases the jaundice is referred to as physiologic, a self-limiting and harmless process and a part of the normal adaptation to extrauterine life. In some infants, high levels of unconjugated bilirubin develop which can be neurotoxic, and treatment with phototherapy and exchange blood transfusions may be needed (32, 46). In infants with hemolytic conditions, such as maternal red cell alloimmunization and other hemolytic conditions, as for example hereditary spherocytosis, the production of bilirubin is very high. This significantly increases the risk for unconjugated hyperbilirubinemia in need of treatment (47, 48).

Sometimes, the jaundice seen in the neonatal period is not due to elevation of the unconjugated bilirubin but rather the conjugated bilirubin, which is a sign of decreased bile flow.

2.2 NEONATAL CHOLESTASIS

2.2.1 Pathophysiology

When bile formation or flow is impaired, this is referred to as cholestasis (49). Cholestasis can occur throughout life, and may have several causes. It may, somewhat simplified, be divided into extrahepatic (or obstructive) or intrahepatic (hepatocellular), depending on whether the impairment of bile flow is due to blocking or absence of bile ducts in the former case, or due to decreased formation or intrahepatic transportation in the latter.

When bile flow is impaired, the compounds normally excreted to the bile accumulate within the hepatocytes and liver. The levels of bile acids and cholesterol then increase within the hepatocyte. The bile acids are amphipathic compounds, designed to dissolve nutritional lipids. With increased concentration during cholestasis in the hepatocytes their detergent properties can cause damage to the function of the cell membranes, and cell organelles containing membranes. The may also promote the formation of reactive oxygen species, within the hepatocytes or through the Kupffer cells, which in turn cause inflammation, fibrosis and cell death (50).
Bile acids may also diffuse and be actively pumped by the hepatocyte back to the sinusoids and the blood stream (51). This leads to an increase of bile acids in the systemic circulation, where cell membranes may be damaged in other parts of the body and inflammation promoted (50).

The classical signs of cholestasis are jaundice, pale stools and high-colored urine, although the condition may also present with pruritus, failure to thrive, coagulopathy and bleeding due to vitamin K deficiency, but in the worst case scenario it may also present with liver failure (49, 52).

2.2.2 Definitions of cholestasis

The joint guidelines from the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and North American Society for Pediatric Gastroenterology,

Hepatology and Nutrition (NASPGHAN) from 2004 recommended that a conjugated bilirubin ≥ 1 mg/dL and ≥ 20 % of the total bilirubin level should be regarded as abnormal and warrant further investigation (53). According to the updated guidelines from 2017, merely a conjugated bilirubin ≥ 1 mg/dL in an infant with prolonged jaundice should be regarded as abnormal (49).

Elevated conjugated bilirubin level in serum is the main route for discovery of cholestasis. In the subsequent investigation, elevated serum bile acids should be confirmed; except for in the rare conditions of primary bile acid synthesis defects where serum bile acid levels, as measured by routine methods, remain within normal limits despite cholestasis (49, 54).

2.2.3 Incidence and etiology

Cholestasis is rare in otherwise healthy term infants, and is described to occur in approximately 1 out of 2500 (55-57). There are multiple known causes for cholestasis in term infants, where biliary atresia accounts for a third to a quarter of cases and is the most common along with equally sized group of idiopathic cholestasis (58-62). In Table 1, from the systematic review by Gottesman et al, the most common causes of neonatal cholestasis found in studies from tertiary referral centers for pediatric hepatology, are shown (58)

Table 1. Summary of etiologies of cholestasis in infancy by disease category.

Diagnosis	**% of total**	**Diagnosis**	**% of total**
Idiopathic neonatal Cholestasis	26.0 %	Choledochal cyst	2.1 %
Biliary Atresia	25.9 %	Hypopituitarism/hypothyroidism	2.0 %
Infection [1]	11.5 %	Hemolysis	1.4 %
TPN-associated cholestasis	6.4 %	Inspissated bile syndrome	1.4 %
Metabolic disease	4.4 %	Progressive Familial Intrahepatic Cholestasis	1.0 %
α -1- antitrypsin deficiency	4.1 %	Alagille's syndrome	1.0 %
Perinatal hypoxia/ischemia	3.7 %	Cystic Fibrosis	0.9 %
Interlobular bile duct paucity	2.5 %	Other	5.9 %

Percentages of etiologies in 1692 cases from 17 studies.
[1] Cytomegalovirus accounted for one third of cases, and bacterial sepsis for one fourth.
Adjusted from Gottesman et al, *BMC Pediatrics 2015* (58), with permission from Springer Nature.

In the study from Gottesman et al. referred to in Table 1 above, the oldest included study was from 1976, and also several others from before the turn of the millennium. Since development of diagnostic methods regarding genetic cholestatic disorders has evolved, the figures then reported would probably be different today. In particular, the relative rate for the group regarded as idiopathic would be expected to be lower (63).
Also, even though the meta-analysis by Gottesman et al. did not include studies with exclusion criteria of certain diagnoses, cases of for example Alagille's syndrome was only reported in 5 out of 17 studies. For α-1-antitrypsin deficiency, the proportions reported differed markedly between studies. No cases were reported in 7 studies, while the highest reported proportion was 17.5% among the 10 studies reporting α-1-antitrypsin deficiency cases. Since the prevalence of α-1-antitrypsin deficiency varies across the world, geographic skewness in the included studies might be of importance.
To conclude, these absolute percentage figures should be interpreted with caution, but provide a rough description of the size ratios (58).

2.2.4 Treatment

Treatment depends on the etiology, but to avoid the detrimental effects of bleeding disorder due to vitamin K deficiency, all infants with cholestasis should be supplemented with Vitamin K and the other fat-soluble vitamin (A, D and E). They often need nutritional support with enough calories and an increased proportion of medium-chain triglycerides to counteract malabsorption and improve growth, and choleretic treatment, most often with UDCA (62-65).

The mechanism of action of UDCA is not fully understood. It is less hydrophobic than the primary bile acids and is thereby thought to counteract the membrane damaging effect of primary bile acids on cholangiocytes during cholestasis. It may also increase the function of BSEP on the canalicular membrane of the hepatocyte through transcriptional and post-transcriptional mechanisms to increase the bile export from the hepatocytes to the bile canaliculi (9, 66, 67).

2.2.5 Outcomes

Cholestasis leads to further liver damage if not treated, but also affects other organs. In liver transplanted children, the majority of studies in the systematic review by Rodijk et al showed neurodevelopmental deficits (68). Both cognitive deficits (69, 70), and in regards to motor skills have been shown (71). In infants with biliary atresia, Caudle et al has shown impaired motor and language skills before transplantation (72), especially evident in girls and possibly associated to higher conjugated bilirubin levels (73).

2.3 CHOLESTASIS IN INFANTS AT RISK

Besides the etiologies described above, neonatal cholestasis has been reported with a much higher incidence in preterm and other sick and high-risk neonates in the neonatal intensive care unit (NICU).

2.3.1 Prematurity

The advances in neonatal care have continuously pushed the boundaries of both survival and long-term morbidities in preterm infants. For example, in the national Swedish cohort study EXPRESS (Extremely Preterm infants in Sweden Study) of extremely preterm infants <27 weeks born 2004-2007, 70% survived to one year of age, and two thirds showed no or mild neurodevelopmental disability at 6.5 years of age (74, 75).

Along with advances as for example the development of the incubator, mechanical ventilation and discovery of surfactant, the introduction of parenteral nutrition has been crucial for this impressive development (76, 77).

Preterm infants are defined as born before 37 full weeks of gestational age, often divided into subgroups. Preterm infants may also be defined according to birth weight. Subgroups of prematurity and low birth weight are described in Table 2 below.

Table 2. Preterm and low birth infants, definitions.

Preterm infants, subgroups	**Gestational age, weeks**	**Low birth weight infants, subgroups**	**Birth weight**
Moderately to late preterm	≥32 to <37	Low birth weight (LBW)	<2500g
Very preterm	≥28 to <32	Very low birth weight (VLBW)	<1500g
Extremely preterm	<28	Extremely low birth weight (ELBW)	<1000g

Abbreviations: LBW, low birth weight, VLBW; very low birth weight; ELBW, extremely low birth weight

2.3.2 Cholestasis in the NICU

When more preterm and other sick infants survive in the neonatal intensive care units, new conditions and morbidities have arisen. One of these conditions is cholestasis, which was first described by Peden et al in a case report regarding a premature infant receiving parenteral nutrition in 1971 (78).

The association between parenteral nutrition and cholestasis has since then been described in numerous publications (79-85).
Cholestasis in these infants may also therefore be referred to by the overlapping terms parenteral nutrition-associated cholestasis (PNAC), parenteral nutrition-associated liver disease (PNALD), with a little wider perspective of the liver, or intestinal failure-associated liver disease (IFALD) if the term also aims to include the consequences of long-term inadequate enteral feedings rather than merely the possible adverse of parenteral nutrition. Terms like total parenteral nutrition-associated cholestasis (TPNAC) and intestinal failure-associated cholestasis (IFAC) are also prevalent. In recent years, increasing focus has been laid on the different lipid emulsions used in parenteral nutrition, and their association with cholestasis (86).

2.3.3 Cholestasis in other high-risk neonates

Cholestasis is also known to be far more common in other high-risk neonates, such as in for example trisomy 21, where incidence was reported to be 3.9% in a population-based setting. In this study, cholestasis was always associated with other organ involvement, such as cardiac, gastrointestinal or bone marrow disease or malformations (87).

Another group of high-risk neonates where an increased risk for cholestasis has been reported are the infants with hemolytic disease of the fetus and newborn (HDFN) (88).

2.3.4 Definitions of cholestasis in infants at risk

As mentioned earlier, the joint guidelines from the ESPGHAN and NASPGHAN recommended that a conjugated bilirubin ≥ 1 mg/dL in an infant with prolonged jaundice should be regarded as abnormal (49).

In research studies, there is heterogeneity in definitions used to define cholestasis in infants at risk. These definitions could be divided into the ones using only a cut-off level of conjugated bilirubin in serum, and those using both a cut-off level of the absolute level of conjugated bilirubin and its proportion of the total level.
Using only a cut-off level of conjugated bilirubin is done by most, but requires that the initial peak of physiologic unconjugated hyperbilirubinemia has passed, and usually an inclusion criteria of parenteral nutrition treatment at least one week or longer ensures this (89-106).

The cut-off level of conjugated bilirubin varies between studies. The most common definition is ≥ 2mg/dL (≈34 μmol/L), but levels may also be set at 1 mg/dL, or even 1.5, 3 or 5 mg/dL in some cases. Other studies use the cut-off of a conjugated bilirubin level, most often 1mg/dL or 2 mg/dL, in combination with proportion requirement of total bilirubin, most often ≥ 20% (88, 107-112).

2.3.5 Incidence of cholestasis in preterm infants

Whereas the incidence of neonatal cholestasis in otherwise healthy term infants is often considered to be approximately 1/2500, in preterm and other sick infants in neonatal intensive care units, the rate is much higher. Depending on the populations studied, and how many risk factors for cholestasis the population holds, incidence rate figures differ.

For example, Christensen et al reported a cumulative incidence rate of 26.6% (369/1384) in their historic cohort study in all infants admitted to four NICUs in Utah during a 4.5 year period, surviving 28 days and receiving parenteral nutrition at least 14 days, defining cholestasis as conjugated bilirubin ≥ 2 mg/dL (≈34 μmol/L) (90).

Tufano and colleagues reported an overall incidence rate of 2.1% (27/1289) of all infants admitted to two NICUs in Naples during a 2.5 year time period, defining cholestasis as conjugated bilirubin ≥ 1 mg/dL (≈17 μmol/L) and ≥ 20% of the total bilirubin level (110).

Champion et al. prospectively examined all infants admitted to their NICU during 14 months for cholestasis, defined as ≥ 17 μmol/L and ≥ 20% of the total level. They categorized infants to a high-risk group if gestational age was < 34 weeks, if born small for gestational age, receiving parenteral nutrition at least 7 days or having surgery. In this high-risk group incidence of cholestasis was 13.7% (112).

In infants in the NICU who had undergone laparotomy and developed short bowel syndrome, Wales et al reported that 62.5% (25/40) developed cholestasis, defining cholestasis as conjugated bilirubin ≥50 μmol/L for at least two weeks (113).

Thus, both the populations studied and the definitions of cholestasis used affect the results. Most studies are single-center studies, whereas population-based settings are lacking.
In Table 3, a number of studies with retrospective observational designs are listed, reporting incidence figures of cholestasis in neonatal wards, focusing on preterm infants or low birth weight infants.

Table 3. Incidence rates of cholestasis reported in preterm and/or low eight infants in NICUs

Authors	Study population	Definition of cholestasis	Incidence
Beale et al 1979 (114)	BW<2000g, any PN.	CB ≥ 1.5 mg/dL	22.6% (14/62)
Baserga et al 2004 (115)	BW<1000g, PN ≥ 7 days.	CB ≥ 2 mg/dL	36.9% (38/103)
Robinson et al 2008 (103)	GA < 34 w, BW < 10th percentile, PN ≥ 7 days	CB ≥ 2 mg/dL	57.5% (23/40)
Hsieh et al 2009 (116)	GA<36 w, PN ≥2 weeks	CB ≥ 1.5 mg/dL (≈26 μmol/L)	17.7% (11/62)
Costa et al 2010 (117)	BW < 1500g, surviving 28 days, PN ≥ 14 days	CB ≥ 2 mg/dL at least twice, consecutive	12.3% (55/445)
Alkharfy et al 2014 (118)	BW<1500g, any PN	CB ≥ 34 μmol/L (≈ 2 mg/dL)	24.1% (74/307)
Yan et al 2017 (93) [1]	GA < 37 w, PN ≥ 14 days	CB ≥ 2 mg/dL	4.9 % (53/1074)
Wang et al 2021 (107)	BW ≤ 1250 g, GA ≤ 32 weeks, PN ≥ 7 days, non-surgery.	CB ≥ 1.5 mg/dL and ≥ 20% of TB	Soy: 20.1% (41/204) Mixed: 10.3% (20/195) [2]

Conjugated bilirubin 1 mg/dL ≈ 17 μmol/L
[1] Yan et al a dual center study, all other single center studies.
[2] Incidences in two populations receiving soy-based or mixed lipid emulsions.
Abbreviations: BW, birth weight; PN, parenteral nutrition; CB, conjugated bilirubin; GA, gestational age, NICU, neonatal intensive care unit; TB, total bilirubin.

2.3.6 Risk factors for cholestasis in preterm infants

The cause of cholestasis in these infants is believed to be multifactorial (79). Several risk factors for cholestasis have been identified, but their relative importance is difficult to establish, since preterm infants and other sick neonates often carry multiple risk factors. Thus, multivariable analyses and results from different studies are needed to be able to draw conclusions.

2.3.6.1 Parenteral nutrition

As mentioned previously, prolonged parenteral nutrition is the strongest risk factor for cholestasis in preterm and other sick infants in the NICU. Christensen et al reported in their large observational single-center study in a 4.5-year-period that in newborn infants receiving parenteral nutrition at least two weeks, the incidence rates increased along with the duration of parenteral nutrition (90). This association between cholestasis and prolonged parenteral nutrition is also reported by many others (96, 110, 112, 118, 119), and it is the risk factor most consistently reported in the literature. In many studies, receiving parenteral nutrition is also a criterion for inclusion.

Lack of enteral feeding is a risk factor closely related to parenteral nutrition, but it is difficult to evaluate its relative importance as the study by Koseesirikul et al. illustrates (101). Veenstra et al. attempted to examine this in their retrospective study of 178 preterm infants with NEC where 96 of them developed cholestasis. They found that enteral feedings initiated within the first week of life was associated with a reduced risk of cholestasis. Shorter time of no enteral feeding was associated with a shorter duration of cholestasis, whereas the time on parenteral nutrition was not (97).
Lack of enteral feedings might aggravate cholestasis through decreased endocrine secretion of cholecystokinin, a gut hormone stimulating gall bladder contraction. Absence of nutrients in the intestinal lumen also leads to less substrates for own metabolism in the enterocytes, so called trophic feeding, which in turn might lead to intestinal mucosal damage and increased permeability for bacteria and their toxins, which is another risk factor for cholestatic development discussed later.

Significant scientific focus has been put on the contents in different parenteral lipid emulsions, generating different hypotheses regarding possible mechanisms. Gura et al reported in 2006 of resolution of cholestasis in two preterm infants depending on parenteral nutrition when the soy-based lipid emulsion was switched to a fish-oil-based (120). Since then, many observational studies have been published comparing fish-oil-containing lipid emulsions, some showing promising results in both preventing cholestasis and resolution of manifest cholestatic liver disease in neonates, infants and children (86, 121-127).
Four groups of lipid emulsions used in neonates are shown in Table 4, and the important contents suggested being relevant for the development of cholestasis are listed.

Table 4. Parenteral lipid emulsions and contents of interest regarding cholestasis.

Lipid source	Examples of brand names	n-6-PUFA	n-3-PUFA	Phytosterol	Tocopherol
Soy-based (soy oil 100%)	Intralipid® (Fresenius Kabi)	High	Low	Contains	Low
Olive-based, (olive oil 80%, soy oil 20%)	Clinoleic® (Baxter)	Medium	Very low	Contains	Low
Fish oil-based (fish oil 100%)	Omegaven® (Fresenius Kabi)	Very low	Very high	No	High
Mixed (soy oil 30%, medium-chain triglycerides 30%, olive oil 25%, fish oil 15%)	SMOFlipid® (Fresenius Kabi)	Medium	Medium	Contains	High

Abbreviation: PUFA, polyunsaturated fatty acids

The main hypothesis regarding the possible mechanism of fish-oil-based lipid emulsions advantage regarding cholestasis versus soy-based, is that it is mediated through their effect on inflammation. The high content in soy oil of ω-6-polyunsaturated fatty acids (n-6-PUFA), from which the downstream metabolism results in arachidonic acid, by further metabolism results in the formation of pro-inflammatory mediators.
Fish oil on the other hand mainly contains ω-3-polyunsaturated fatty acids (n-3-PUFA), which by metabolism results in eicosapentaenoic acid, with less inflammatory derivatives (128).

In Figure 4, the structures of different important dietary lipids are shown.

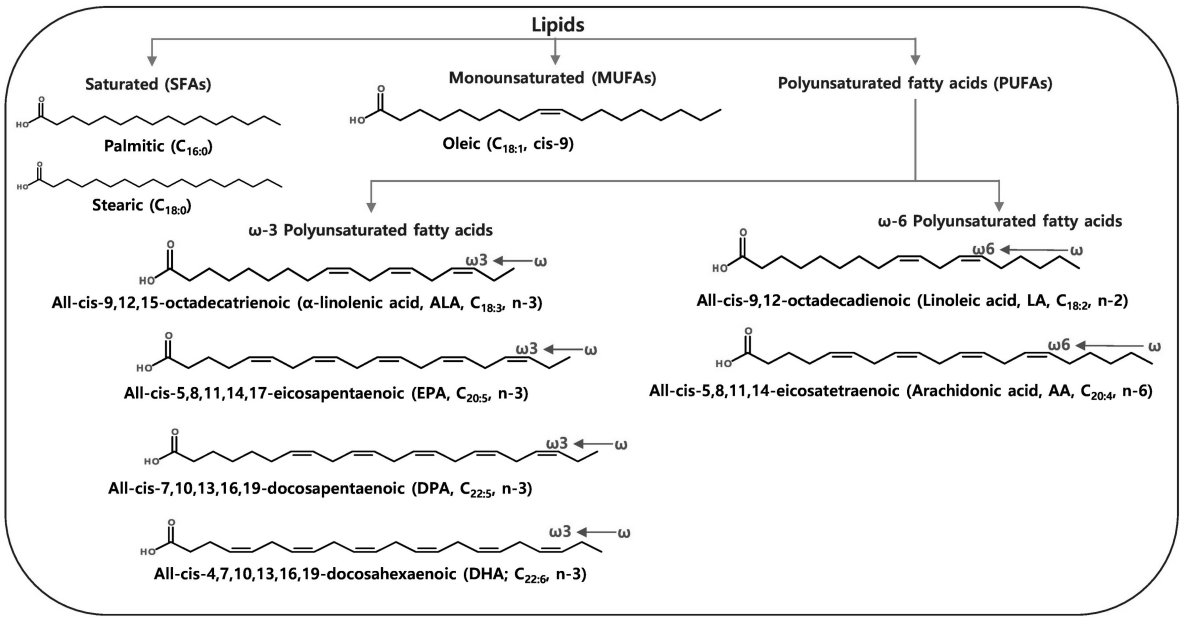

Figure 4. The main dietary fatty acids. From Saini et al. *Life Sciences 2018* (129), with permission from Elsevier.

In a randomized trial by Levit et al., using either a low dose of soy-based parenteral lipid emulsion of 1g/kg/day versus a standard dose of 3 g/kg/day in preterm infants (<30 weeks) did not affect the rate of cholestasis (130).

Less research interest has focused on the 20% soy and 80% olive oil-containing lipid emulsion available for treatment of term and preterm infants (Table 4). Olive oil mainly contains oleic acid, a monounsaturated long-chain fatty acid with less pro- or antiinflammatory properties than the PUFAs. As compared to entirely soy-based lipid emulsions, the hypothesis would be that containing less pro-inflammatory n-6-PUFA and more inert oleic acid, olive-based lipid emulsion would have less inclination to promote cholestasis.

A few randomized trials have been performed comparing soy-based to soy- and olive based lipid emulsions in preterm infants. One study investigated inflammatory response pathways, and found that in the soy-based group more T-cell-mediated interleukin 6 was produced. However, liver toxicity was not evaluated (131). Two other studies evaluated cholestasis, but the follow-up was only 5 days and 6 days, respectively, and no differences were observed within this short time (132, 133).

Nutrition with olive-based lipid emulsion or with mixing 50% olive-based and 50% fish oil-based was reported in one randomized trial in very preterm infants to lower the risk both for retinopathy of prematurity and cholestasis (134). Olive-based lipid emulsion has been compared to a mixed lipid emulsion in a randomized trial in very preterm infants, showing lower incidence of cholestasis in those receiving the mixed lipid emulsion (135).

Another hypothesis is that high content of phytosterols (a compound similar to cholesterol), in plant-based lipid emulsions such as soy-based lipid emulsion promotes cholestasis, whereas fish oil lacks phytosterols (136). Olive-based lipid emulsion contains about two thirds of the phytosterol content of soy-based. Preterm infants receiving soy-based lipid emulsion have been shown to have higher serum levels of phytosterols than those receiving a fish-containing mixed lipid emulsion, but the link to cholestasis remains hypothetical (137).

Insufficient levels of alfa-tocopherol, a form of Vitamin E, is another element in parenteral lipid emulsions proposed to play a role in the development of cholestasis. Based on animal studies, it is proposed that deficiency in Vitamin E, resulting in decreased antioxidant capacity, could contribute to cholestasis (128, 138, 139). Cholestasis in itself causes malabsorption of dietary lipids such as alfa-tocopherol, which might cause a vicious circle of further exaggeration of cholestasis.

To summarize, the main hypotheses regarding the role of the contents in parenteral lipid emulsions in the development of cholestasis are presented in Table 5.

Table 5. Summary of hypotheses on lipid emulsions and development of cholestasis

Excess of n-6-PUFA	Increasing inflammatory response from high levels of proinflammatory fatty acids leading to cholestasis
Deficiency of n-3-PUFA	Deficiency of fatty acids with antiinflammatory properties, promoting cholestasis
Ratio n-6-PUFA/n-3-PUFA	Not only the absolute levels, but a high ratio of proinflammatory versus antiinflammatory PUFAs promotes cholestasis
Phytosterols	The steroid compound found in plant-derived lipid emulsions blocks the FXR receptor and prevents the negative feedback from bile acids, promoting cholestasis
Alfa-tocopherol (Vitamin E)	Deficiency leads to increased susceptibility to oxidative stress, which promotes development of cholestasis

Abbreviations: PUFA, polyunsaturated fatty acids; FXR, Farnesoid X receptor.

However, in a meta-analysis in 2016 by the ESPGHAN Committee on Nutrition, not enough evidence was found in randomized trials for the advantage of any of the lipid emulsions used in regards to hepatotoxicity in preterm infants (81). In an updated Cochrane review from 2019 regarding parenteral lipid emulsions in preterm infants, the authors found insufficient evidence in randomized trials of any advantage for any of the different lipid emulsions used,

neither for the prevention cholestasis or the resolution of manifest cholestasis (140). Similar conclusions were made in the accompanying Cochrane review regarding late preterm and term infants with surgical conditions and/or cholestasis (141).

Considering the many studies showing promising results regarding fish oil-containing lipid emulsions, larger randomized trials with enough power, aimed at examining cholestasis as an outcome in neonates receiving different lipid emulsions are needed.

2.3.6.2 Gestational age and birth weight

Low birth weight and low gestational age are variables that obviously correlate to a high extent and therefore usually one of them is focused on in multivariable analyses of risk factors. Low birth weight has been reported by several as a risk factor for cholestasis in the NICU (90, 95, 118), which in line with that other report low gestational age as an important risk factor for cholestasis (110).

Small for gestational age has been reported by some to be an independent risk factor for cholestasis, but not others. One could hypothesize that impaired hepatic perfusion in utero could affect the development of bile acid metabolism in the liver (99, 103, 112, 117). In an autopsy series of deceased newborns on parenteral nutrition, small for gestational age was found in 60% (5/8) of infants with more severe histopathological liver findings, as compared to 6% (1/16) with milder findings (142).

2.3.6.3 Necrotizing enterocolitis and other intestinal disease

Several studies have identified intestinal disease as an important risk factor for cholestasis, not only as a reason for prolonged use of parenteral nutrition. In preterm infants, necrotizing enterocolitis is the most common intestinal disease associated with cholestasis, followed by spontaneous intestinal perforation (95, 119, 143). In near term or term infants, different gut malformations dominate (90, 108). The need for surgery is a marker for more severe intestinal disease, but may also be a risk factor itself in aggravating cholestasis (101, 112, 119, 144, 145), for example by decreased gut motility and reduced enterohepatic circulation.

The role of bile acids has been implied in the development of NEC (146-150). There is a link between NEC and risk for cholestasis (97). This is implying that there is a possible link between the liver, enterohepatic circulation and metabolism of bile acids, and development of NEC in preterm infants.

2.3.6.4 Bacteria

Parenteral nutrition is one of the most important reasons for use of central vascular catheters for a long period of time in neonates. This increases the risk for infections, and the rate has been estimated to 0.6-2.5 per 1000 catheter days in neonates (151). The highest rate of sepsis is seen in the most preterm infants (152, 153).

Several studies report neonatal sepsis as a risk factor for cholestasis in infants in NICUs (93, 95, 110, 154, 155). Hermans et al. reported that in children on long-term parenteral nutrition from the neonatal period, a more pronounced fibrosis in liver biopsies was associated with earlier catheter-related sepsis than in those with a milder fibrosis, even though the group with milder fibrosis had longer duration of parenteral nutrition (156).

Gram-negative sepsis has been shown to cause liver enzyme abnormalities more often than the gram-positive coagulase negative staphylococci, the most common late onset infection in preterms (157). It has been hypothesized, that the endotoxin (lipopolysaccharide) from these bacteria, through inflammatory response of Kupffer cells, down-regulate the bile flow from the hepatocytes (158), possibly mediated by the FXR, increasing bile acid production, but also by down-regulating the BSEP, the canalicular transport protein.
Veenstra et al. found that in preterms with NEC, a concomitant bacterial infection (predominantly gram negative sepsis) was associated with increased risk of developing cholestasis, and remaining cholestatic at discharge (97).

Endotoxin has also been hypothesized to reach the liver through translocation from the gut microbiome over a damaged intestinal mucosa from for example necrotizing enterocolitis, or through shortage of intestinal feedings, as supported by data from animal models (159-161). In infants with short bowel syndrome from the neonatal period, bacterial overgrowth from the colon to the ileum has been identified, and it remained to a higher extent in those who were not weaned from parenteral nutrition (162).

In 2017, Wang and colleagues reported on 18 infants with short bowel syndrome following neonatal intestinal diseases requiring surgery. A significant shift in the gut microbiota towards gram negative species was seen in those infants who developed the complications of cholestasis and catheter related infections, and the authors postulate that endotoxin from these bacteria plays an important role in he development of cholestasis (163).
However, Parm and colleagues found that more colonization from possibly pathogenic gram-negative strains was found in rectal swabs in those premature infants that received early enteral feeds than in those on total parenteral nutrition, thus complicating the picture (164).

2.3.7 Cytomegalovirus and cholestasis

Cytomegalovirus (CMV) infection is one of the most common viral infections in the perinatal period (165). It can transmit vertically during pregnancy (resulting in congenital infection) and labor, through breast-milk or horizontally (166).
The seroprevalence of CMV in adult women ranges from 40% to 90%, with higher rates in low-income countries (167). The highest risk of vertical transmission is noted after primary infection in previously seronegative mothers, but maternal reactivation of the virus or reinfection with a different strain may also result in congenital infection (168-170).

The overall prevalence of congenital CMV infection has been estimated to 0.62%, this figure is also higher in low-income than in high-income settings (166). The prevalence of congenital CMV infection in preterm in comparison to term infants is less clear (171, 172). The

symptoms of congenital infection may vary from asymptomatic in most cases to severe and sepsis-like in some cases. At birth, the neonate may present with, among other things, low birth weight, microcephaly, retinitis, liver disease, hearing loss and later developmental delays (167).

Treatment of the congenitally infected infants with moderate or severe disease is possible with the intravenous antiviral ganciclovir or its orally administered prodrug valganciclovir for 6 weeks or 6 months, respectively (173). Adverse effects must be monitored, as for example myelosuppression and hepatotoxicity. The possible adverse effects of carcinogenicity and decreased fertility must be taken into consideration before treatment. In infants with congenital CMV, an improvement or stabilization of hearing has been reported, and possibly also an improvement in neurodevelopmental outcome (174-178).

Postnatal CMV infection is less studied than congenital. In a recent meta-analysis of breast-fed preterm infants, 16.5% were infected if the mother was seropositive, with higher rates if fresh milk was used than if freeze–thawed milk was at least partially used. In breast-milk, 80.5% of the mothers shredded CMV if they were seropositive (179).
Even though breast-milk is the most obvious and important route of transmission, the virus can be found in almost any body fluid during shredding, for example saliva (180), and therefore transmission from other close contacts than the mother may be possible in the neonatal period, even in a neonatal ward.
To decrease the risk of transmission from blood products, filtering out white blood cells and exposing donated products to radiation is performed (181). In a retrospective review of all very low birth weight infants tested for postnatal CMV, Mukhopadhyay and colleagues found that 19% were positive (182).

Postnatal CMV infection in term infants has been considered mild and with few complications. However, in preterm infants it has been associated with poorer outcome regarding bronchopulmonary dysplasia in a large retrospective cohort study from 2015 (183). In a recent systematic review, the authors concluded that preterm infants with postnatal CMV infection likely have an increased risk for pulmonary and neurologic complications, but they did not find enough evidence of an association with necrotizing enterocolitis, or visual or hearing impairments (184).

CMV is known to affect the liver in patients having symptomatic infection. The virus may cause hepatitis and cholestasis in infants and is an important differential diagnosis in examinations of term infants presenting with cholestasis (58, 185-187).
In an autopsy series including infants with CMV infection, a common histopathological finding was cholestasis, as well as cytomegalic inclusion bodies in hepatocytes, but it was also a common finding in the bile duct epithelium in contrast to the findings in adults (188).

Goel et al. reported in 2018 that in liver biopsies from 31 infants evaluated for neonatal cholestasis, CMV DNA was found by polymerase chain reaction (PCR) in almost half of the cases, with similar rates in those with biliary atresia and other causes of neonatal cholestasis

(189). CMV has been implied in the development and outcome of biliary atresia in several studies, but its role remains unclear (185, 186, 189-196).

In preterm infants, often receiving parenteral nutrition and developing cholestasis in the NICU, the role of CMV is unclear.

2.3.8 Course, treatment and outcome of cholestasis in preterm infants

2.3.8.1 Onset, peak and duration of cholestasis

The natural course of cholestasis is difficult to describe since the settings are so different between studies, and interventions are always performed except for in the mildest cases.

In the published research, the age at onset of cholestasis is usually not reported, due to the fact that inclusion criteria of minimum time on parenteral nutrition is often used. Tufano and colleagues did not have such an inclusion criterion, and reported that in all infants admitted to their NICU in the study period of 2 years and 8 months time, the median age of onset of cholestasis was 20 days (range 2-90) (110). Champion et al. found that age of onset of cholestasis was 15 days in a prospective study of all admitted neonates to their NICU during 14 months (112).

The duration of cholestasis depends on the population studied, and the success of treating the underlying conditions, especially terminating parenteral nutrition. After cessation of parenteral nutrition, a slow normalization of conjugated bilirubin in at least several weeks is reported in one study (197). In the large retrospective cohort study by Christensen et al. of all neonates receiving parenteral nutrition for at least two weeks, cholestasis duration ranged from fewer than 10 days to more than 440 days in 357 cholestatic infants (90).

The peak level of conjugated bilirubin is most commonly used to describe the severity of cholestasis. Christensen and colleagues found an association with higher peak conjugated bilirubin and increased mortality (90).
Willis and colleagues reported that high mortality and morbidity rates were associated with high conjugated bilirubin levels in patients with prenatally diagnosed intestinal malformations and in referred necrotizing enterocolitis patients. In cholestatic patients who had also received ursodeoxycholic acid treatment, they found that 38% (8/21) of infants with conjugated bilirubin exceeding 10 mg/dL (≈170 μmol/L) died or needed a transplant, whereas only 7% (3/45) of infants with a lower peak of conjugated bilirubin suffered these consequences within the first year of life.

2.3.8.2 Growth

Niccum et al. reported in 2019 growth data from a retrospective study comparing cholestatic and non-cholestatic neonates with average gestational age 32-33 weeks in their NICU. Weight percentiles did not differ between the groups within the first 8 weeks of life, but at discharge and at 6 months of age the cholestatic weight percentiles were significantly lower (91). The authors did not state at what age full enteral feedings were achieved, but 89% were

still cholestatic at discharge when parenteral nutrition certainly had been terminated. Malabsorption of dietary fat in infants still cholestatic but without the earlier support of parenteral nutrition could be speculated to explain these findings.

2.3.8.3 Treatment

The most important treatment of cholestasis in these infants is the cessation of parenteral nutrition. Changing lipid emulsion to fish-oil-containing is often tried, but convincing evidence from randomized trials is lacking (140, 141). Cycling of parenteral nutrition in less than 24 hours per day, or decreasing the total lipid dose are also methods used to prevent cholestasis, but their efficiency has not been verified in randomized trials (100, 130).

Treatment with cholecystokinin in order to stimulate gall bladder contractions and increase bile flow was not successful in a randomized trial (198). Erythromycin that can stimulate intestinal motility and increase feeding tolerance has not been demonstrated to decrease the rate of cholestasis (199).

Ursodeoxycholic acid is widely used in other cholestatic disorders, and often used in these infants without any serious side effects. Some observational studies have reported encouraging results, but the efficacy remains to be proven in randomized trials (200-204).

2.3.8.4 Outcomes

Mortality is consistently higher in cholestatic infants than in non-cholestatic in the literature, and associated to a more severe cholestasis, as described above (95, 102). However, it is often hard to determine the role of cholestasis as the primary cause of death in the presence of all the other accompanied morbidities. Studies are generally also designed to study cholestasis as the outcome and not as a risk factor for death before discharge.

In 2004, Zambrano and colleagues published a case series of 24 autopsies in deceased newborns that had received parenteral nutrition. Histopathological liver examination showed that periportal inflammation, cholestasis, bile duct proliferation, extramedullary hematopoiesis and fibrosis were the most common findings, which was found in 17-19 of the 24 cases. Cirrhosis was found in 3/24 patients, and iron deposition in 22/24. Patients were divided in two groups depending on severity of liver findings. All infants in the group with more severe liver findings (n=8) had experienced conjugated hyperbilirubinemia, versus 25% in the group with milder liver findings (n=16). Being small for gestational age was also far more common in the severe group, as well as bronchopulmonary dysplasia (142).

Reports on long-term liver outcomes in surviving cholestatic preterm infants are lacking.

2.3.9 Cholestasis and hemolytic disease of the newborn

Another group of neonates at high risk and need for neonatal care are the ones born to mothers with red cell alloimmunizations. The fetus and newborn can develop hemolytic disease of the fetus and newborn (HDFN), or hemolytic disease of the newborn (HDN) if only the neonatal consequences are referred to.

2.3.9.1 Antibody types

IgG antibodies from the mother directed at different surface antigens on the erythrocytes of the fetus causes early destruction of the fetal erythrocytes with risk for fetal anemia in HDFN (205). Antibodies directed against the D-antigen within the Rhesus (Rh)-system is the most common antibody to cause severe HDFN, but also c-antibodies within the Rh-system and K-antibodies within the Kell-system are known to cause severe disease (206, 207).
Untreated, severe HDFN often leads to intrauterine death or the severe condition of hydrops fetalis if surviving until birth.

If there are several antibodies present, they are ordered as primary, secondary and so on, in order of their concentration (or titer). Most research has focused on the primary antibodies. Higher concentrations are related to higher risk for disease and a more severe clinical course, but also depends on the specificity of the primary antibody found as described above.

2.3.9.2 Multiple antibodies

In about one out of four alloimmunized mothers, there are multiple maternal antibodies present (208). Several studies have reported synergistic effects with a more severe clinical course in such cases. In particular, there is a need for more intrauterine transfusions due to a more severe anemia, when compared to single immunizations (208-212).
In a Chinese cohort study, the authors did not find an increased risk for fetal anemia with anti-D in combination with other antibodies. Due to genetic differences the anti-K antibodies were not present in this population, and anti-c was very rare (213).

In Table 6, the most common and important antibodies causing HDFN are listed.

Table 6. Correlation of red cell alloantibody specificities with occurrence HDFN.

Antibody type		**Risk for HDFN** [a]	**Course of disease** [b]
ABO		Low	In general mild, incidentally severe
Rh	D	High	Often (very) severe, otherwise mild
	c	High	(Very) severe or mild
	E	Medium	Sometimes severe, mostly mild
	Other Rh antigens	Medium	Incidentally severe, mostly mild
Kell	K	High	(Very) severe or mild
	Other Kell	Medium	Mild to severe
Duffy	Fya/Fyb	Medium	Mostly mild
Kidd	Jka/Jkb	Low	Only mild
MNS	M, N, S, s	Low	Mostly mild, very rarely severe
	Other antigens	Low	Mostly mild, very rarely severe
	I, Le, P1, Lu, Yt	No risk	
Other systems		Very low	Very rarely severe

[a] High risk: >50%, medium risk >10–50%, low risk 1–10%, very low/incidentally

[b] Very severe disease: Need for intrauterine treatment and/or exchange transfusion after birth. Severe disease: Need for intrauterine treatment and/or preterm induction of labor and/or blood transfusions after birth. Mild disease: Only treatment with phototherapy is needed. Adjusted from de Haas et al, *Vox Sanguinis 2015* (205), with permission from John Wiley and Sons.

2.3.9.3 Treatment

Fetuses who develop anemia may need intrauterine blood transfusions to survive (214). The transfusions are life-saving, but create a state of iron overload in the fetuses and neonates that can persist for months (215). After birth, these infants have low hemoglobin levels, and low reticulocyte counts and may need further transfusions due to a refractory bone marrow with inadequate hematopoiesis following repeated intrauterine blood transfusions.

As a newborn infant with HDFN, the risk for severe unconjugated hyperbilirubinemia due to continuing hemolysis is greatly increased, and intensive phototherapy and even exchange transfusions are often needed to prevent neurological injury and impairments (32, 47).

2.3.9.4 HDFN and cholestasis

Since almost a century, there have been repeated case reports of infants with HDFN who develop conjugated hyperbilirubinemia and cholestasis (216-219). In 1952, Hsia and colleagues described all infants with prolonged obstructive jaundice in the previous twelve years in the Children's medical center in Boston, and found that a major cause was inspissated bile syndrome due to HDFN (220).

The hypothesis is that the massive flow of bilirubin in infants with HDFN renders the bile to become more viscous, which causes cholestasis. The syndrome of inspissated bile, where viscous bile caused bile plugs, sludge and obstruction in infants with prolonged jaundice but with continuous biliary ducts had been described earlier (221). To indirectly support this hypothesis it is noted that cases of cholestasis have also been described in infants with non-immune hemolytic disorders, such as for example glucose-6-phostatase deficiency, pyruvate kinase deficiency, congenital dyserythropoietic anemia, and hereditary spherocytosis (222-225).

Despite several case reports, very few actual studies have been published on the subject of cholestasis in HDFN, but in 2012 Smits-Wintjens and colleagues published a large observational study from the Netherlands. They found that cholestasis, defined as conjugated serum bilirubin ≥1 mg/dl (≈ 17 μmol/L) and ≥20% of the total bilirubin level, occurred in 13% (41/313) of the term or near term infants with HDFN admitted to their referral center over a ten-year-period.
Primary antibodies were reported, and D immunization was the most common (77%) followed by Kell (12%) and c (8%), as expected. Intrauterine transfusions had been given in 66%. D immunization and treatment with at least one intrauterine blood transfusion were the independent risk factors in multivariable regression analysis.
In 12% of cholestatic infants, ursodeoxycholic acid treatment, fat-soluble vitamins and nutrition with medium chain triglycerides was needed. Resolution of cholestasis was seen within three months at longest in the 44% of infants where follow-up was available to the researchers, in the remainder data regarding follow-up was missing.

In 2013, Takci et al reported on a more selected group of infants, i.e. the ones with hydrops fetalis, often referred to their tertiary center from rural areas in Turkey, where the prenatal management of HDFN had not been optimal. The found that 60% (18/30) of such infants developed cholestasis, defining cholestasis as ≥2 mg/dl (≈34 μmol/L) and ≥20 % of the total bilirubin level, and was present at birth in 27%.
Although mortality was as high as almost 40% in the cholestasis group due to complications of hydrops fetalis, all the survivors had resolution of their cholestasis within 3 months of age.

The state of knowledge is thus scarce, and there is a need for population-based studies with a long-term perspective.

3 RESEARCH AIMS

3.1 GENERAL AIMS

The overall aim of this thesis is to increase the knowledge about cholestasis in newborn infants, targeting infants at risk for severe morbidity and adverse neonatal outcomes.

3.2 SPECIFIC AIMS

- To evaluate the population-based incidence and outcome of cholestasis in very preterm infants and identify the most important risk factors.

- To investigate the role of two different parenteral lipid emulsions in the development and outcome of cholestasis in very preterm infants.

- To investigate the prevalence and importance of cytomegalovirus infection in association with cholestasis in preterm infants.

- To evaluate the population-based incidence, risk factors and outcome for cholestasis in term and preterm infants with hemolytic disease of the fetus and newborn (HDFN).

- To investigate if chronic liver diseases in childhood may have an early presentation in the neonatal period as a transient cholestatic episode after preterm birth, or in infants with HDFN.

4 MATERIALS AND METHODS

4.1 STUDY SUBJECTS AND DESIGN

Subjects were included in the four papers according to the criteria described under each subheading below. The time periods of inclusion and possible overlap of subjects are illustrated in Figure 5.

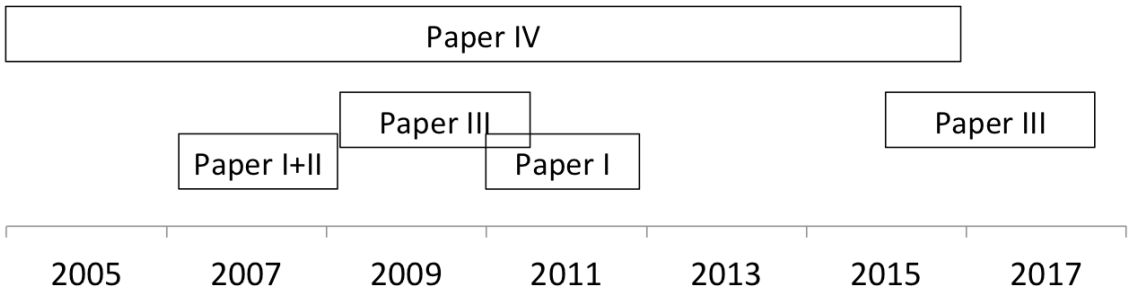

Figure 5. Timeline of subjects included in all papers.

Paper II is based on the population born in 2006-2008, also included in paper I.
In paper III, a total of 10 infants from paper I were also included.

4.1.1 Study subjects and design: Paper I and II

Paper I was a retrospective case-control study based on two populations of preterm infants with a gestational age of less than 30 full weeks (≤29 weeks and 6 days), who were born and treated in Stockholm County. All infants surviving the first 28 days of life and not transferred to other regions were included. The first population consisted of preterm infants born between March 2006 and February 2008 (referred to as the SOY population in Paper I). The preterm infants in the second population were born between January 2010 and December 2011 (referred to as the OLIVE population in Paper I). For every cholestatic case, two non-cholestatic controls matched on gestational age were randomly selected from each population.

In the SOY population, out of a total of 296 preterm infants, 250 were included and 46 excluded due to short life span or being transferred to other regions. In the OLIVE population, out of a total of 319 preterm infants, 268 were included and 51 excluded. Altogether, 518 infants were included in the study.

In paper II, the study population was based on the same population of preterm infants born between March 2006 and February 2008. In this cohort, more detailed data was available on infections and long-term follow-up.

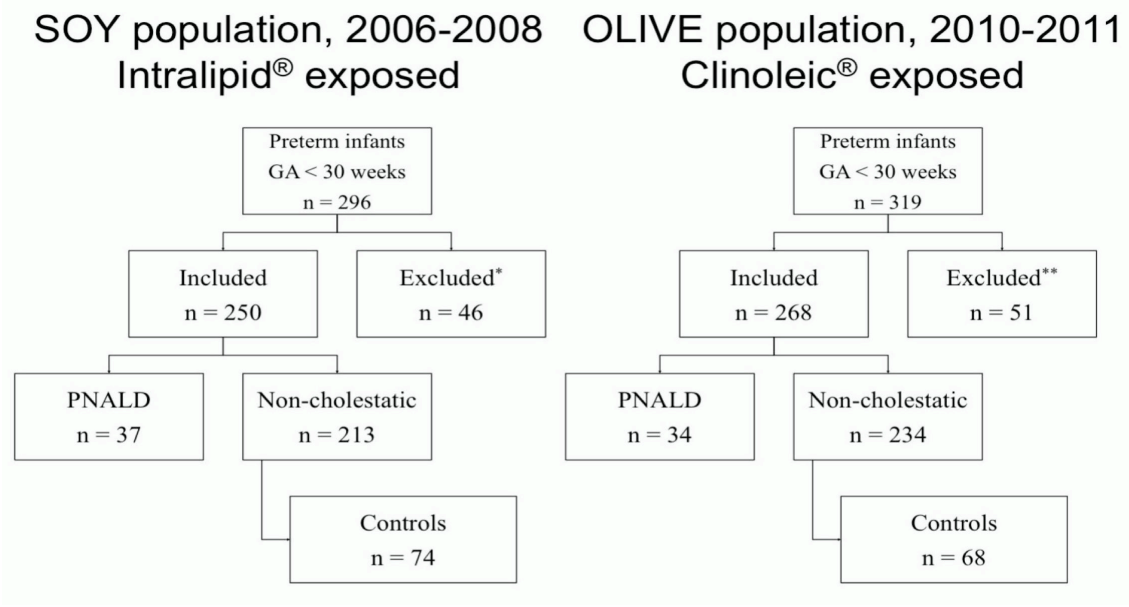

Figure 6. Flowchart of populations, patient inclusion, exclusion and outcome in paper I & II.
* Transferred, other region: n=22; death at age < 28 days: n=24.
** Transferred, other region: n=23; death at age < 28 days: n=28.
From Teng et al. *J Pediatr Gastroenterol Nutr 2015* (226) with permission from Wolters Kluwer Health, Inc.

4.1.2 Study subjects and design: Paper III

This case-control study was performed prospectively at the neonatal units at Karolinska University Hospital, Stockholm, Sweden. Preterm infants (gestational age < 37 weeks) admitted to the neonatal units from March 2008 to July 2010 who subsequently developed cholestasis during the neonatal period were included.

A total of 45 cholestatic preterm infants were included. A control group of preterm infants without cholestasis was subsequently recruited from the same neonatal units and matched for gestational age. The infants in the control group were born from January 2015 to September 2017.

Initially, the aim was to include one control infant per cholestatic case, matched on gestational week at birth, but due to difficulties in including enough non-cholestatic infants within a reasonable time, inclusion had to be terminated when 24 control infants had been included.

4.1.3 Study subjects and design: Paper IV

Paper IV was a population-based cohort study with retrospective data collection from hospital charts. The study population consisted of all alloimmunized pregnancies in the Stockholm region from 2004 to 2015 that resulted in a live born child with hyperbilirubinemia caused by HDFN. The pregnant women and their children were identified through the GravImm register (www.gravimm.se).

Within the study period, 170 live born infants from mothers in the GravImm register were diagnosed with hyperbilirubinemia caused by HDFN. Of these, 21 had never been tested for conjugated bilirubin levels during their neonatal period. The remaining 149 infants were included in the study.

4.2 METHODS

4.2.1 Definitions

Common definitions in all papers for variables and outcomes: Small for gestational age, z-score for birth weight < -2 standard deviations from expected (227); Necrotizing enterocolitis, ≥ stage 2 according to Bell's criteria (228); Retinopathy of prematurity, stage according to guidelines from the International Committee for the Classification of Retinopathy of Prematurity (229), Bronchopulmonary dysplasia, as moderate or severe as defined by the consensus document by the National Institute of Health (230); Intraventricular hemorrhage, according to grade (231); Duration of treatments (parenteral nutrition, mechanical ventilation etc.), as initiated days of treatment; sepsis as blood culture positive with typical clinical symptoms, laboratory test findings and a diagnosis from the treating neonatologist; Death, as death before discharge from hospital care.

4.2.2 Methods: Paper I

As described previously, two sub-populations of preterm infants receiving either a soy-based parenteral lipid emulsion (SOY population) or an olive-based (OLIVE population) as the default lipid emulsion in different time periods were compared. The primary lipid emulsion was changed between these time periods in all NICUs in Stockholm.

Stockholm County's guidelines for parenteral nutrition in newborns remained unchanged between the two time periods studied, except for the lipid emulsion used. Lipid emulsion infusion was started on the first day of parenteral nutrition with a dose of 0.5 to 1 g/kg/day and increased for 3 days to 3.5 g/kg/day for infants with a birth weight <1000 g, and to 3g/kg/day for infants with a birth weight >1000 g. In the absence of contraindications, early enteral trophic feeding using the mother's breast milk or donated bank milk was started alongside parenteral nutrition on the first or second day of life. Enteral nutrition was then gradually increased, analyses of the breast milk were performed, and nutrition individually fortified to meet the recommendations by ESPGHAN (232).

Cholestasis was defined as conjugated serum bilirubin ≥30 μmol/L (≈1.8 mg/dL) on at least 2 occasions, with a ratio of conjugated and total serum bilirubin exceeding 20%. The non-cholestatic infants in the two cohorts were divided in separate subgroups according to week of gestational age. Two non-cholestatic controls per case born in the same gestational week were randomized from the corresponding cohort.

Cholestatic cases and controls were compared. In a multivariate model, using the stepwise forward logistic regression method, independent risk factors for cholestasis were identified.

4.2.3 Methods: Paper II

We performed a population-based retrospective case-control study of all preterm infants born and treated in Stockholm County during a 2-year period, using the same sub-population (SOY) as described in paper I. The infants and their mothers were identified through the Swedish neonatal quality register (SNQ). Cholestatic infants were detected through medical chart reviews. For every cholestatic case, two non-cholestatic control subjects born in the same gestational week were randomized from the population. Data from the perinatal period were primarily acquired from medical chart reviews. The same definition of cholestasis as described in paper I was used.

The onset of cholestasis was defined as the age when the bilirubin levels fulfilled the cholestasis criteria for the first time. The grade of cholestasis was arbitrarily dichotomized as low, if peak conjugated bilirubin <100 μmol/L, and as high, if peak conjugated bilirubin was ≥100 μmol/L. The duration of cholestasis was defined as the time elapsed from onset of cholestasis to when conjugated bilirubin no longer exceeded 30 μmol/L.

The development of chronic liver disease of clinical significance was defined by the infant being enrolled at any time, after discharge from neonatal care, as a patient until the age of 10 years at the tertiary center for pediatric hepatology at Karolinska University Hospital in Stockholm or at any other hospital based pediatric service in the country.

The proportion of cholestatic infants in each week of gestational age in the entire population was examined and compared. Cholestatic cases and non-cholestatic controls were compared. The outcomes of bronchopulmonary dysplasia (≥moderate) and retinopathy of prematurity (≥ grade 3) were examined in a multivariate model using logistic regression.

4.2.4 Methods: Paper III

Cholestasis was defined as conjugated serum bilirubin ≥ 30 μmol/L, and more than 20% of the total serum bilirubin level.

Blood and urine samples were collected from all infants as well as blood samples from the mothers to analyze the presence of CMV DNA and CMV IgG and IgM in serum. In the cholestatic group these samples were collected as soon as possible after cholestasis was detected. Samples from the reference group were collected at 3-6 weeks postnatal age. The

urine sample was if possible collected from a consecutive urine portion after blood sampling. Maternal blood sampling was done on the same day as infant blood sampling.

Maternal plasma samples were tested for CMV-specific IgG and IgM. DNA was extracted from infant plasma and peripheral blood mononuclear cell (PBMC) samples, as well as urine samples. Approximately 100 ng of DNA per sample were amplified using quantitative polymerase chain reaction (qPCR) with specific CMV-IE (cytomegalovirus immediate early gene) DNA primers as described by Xu et al (233). The ΔCT method was used for calculation of cycle threshold (CT) values. The CT threshold for the quantitative PCR was set at 35 for a positive test. Positive PCR results were recorded for each sample: PBMC, plasma and urine. Infants who were positive in any test were categorized as CMV positive and all others were considered CMV negative. If there were missing data for any CMV DNA sample, the result for that sample was assumed to be negative to avoid overestimating the results.

Clinical data were collected from hospital medical records and the Swedish neonatal quality register (SNQ). All clinical data were collected before registering the results of the CMV PCR tests in the database in order to blind the researchers as much as possible and reduce the risk for bias. Comparisons were made between the cholestatic infants and the non-cholestatic reference group, and within the cholestasis group between the CMV positive and CMV negative infants. Being CMV positive as a risk factor for cholestasis was evaluated in a multivariate model adjusting for other risk factors.

4.2.5 Methods: Paper IV

Data describing antibodies and the pregnancy were obtained from the GravImm register and obstetric medical charts, additional data were collected pediatric medical charts and transfusion medicine registers. All erythrocyte antibody types present were recorded and variables created to denote if a specific antibody was present alone or in combination with at least one other antibody (multiple antibodies).

For the neonates, the results for every serum analysis of bilirubin, liver enzymes and bile acids levels during the first 90 days of life were extracted from laboratory records together with the time and date of the assay. Medical charts were reviewed up to two years of age to identify children who had any diagnosis of chronic liver disease.

Cholestasis was defined as serum conjugated bilirubin exceeding 34 μmol/L (≈ 2 mg/dL) and exceeding 20% of serum total bilirubin during the study period of the first 90 days of life. Three other common definitions of cholestasis were also explored: conjugated bilirubin ≥17 μmol/L; conjugated bilirubin ≥17 μmol/L and ≥ 20% of total bilirubin; conjugated bilirubin ≥ 34 μmol/L.

The age when first fulfilling the criteria for these diagnoses was examined as a “time-to-event” outcome. Any cholestasis diagnosis was analyzed as a dichotomous outcome, with patients with no diagnosis of cholestasis serving as reference. Liver disease of clinical

significance by two years of age was defined as enrollment at any clinic in the Stockholm region with a diagnosis of liver disease until two years of age.

Univariate odds ratios for different risk factors were calculated for developing cholestasis, and multivariable model including the most important risk factors from the univariate analysis was created.

4.3 STATISTICAL ANALYSES

SPSS for Mac (IBM, New York, USA) was used for data management and statistical analyses in all papers.
Fisher exact test was used as the significance test for proportions. Independent samples t-test was used for comparing means for normally distributed continuous variables. When medians and interquartile range was reported for continuous variables, Mann Whitney U test was used as the significance test. For trends, Mantel-Haenszel test of trend was used in paper II. In paper IV Spearman's correlation was used for correlations and the time to event (cholestasis) was investigated using Kaplan-Meier analysis and the cumulative incidence presented graphically.
Logistic regression was used for calculating odds ratios, and adjusted odds ratios were calculated in multivariable models, in paper I using the stepwise forward method, in all other papers the enter method.
Significance level was always set at 5%.

4.4 ETHICAL CONSIDERATIONS

All studies within this thesis were performed in accordance with the Helsinki declaration, and the regional ethical review board in Stockholm approved all studies.

4.4.1.1 Ethics: Paper I and II

Patient charts were reviewed retrospectively. Informed consent was not obtained from the caretakers, since no extra sampling or data collection was performed, other than data collection from the medical charts and the national neonatal quality register. Also, a non-negligible proportion of the infants were deceased which contributed to the decision not to seek informed consent by the caretakers.

4.4.1.2 Ethics: Paper III

Informed consent was achieved from all caretakers for the blood and urine sampling from the infants and their mothers. To cause as little pain as possible to the infants, blood sampling was always performed at the same time as blood sampling was scheduled for clinical reasons. To minimize the negative impact of multiple blood samplings in the infants regarding blood

volume and iron status, only the very small amount of 0.5 mL was drawn for the research purpose.

4.4.1.3 Ethics: Paper IV

In this register-based cohort study, data was collected from the GravImm register, the Swedish neonatal quality register (SNQ), the obstetric charts and the medical charts of the infants and their mothers. No additional consent other than the initial approval from the mothers to be included in the GravImm register, including approving the research purposes of the register, was obtained from the caretakers for participation in the study, since no additional examinations were performed, or samples collected from the study subjects.

5 RESULTS AND DISCUSSION

5.1 RESULTS AND DISCUSSION: PAPER I

In the cohort of 518 very preterm infants born and treated in Stockholm, with gestational age < 30 weeks and surviving the neonatal period, born in two different two-year periods when a fully soy-based lipid emulsion was used (SOY period) or a 20% soy and 80% olive oil-based lipid emulsion was used (OLIVE period), we made the following findings.

Main findings:

- ❖ Cholestasis occurred in 14.8% of preterm infants during the SOY period versus 12.7% during the OLIVE period (p=0.52).

Other findings:

- ▪ Necrotizing enterocolitis (NEC) and length of parenteral nutrition were strong risk factors for cholestasis:

Table 7. Independent risk factors for PNALD: Logistic regression analysis, stepwise forward method including cholestatic cases (n=71) and GA-matched non-cholestatic controls (n=142).

		OR	95% CI for OR	p
NEC	No	1		
	Yes	10.39	3.23-33.37	<0.001
Parenteral nutrition, duration (OR per 1 day increase)		1.11	1.07-1.15	<0.001
Population	OLIVE	1		
	SOY	2.96	1.19-7.35	0.02

Final model. PNALD = parenteral nutrition-associated liver disease, GA = gestational age, OR = odds ratio, CI = confidence interval, NEC = necrotizing enterocolitis.

From Teng et al, *J Pediatr Gastroenterol Nutr 2015* (226), with permission from Wolters Kluwer Health, Inc.

As mentioned earlier, the incidence of cholestasis in preterm and other sick neonates differ depending on the population studied. We report the population-based incidence of cholestasis in a large cohort of preterm infants, surviving the neonatal period. We found that cholestasis

is common; it affects approximately 1 out of 7 of preterm infants with gestational age <30 weeks, if a soy-based or predominantly olive-based parenteral lipid emulsion is used. The difference in incidence of cholestasis between the time periods, but when adjusted for NEC and time on parenteral nutrition, being treated during the soy-period was a significant risk factor, suggesting that there may be a small advantage in using a predominantly olive-based lipid emulsion over entirely soy-based.

To our knowledge, no other studies have compared cholestasis using these lipid emulsions. Small, randomized trials on olive oil-based lipid emulsion in preterms has either not focused on liver toxicity or had a short follow-up (131-133, 234).

Further prospective research is needed, but according to our results, with a single objective to significantly reduce the incidence of cholestasis, a predominantly olive oil-based lipid emulsion is at best marginally better than an entirely soy-based.

5.2 RESULTS AND DISCUSSION: PAPER II

Main findings:

- Mortality was higher among cholestatic infants, and highest in those with high-grade cholestasis:

Table 8. Mortality rates reported in paper II.

	Cholestasis		Non-cholestatic controls	p
Mortality	14% (5/37)		2.7% (2/74)	0.040
	High-grade	Low-grade		
Mortality	26% (5/19)	0% (0/18)		0.046

High-grade cholestasis defined as peak conjugated bilirubin ≥100 μmol/L and low-grade as <100 μmol/L.

Other findings:

- The risk of developing cholestasis was higher in lower gestational ages

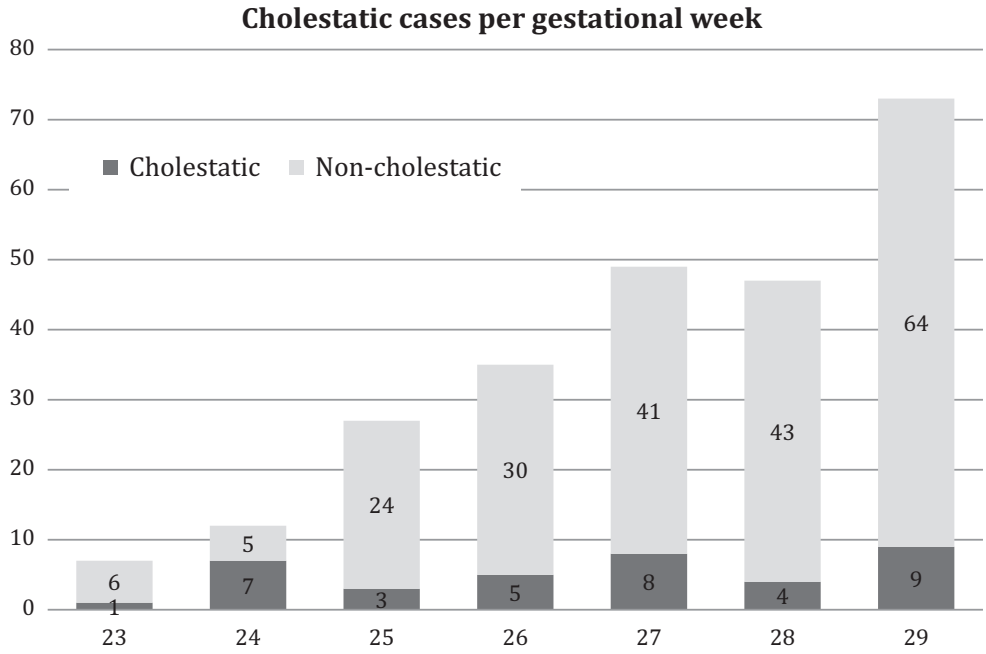

Figure 7. Proportion of cholestatic cases per gestational week. Significant trend of higher proportion of cholestasis in lower gestational weeks (p = 0.034*, Mantel-Haenszel test of trend). From Teng et al, *Acta Paediatrica,* 2021 (235), with permission from John Wiley and Sons.

- There were no cases of chronic liver disease at ten years of age in either cholestatic infants nor non-cholestatic controls.

- Rates of ROP, stage 3 or more, (36% versus 14%, p=0.011) and BPD (54% versus 30%, p=0.022) were higher in cholestatic infants than in non-cholestatic controls. When calculating crude and adjusted odds ratios, adjusting for gestational age, gender and mechanical ventilation >2 weeks, the differences remained.

Our findings of high mortality rates in cholestatic infants, with even higher rates associated with higher peak conjugated bilirubin levels, are in line with previous reports (90, 102). In our study, as many others, it is difficult to assess the role of cholestasis as the actual cause of death. The finding that higher incidence of cholestasis occurs in lower gestational ages is also affirmative of previous findings (110).

To our knowledge, a long-term perspective on liver disease in preterm infants with transient cholestasis up to ten years of age has not been reported previously. Even though our definition of chronic liver disease later in childhood does not include any liver function tests, we can conclude that it is unlikely that liver disease with any other underlying cause would constitute a significant part of those being transiently cholestatic as preterm infants in the NICU. In order to follow through on this research question, liver function tests could be investigated prospectively as a part of a follow-up-program for preterm infants.

The risk for ROP and BPD were higher in cholestatic infants, and we speculate that there might be a common pathway in these conditions, possibly mediated through an inadequate inflammatory response. Inflammation is suggested to play a role in the development of BPD (236), as well as ROP (237) and early inflammatory markers have been associated with development of cholestasis (238). Immune system development and immune responses in preterm infants are currently being mapped out (239) and will need to be further explored to reveal possible common pathways for various neonatal morbidities.

5.3 RESULTS AND DISCUSSION: PAPER III

Main findings:

- In 69 % (31/45) of cholestatic preterm infants CMV DNA was found in at least one sample from plasma, peripheral blood mononuclear cells (PBMC) or urine.
  In 13 % (3/24) of non-cholestatic preterm infants CMV DNA was found in at least one of these samples ($p<0.00001$).

- In a multivariable model, using logistic regression and calculating crude and adjusted odds ratios adjusting for necrotizing enterocolitis, prolonged parenteral nutrition and gestational age, being CMV DNA positive in any sample type remained independantly associated with cholestasis.

Other findings:

- In cholestatic infants, when comparing the CMV positive to the CMV negative in any sample, the groups did not differ much except regarding mortality and NEC rates:

Table 9. Mortality and NEC rates in paper III.

	Cholestatic (n=45)		
	CMV positive (n=31)	CMV negative (n=14)	
NEC	55% (17/31)	21% (3/14)	p=0.054
Mortality	26% (8/31)	0% (0/14)	p=0.044

Difference in mortality rate just reaching statistical significance, and NEC rate bordering on significance.

These findings have to be confirmed by others. Sampling should then be done from several locations. If CMV is implicated in the development of cholestasis of preterm infants, studies including antiviral treatments could be considered.

5.4 RESULTS AND DISCUSSION: PAPER IV

In this population-based retrospective cohort study, we describe the incidence and risk factors for cholestasis in infants with hemolytic disease of the fetus and newborn (HDFN).

Main findings:

- Cholestasis was found in 7% (11/149) of infants with HDFN.

- Intrauterine erythrocyte transfusions (IUT) and maternal alloimmunization with D-, c- or K-antibodies in combination with at least one other antibody of any type (multiple antibodies) were independent risk factors for cholestasis.

Other findings:

- The median age at onset of cholestasis was 1.1 days.
- The majority of the cholestatic infants had peak conjugated bilirubin level ≥100 μmol/L and were treated with ursodeoxycholic acid.
- Postnatal peak ferritin levels correlated strongly to number of IUTs (Person's r =0.79, p<0.01), but also to peak conjugated bilirubin level (Person's r =0.78, p<0.01).
- By two years of age, none of the infants had chronic liver disease.

To our knowledge, this is the first report with a population-based approach to cholestasis in these infants. Our results, both regarding incidence figures and risk factors are in line with the only similar previous study from the Netherlands (88). The only significant difference is the fact that we found that multiple red cell antibodies in the mother increased the risk for cholestasis, which should be taken into account in future research.

Since onset of cholestasis is early, often already at birth, we stress that when infants with HDFN are followed up postnatally with repeated total bilirubin measurements should also are screened with conjugated bilirubin, at least in the first week of life. This would increase the probability of identifying these infants, and to ensure a correct management. In the future, a prospective multicenter study would be beneficial. As a suggestion, umbilical blood samples should be obtained at birth, to evaluate conjugated bilirubin and bile acids, due to the early onset we found in this study.

Although the course of cholestasis is often resolving as the hemolytic disease process declines over time, more than half of the cholestatic infants had conjugated bilirubin reaching levels exceeding 100 μmol/L, and were treated with ursodeoxycholic acid and received Vitamin K.

The cholestatic disease process has an early onset or may be present at birth, but worsening of cholestasis may occur postnatally. Since inspissated bile is suspected to contribute to the disease process, theoretically, the choleretic properties of UDCA could be advantageous in increasing bile fluidity, but randomized trials will be difficult to perform, and need a multi-center setting to reach enough power.

5.5 METHODOLOGICAL CONSIDERATIONS

5.5.1 Strengths

- The relatively large study populations in all papers, compared to similar studies.
- The population-based approaches in paper I, II and IV,
- Looking for cholestasis in larger cohorts of infants at risk rather than looking for specific etiologies in smaller groups of infants with cholestasis.
- In the absence of larger randomized trials, comparing two eras of using soy-based and olive-based parenteral lipid emulsions in study I, has added to the knowledge on their association to cholestasis.
- The long-term perspectives on liver disease later in childhood in studies II and IV.

5.5.2 Weaknesses

- Retrospective observational design, as in paper I, II and IV, always includes the risk of unknown bias, and limits the possibility of drawing conclusions on causation, which has been clarified and done only with caution in all papers.
- Defining absence of long-term liver disease as absence of diagnosis is a rough measure as compared to performing liver function tests.
- In paper III, the inclusion of a control group in another time period than the cases is a weakness as well as the fact that there were missing data regarding some samples.
- In paper IV, the retrospective design affects the results when conjugated bilirubin tests were not always performed at adequate time points, or not at all in some cases. Incidence of cholestasis may thus be underestimated, and age at detection as a measure of age at onset, may be overestimated. This was taken into consideration when conclusions were drawn from the results.

6 CONCLUSIONS

- **Aim**: To evaluate the population-based incidence and outcome of cholestasis in very preterm infants and identify the most important risk factors.

Conclusions: Cholestasis in preterm infants is a common problem in neonatal intensive care units. As many as one out of seven of infants born before 30 full gestational weeks who survive the neonatal period could be expected to develop cholestasis, at least if a soy-based or olive-based parenteral lipid emulsion are the primary lipid emulsions used.
Cholestasis is more than 300 times more common in these very preterm infants than in term infants in general. Cholestasis in these infants usually develops at a few weeks of postnatal age. Intestinal disease, such as necrotizing enterocolitis, with need for prolonged parenteral nutrition increases the risk for cholestasis markedly.
Cholestasis in preterm infants may in some cases be associated with a more severe outcome, regarding retinopathy of prematurity, bronchopulmonary dysplasia and mortality, possibly through a common inflammatory pathway. Severe cholestasis is associated with higher mortality in this patient group.

- **Aim**: To investigate the role of two different parenteral lipid emulsions in the development and outcome of cholestasis in very preterm infants.

Conclusions: Switching from a soy-based parenteral lipid emulsion to a predominantly olive-based does not affect, or at best slightly reduces, the incidence of cholestasis.
Further studies are needed regarding treatment interventions of cholestasis.

- **Aim:** To investigate the prevalence and importance of cytomegalovirus infection in association with cholestasis in preterm infants.

Conclusions: Cytomegalovirus may be associated with an increased risk for cholestasis, and further studies of its role in the pathogenesis of cholestasis as well as the possible implications with regard to use of antiviral treatment are warranted.

- **Aim**: To evaluate the population-based incidence, risk factors and outcome for cholestasis in term and preterm infants with hemolytic disease of the fetus and newborn (HDFN).

Conclusions: In term and preterm newborn infants with hemolytic disease of the fetus and newborn, the incidence of cholestasis is almost 200 times higher than in term infants in general. The onset of cholestasis is usually early, within the first days of life and is often

present at birth.
In infants with HDFN, screening with total serum bilirubin tests are often performed for early detection of significant hemolysis in need of treatment. Conjugated bilirubin tests should also be performed, at least in the first week of life, to detect cholestasis for correct diagnosis and treatment. Further studies on cholestasis in infants with HDFN focusing on pathophysiology are most warranted, since the knowledge is still scarce.

- **Aim**: To investigate if chronic liver diseases in childhood may have an early presentation in the neonatal period as a transient cholestatic episode after preterm birth, or in infants with HDFN.

Conclusions: If patients survive the neonatal period, cholestasis is rarely an early sign of liver disease later in childhood for at least up to ten years of age in very preterm infants, or two years or longer in infants with HDFN.

7 POINTS OF PERSPECTIVE

7.1 CLINICAL IMPLICATIONS

Efforts should be made to diagnose and treat cholestasis when it occurs in preterm infants, since our findings suggest that it may be associated with poorer outcome, even though further research is needed to evaluate these potential associations. Neonatal wards should have routines to screen for cholestasis in preterm infants, when treated with parenteral nutrition.

For the sole purpose of significantly reducing cholestasis in preterm infants, soy-based parenteral nutrition should not be replaced with a predominantly olive-based, but there may be other reasons for selecting the primary parenteral lipid emulsion that are not examined within this thesis.

Cholestasis should be screened for in infants with HDFN, at least within the first week of life for correct diagnosis and treatment of jaundice.

7.2 FURTHER RESEARCH

Research is ongoing regarding the composition of parenteral lipid emulsions and their role in the development and reversal of cholestasis in preterm and other sick neonates. Larger randomized trials comparing different parenteral lipid emulsions are needed, especially to establish the benefits of fish-oil-containing lipid emulsions.

Enteral supplementation with essential lipids has recently shown very promising results regarding preventing ROP in a randomized trial, and is safe in preterm infants (240). Evaluating cholestasis as an outcome using a similar approach in infants tolerating enteral feeding could be a future proposed study.

The fact that some, but not all, preterm infants develop cholestasis raises the question of genetic predisposition. Investigating preterm infants with cholestasis for less severe biallelic mutations, or for heterozygous severe mutations among the increasing number of known mutations that can cause progressive familial intrahepatic cholestasis (PFIC) could increase our knowledge and understanding.

Further research should in prospective studies focus on the long-term outcomes of cholestasis as a preterm infant, for example the effect on growth and neurocognitive development.

Further studies regarding the role and possible treatment of CMV in cholestatic neonates are also warranted.

In HDFN, prospective studies regarding cholestasis are needed. Analyses of bile acids along with conjugated bilirubin in chord blood and regularly in at least the first week after birth would be suggested. If possible, to evaluate bile acids and conjugated bilirubin in fetuses with

HDFN receiving intrauterine transfusions at the same time as blood samples for hemoglobin levels are drawn, would be very relevant to evaluate the fetal metabolism of bile and to examine if the disease process of cholestasis may already be initiated in the intrauterine life.

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