The role of vitamin A in bile acid synthesis and transport and the relevance for cholestatic liver disease
Hoeke, Martijn Oscar

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Hoeke, M. O. (2013). The role of vitamin A in bile acid synthesis and transport and the relevance for cholestatic liver disease. Groningen: s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 1

The Interrelationship Between Bile Acid and Vitamin A Homeostasis

Martijn O. Hoeke and Klaas Nico Faber

Department of Gastroenterology and Hepatology, University Medical Center Groningen, Groningen, University of Groningen, The Netherlands
Abstract

This thesis explores the regulatory interaction between vitamin A and bile salts. Bile salt homeostasis is of crucial importance to maintain vitamin A homeostasis. Bile salts are the emulsifying components of bile that are required for the effective absorption of the fat-soluble vitamin A in the intestine, most of which is stored in stellate cells in the liver. Dysregulation of bile salt homeostasis, for instance in patients with cholestasis, causes vitamin A malabsorption. At the same time cholestatic liver disease promotes the release of vitamin A from hepatic stellate cells, promoting depletion of vitamin A from the body. A functional relationship in the opposite direction, in which vitamin A influences bile salt homeostasis, has only recently emerged. Complex regulatory pathways ensure that bile salt homeostasis is maintained. These processes involve bile acid sensors, which are ligand-activated transcription factors. This way, bile salts regulate their own synthesis and transport at the transcriptional level. These bile acid sensors need to physically interact with a vitamin A sensor in order to regulate transcription. In this thesis, we show that vitamin A is required to maintain bile salt homeostasis and protects the liver during obstructive cholestasis. This chapter introduces the reader to the origin and function of bile salts and vitamin A and the signalling pathways they control.

1. The enterohepatic circulation of bile acids

The liver is the largest solid organ of the human body and well-known for its regenerative capacity. The liver consists of various cell types, including hepatocytes, stellate cells, cholangiocytes, endothelial cells, Kupffer cells (hepatic macrophages), and oval or progenitor cells, each with their specific role in the function of the liver. Hepatocytes (the hepatic parenchymal cells) make up 80% of the liver mass and fulfil most of the primary functions of the liver. Hepatocytes are arranged in plates that form a physical barrier between the blood in the sinusoid and the bile in the canaliculus. Approximately 80% of the hepatic blood supply enters via the portal vein and is rich in nutrients originating from the intestine. Oxygen-rich blood enters via the hepatic artery, mixes with the portal blood and flows along the hepatic sinusoids and exits via the central vein. The sinusoids (Figure 1.1) are lined with a sheet of endothelial cells, that allows for the exchange of small molecules between blood and hepatocytes. Kupffer cells are integrated into the sheet of endothelial cells. Stellate cells reside in the space of Disse, the plasma-filled space between hepatocyte and sinusoid. Stellate cells (Ito cells, fat-storing or vitamin A-storing cells) are myofibroblast-like cells that contain large cytoplasmic lipid droplets. Cholangiocytes are the bile duct epithelial cells and form approximately 3% of the liver mass (1).

In broad terms, the physiological functions of the liver can be described as, synthesis, storage, metabolism, catabolism and excretion. Proteins synthesized by the liver include serum proteins such as albumin, different coagulation factors (such as fibrinogen) and the retinol binding protein 4 (RBP4).
The Interrelationship Between Bile Acid and Vitamin A Homeostasis

Cholesterol and triglycerides are synthesized by the liver. Moreover, it is the main storage site of glucose (in the form of glycogen), iron and vitamin A. Another important function of the liver is detoxification. Endogenous waste products (such as bilirubin) and exogenous harmful compounds (drugs and toxins) are metabolized by the liver. This makes them less toxic and increases water solubility, which enhances clearance of these compounds. The liver is also the largest gland of the human body, amongst others synthesizing and excreting the hormone insulin-like growth factor 1 (IGF-1) and producing bile. Bile aids in the digestion and absorption of nutrients and is the main route for the excretion of waste products via the intestine. Major components of bile are bile salts, phospholipids, cholesterol, bilirubin, alkaline hydrogen carbonate ions ($\text{HCO}_3^-$) and water. Bile salts are the components of the bile that give it its fat-emulsifying properties. Bile salts are synthesized by hepatocytes using cholesterol as substrate. The liver secretes bile via the bile ducts and the gallbladder into the intestinal lumen (duodenum). Here, bile fulfils its function in the digestion and absorption of fats and fat-soluble dietary compounds. In the terminal ileum, bile salts are reabsorbed and transported back to the liver via the portal vein. This shuttling of bile salts between liver and intestine is called the enterohepatic circulation of bile salts and is a very efficient process as per cycle only 5% of the bile salts are lost in the feces. *De novo* synthesis of bile salts in the liver compensates for this loss and maintains a balanced amount of bile salts in the enterohepatic cycle.

**Figure 1.1. Structure of the liver sinusoid**
The liver possesses a dual blood supply. Nutrient-rich blood coming from the portal vein and oxygen-rich blood coming from the hepatic artery merge upon entering the sinusoid. The sinusoids are lined with endothelial cells and Kupffer cells. The plasma-filled space of Disse separates the endothelial cells from the hepatocytes. Bile produced by the hepatocytes is excreted into the bile canaliculi that empty into the bile ductule, which is formed by cholangiocytes. Vitamin A-storing stellate cells reside within the space of Disse. Reprinted from Frevert (2004) (361).
1.1. Bile Acid/Salt Biosynthesis
Bile acid synthesis produces two main types of primary bile acids: cholic acid (CA) and chenodeoxycholic acid (CDCA). These are conjugated to either glycine or taurine, yielding the bile salts taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA) and glycochenodeoxycholic acid (GCDCA). Bile salts that are not absorbed at the terminal ileum move to the colon where resident bacteria may convert them to secondary bile acids, such as lithocholic acid (LCA) and deoxycholic acid (DCA). A significant fraction of these secondary bile acid are also absorbed into the circulation. DCA accumulates in the bile acid pool, LCA to a much lesser extent as it is sulfated and subsequently excreted into bile (reviewed in (3)). The term bile “acid” refers to the protonated (-COOH) form, while bile “salt” refers to the deprotonated or ionized (-COO⁻) form. Bile acids conjugated to glycine or taurine are largely deprotonated at the physiological pH, and should thus be regarded as bile salts. The ionized conjugated bile salt is more hydrophilic when compared to the unconjugated bile acid. Conjugated bile acids are therefore more efficient in emulsifying fats than their unconjugated counterparts. Cholesterol is oxidized to bile acids through a cascade of enzymatic conversions involving at least 13 different enzymes (see for a review (4)). Although the first steps of the conversion from cholesterol to bile acids may take place at extra-hepatic locations, the production of the end products, e.g. bile salts, is restricted to the hepatocytes in the liver. Two enzymes, cholesterol 7-alpha-hydroxylase (CYP7A1) and sterol 12-alpha-hydrolase (CYP8B1) are of particular relevance. CYP7A1 is considered the rate-limiting enzyme in bile acid synthesis (5), while CYP8B1 converts CDCA to CA and thus determines the relative hydrophobicity of the bile acid pool (6). The final step in bile acid synthesis is the conjugation of taurine or glycine and is exclusively performed by the peroxisomal enzyme bile acid coenzyme A: amino acid N-acyltransferase (BAAT) (7). Bile acid synthesis is increased following feeding and glucose and insulin are important factors that induce CYP7A1 gene expression (8, 9).

1.2. Transporter Proteins Involved in (Bile Formation and) Bile Salt Transport
Once conjugated, membrane translocation of bile salts requires active transport by membrane-imbedded transporters. These transporters reside in the membranes of polarized hepatocytes, cholangiocytes and enterocytes and keep the bile salts in the enterohepatic circulation. The transporter proteins involved in the enterohepatic circulation have been the subject of multiple reviews (10-13). A schematic overview of the enterohepatic circulation of bile salts and the transporter proteins involved is depicted in Figure 1.2.

1.2.A. Hepatic Bile Salt Transport Mechanisms

**Hepatic Basolateral Transport/Bile Salt Import**
Bile salts returning from the intestine via the blood circulation are absorbed into the hepatocyte by the sodium/taurocholate co-transporting polypeptide (NTCP) (15, 16)
and at least three members of the organic anion transporting polypeptide (OATP) family (17). A recent study showed that also unconjugated bile acids are subject to active transport via OATP1B2 rather than entering the cell by passive diffusion (18). NTCP (SLC10A1) belongs to the SLC10 family, which comprises transport proteins that transport organic salts in a sodium-dependent manner. The human NTCP protein consists of 349 amino acids with a molecular mass of 56 kDa. In the liver, NTCP is expressed exclusively at the basolateral membrane of the hepatocyte and preferentially transports taurine conjugated bile acids. (15, 19, 20). However, also various other physiological bile salts and the steroid conjugate estrone 3-sulfate are substrates of NTCP (21). Expression of NTCP in pancreatic acinar cells has also been reported (22).

The superfamily of OATPs comprises a family of transporters with a broad spectrum of substrates. The substrate specificity of OATPs is reviewed in detail by König et al. (23). While most of the OATP proteins are expressed in multiple tissues, OATP1B1 (OATP2/OATP-C/SLCO1B1/SLC21A6) and OATP1B3 (OATP8/SLCO1B3/SLC21A8) are predominantly if not exclusively expressed in liver at the basolateral membrane of hepatocytes. OATP1B1 consists of 691 amino acids with a molecular mass of 84 kDa. OATP1B3 consists of 702 amino acids and represents a glycoprotein with a molecular mass of 120 kDa. Both of these OATPs were shown to transport taurine- and glyco-conjugated CA (10, 23-25). OATP1A2 (OATP-A/SLCO1A2) is mainly expressed in brain and liver. It has a molecular mass of 85 kDa and consists of 670 amino acids. The rodent ortholog is OATP1A1, which has 12 putative transmembrane spanning domains. OATP1A2 is localized to the basolateral membrane of the hepatocyte and is reported to transport both conjugated and unconjugated CDCA and CA (10, 26, 27).

**Hepatic Intracellular Transport of Bile Acids**

The cytosolic liver fatty-acid-binding protein (L-FABP) is involved in fatty acid metabolism and transport. Moreover, L-FABP was found to bind bile acids in a 1:2 ratio and to be excreted into bile (28, 29). Although bile acid metabolism is altered in *L-fabp*−/− mice, the physiological role of L-FABP in bile acid trafficking remains to be determined (30).

Part of the bile salt pool that enters the liver is deconjugated by intestinal flora when passing through the intestine. Before excretion into bile, *de novo* and recycled bile acids need to be (re-)conjugated. The enzyme responsible for bile acid conjugation (BAAT) resides solely in peroxisomes, which suggests the existence of intracellular bile acid/salt transporters. Although no intracellular transporter has been identified to date, recent data from our laboratory strongly supports the existence of such bile salt transporters in the peroxisomal membrane (4, 7, 31).
The enterohepatic circulation of bile salts (BSs) requires dedicated transporter proteins on both sides of polarized hepatocytes, cholangiocytes and enterocytes. Bile salts returning from the intestine via the blood circulation are absorbed into the hepatocyte by the sodium/taurocholate co-transporting polypeptide (NTCP) and at least three members of the organic anion transporting polypeptide (OATP) family. Conjugated monovalent BSs are excreted from the hepatocyte via the canalicular bile salt export pump (BSEP). Bivalent BSs, together with organic anions (OA\(^{-}\)), are excreted via the canalicular multidrug resistance-associated protein 2 (MRP2). The ABC half-transporters ABCG5/G8 secrete cholesterol into bile. The multidrug resistance protein 3 (MDR3) and the familial intrahepatic cholestasis type 1 (FIC1) protein excrete phosphatidylcholine (PC) and phosphatidylserine (PS), respectively, which form mixed micelles in bile together with cholesterol and BS\(^{-}\). Organic cations (OC\(^{+}\)) are excreted by the efflux pump for xenobiotic compounds (MDR1). Cholangiocytes and enterocytes use similar transport mechanisms for BS\(^{-}\) shuttling. At the apical membrane, BS\(^{-}\) are taken up by the ileal bile salt transporter (ASBT). Cellular transport of BS\(^{-}\) in the enterocyte is facilitated by the ileal bile acid binding protein (IBABP). Excretion of BS\(^{-}\) is primarily performed by the organic solute transporter dimer (OST\(\alpha/\beta\)). Besides excreting OA\(^{-}\), the multidrug resistance-associated protein 3 (MRP3) provides an alternative route for the elimination of BS\(^{-}\). Both OST\(\alpha/\beta\) and MRP3 are also expressed at...
The interrelationship between bile acid and vitamin A homeostasis

The basolateral membrane of hepatocytes, in particular in pathological conditions. Here they provide an alternative route for the elimination of BS and other OA. MDR1 is also present in the cholangiocytes and enterocytes at the apical membrane, while MRP2 is also expressed in ileum. BS excreted by the enterocytes/cholangiocytes return to the liver via the blood, closing the enterohepatic circulation. Inspired by Trauner et al. (2003) (11) and Zollner et al. (2006) (276).

Hepatic canalicular bile salt export

Conjugated recycled and de novo synthesized bile salts are excreted into bile by the bile salt export pump (BSEP/ABCB11). The 160 kDa BSEP-protein is exclusively expressed at the canilicular membrane of hepatocytes and belongs to the B subfamily of ATP-binding cassette (ABC) transporters. A total of 48 ABC type transporters have been identified in the human genome. BSEP contains 12 membrane spanning domains and two intracellular loops that contain nucleotide/ATP binding motifs, which is the typical build-up of an ABC transporter. Mutations in the BSEP gene are associated with progressive familial intrahepatic cholestasis type 2 (PFIC2), which is characterized by severe and progressive liver disease. In addition, mutations in BSEP may cause benign recurrent intrahepatic cholestasis (BRIC) that is characterized by intermittent attacks of cholestasis without permanent liver damage (32-37).

The multidrug resistance-associated protein 2 (MRP2/ABCC2) transports a variety of organic anions at the canalicular membrane into bile, including conjugated bilirubin (38). MRP2 belongs to the C subfamily of ABC transporters (MRPs), of which ten members (MRP1 to 9 and pseudo gene ABCC13) have been identified (39). Besides the liver, MRP2 is expressed in the intestine (duodenum) and kidney (40). MRP2 is capable of transporting bivalent glucuronidated and sulfated bile salts, and was recently also shown to transport tauroursodeoxycholate (41). The role of MRP2 in maintaining bile salt homeostasis may differ between species. Mutations in MRP2 lead to Dubin-Johnson syndrome in humans (42), a benign form of jaundice (43, 44), transport of bile salts into bile is not disturbed in these patients. However, Groningen yellow (GR/TR) rats, which lack a functional MRP2 protein, display an impaired bile flow (45).

Additional hepatic canalicular transporter proteins that contribute to bile formation

The multidrug resistance protein 3 (MDR3/ABCB4; the rodent ortholog is MDR2) and the ABCG5/ABCG8 dimer transport phospholipids and cholesterol, respectively, from the inner to the outer leaflet of the canalicular membrane. Bile salts excreted into the canaliculus interact with the outer leaflet of the hepatocyte's apical membrane and extract cholesterol and phospholipids thereby forming mixed micelles (46). Like BSEP, MDR3 belongs to the B subfamily of the ABC transporter family and shares the 12 membrane spanning domain structure. Defects in the MDR3 gene cause progressive familial intrahepatic cholestasis type 3 (PFIC3). In this disease, phospholipid transfer to bile is disturbed. Lack of phospholipids hampers the formation of the mixed micelles, increasing the concentration of pure micelles in bile, resulting in damage to the bile duct epithelium and cholestasis (47, 48).
Another member of the B subfamily of ABC transporters is \textit{ATP8B1}, it encodes FIC1, named after the associated disease familial intrahepatic cholestasis type 1 that is caused by mutation in the \textit{ATP8B1} gene (reviewed in (49)). The FIC1 protein acts as a flippase for phosphatidylserine. Although FIC1 does not transport bile salts, mutations in the \textit{ATP8B1} gene cause cholestasis. The proposed mechanism for this observation is that in the absence of functional FIC1 protein the membrane gets depleted of lipids (including cholesterol) in the process of bile formation. Lack of cholesterol in the membrane limits the function of BSEP resulting in intracellular bile salt accumulation (49).

ABCG5 and ABCG8 are members of the G subfamily of the ABC-transporters. They are so-called half-transporters that form a heterodimer to become a functional cholesterol transporter. ABCG5/G8 is localised to the apical membrane of hepatocytes, and cholangiocytes and enterocyte (50, 51). Defects in either the murine \textit{Abcg5} or \textit{Abcg8} gene are associated with decreased excretion of cholesterol in bile and an increased absorption of cholesterol in the intestine (52).

The multidrug resistance-1 P-glycoprotein (MDR1/ABCB1) is an efflux pump for xenobiotic compounds and has a broad substrate specificity. It is associated with the resistance of tumor cells against chemotherapeutic drugs. MDR1 is expressed at the canalicular membrane of hepatocytes and the apical side of the bile ductual and intestinal epithelium (53). Apart from metabolites of LCA, bile salts do not seem to be substrates for MDR1 and malfunctioning of MDR1 does not result in cholestatic liver disease (54). However, accumulation of certain drugs and substrates for MDR1 may inhibit the function of BSEP (12, 55). Interestingly these mice have increased MDR1A expression while expression of MDR2, MRP2, and MRP3 (the latter will be discussed later) was only slightly increased (57). This suggests that MDR1A in mice might be involved in bile salt transport in the absence of BSEP.

\textbf{1.2.B. Bile Ductular and Intestinal Bile Transport Mechanisms}

After hepatobiliary secretion, bile salts travel through the bile ducts and are stored in the gallbladder. Triggered by food ingestion, the gallbladder contracts and bile is released into the duodenum where bile salts fulfil their function in the digestion and absorption of fat-soluble nutrients. At the terminal ileum, they are efficiently reabsorbed to the circulation and returned to the liver via the portal tract. The bile ductular and distal ileum epithelial cells, cholangiocytes and enterocytes, contain highly similar bile salt transport mechanisms (\textbf{Figure 1.2}).

\textbf{Apical Sodium-dependent Bile Salt Import}

The apical sodium-dependent bile salt transporting protein (ASBT/SLC10A2) was initially identified as the intestinal analogue of NTCP, belonging to the same SLC10 family of sodium-dependent organic ion transporters. Subsequently, ASBT was also shown to facilitate bile salt absorption at the apical domain of rat cholangiocytes.
The membrane topology and transport characteristics are similar to NTCP. The ASBT glycoprotein consists of 348 amino acids and has a molecular mass of 48 kDa (12, 19, 58-60). Mutation in the SLC10A2 gene have been associated with primary bile salt malabsorption and patients suffer from diarrhea and fat malabsorption (61).

**Intracellular Bile Salt Transport**

The intestinal bile acid binding protein (IBABP/FABP6) is expressed in enterocytes and cholangiocytes. IBABP represents a protein of 128 amino acids with a molecular mass of 14-15 kDa. IBABP is proposed to be the most important protein for transcellular bile salt movement in enterocytes. However, its physiological function remains elusive, as the absence of IBABP, as observed in Fxr-/- mice, does not affect the turnover rate and cycling time of the cholate pool (62). Recent data obtained with Fabp6-/- mice show that IBABP is not prerequisite for ileal bile acid absorption but that it does contribute to efficient shuttling of bile acids through the enterocyte (63). Additionally, binding of bile salts by IBABP has also been suggested to prevent intracellular toxicity (12, 64, 65).

**Basolateral Bile Salt Efflux Mechanisms**

The identity of the transporter that facilitates the efflux of bile salts from ileal enterocytes across the basolateral membrane to the circulation has long been unclear. Initially, it was proposed that this activity was performed by a truncated form of ASBT (t-ASBT) that is a product of alternatively splicing of the corresponding transcript. T-ASBT was shown to be capable of transporting TCA in vitro and to be expressed at the basolateral membrane. T-ASBT was identified in ileum, kidney, and bile ductual epithelial cells but not in hepatocytes (12, 66). A second transporter that was proposed to export bile salts from enterocytes was the multidrug resistance-associated protein 3 (MRP3/ABCC3). MRP3 is expressed in various tissues of the gastrointestinal tract, including liver and ileum, where it was shown to be expressed at the basolateral membrane of cholangiocytes and enterocytes, respectively. MRP3 was shown to be capable of transporting various endogenous bile salts. However, mice lacking Mrp3 do not show altered bile salt physiology. This is in line with the observation that MRP3 expression is rather low in human liver under healthy conditions (39, 67-71).

With the identification in 2005 of the organic solute transporter alpha/beta (OSTα/β) as the main functional basolateral bile salt transporter in the mouse ileum, the missing link in bile salt circulation was identified (72, 73). OSTα/β consists of an α- and a β-subunit that together form a functional transporter at the basolateral membrane. OSTα/β was initially identified as an organic solute and steroid transporter in the marine vertebrate little skate (74). OSTα is a 340 amino acid protein, and is predicted to contain seven transmembrane domains. The β-subunit is a much smaller protein of 128 amino acids predicted to have a single transmembrane domain (75). The human/rodent homologs were found to export various endogenous bile salts. OSTα/β is predominantly expressed at the basolateral membrane of intestinal epithelium and cholangiocytes in liver, with less but detectable amounts also in renal proximal tubu-
lar cells in kidney and hepatocytes in the liver (76). Interaction between the α- and β-subunits is essential for protein stability and efficient trafficking through the Golgi apparatus to the plasma membrane. Ostα/β mice have normal Ostβ mRNA levels, but the OSTβ protein is absent (77). Emerging evidence suggests a pleiotropic function of OSTα/β that is not limited to bile salt transport (78). Bile salts that are excreted to the circulation by the enterocytes are transported back to the liver, closing the circle of the enterohepatic circulation.

1.3. The Adaptive Response to Bile Salt Accumulation

Bile salt homeostasis is maintained by coordinated regulation of bile salt synthesis and transport. When bile acid concentrations increase, for instance when mice are fed a bile salt-containing diet, expression of both Cyp7a1 and Cyp8b1 is reduced, which represses bile acid synthesis. At the same time expression of Ntcp is lowered and Bsep expression is increased (79). In obstructive cholestasis, as in patients with primary biliary cirrhosis (PBC) and in bile duct-ligated rodents, hepatic expression of OSTα, OSTβ, MRP3 and multidrug resistance-associated protein 4 (MRP4/ABCC4) is increased (80-83). Intestinal expression of ASBT mRNA is lowered by elevated bile salt levels (84). The fact that Mrp4/− mice have an impaired cytoprotective response in obstructive cholestasis exemplifies that these adaptive responses are crucial to prevent excessive liver damage when bile salt flow from the liver is impaired (83). Although MRP3 was shown to transport (secondary) bile salts in vitro, bile duct-ligated Mrp3−/− mice do not suffer more liver damage than wild type mice (69). In this case MRP4 may be sufficient to prevent excessive liver damage. These mechanisms protect the hepatocyte and enterocyte from cytotoxic accumulation of bile acids. Regulation of these adaptive mechanisms will be discussed later.

2. Vitamins, essential micronutrients

In 1916, vitamin A was one of the first vitamins that was discovered (85). Historically, the word “vitamine” was used to describe a growth factor that is present in food and that is essential for life. The combination of the words “vital” and “amine” yielded “vitamine”. As it became apparent that there were multiple vitamins, McCollum divided them into two classes: “fat-soluble factor A” and “water-soluble factor B”. Following the discovery that not all of these micronutrients shared the amine-group in their molecular structure, Drummond changed the name to “vitamin”, dropping the final “e”. He renamed McCollum’s compounds into vitamin A and vitamin B and new members of these series of factors were named vitamin C, vitamin D etc. (85, 86). Water-soluble vitamins are readily expelled from the body, hence, regular (daily) intake is required. In contrast, fat-soluble vitamins are stored in the liver and adipose tissues. Stable levels of these vitamins can therefore be maintained for a longer time period. The downside of this is that excessive intake of the fat-soluble vitamins A, D, E and K poses a greater risk of vitamin intoxication than vitamins B and C. The uptake of fat-soluble vitamins requires the presence of bile salts in the intestinal lumen. Cholestatic liver diseases, such as PBC, primary sclerosing cholangitis (PSC), bilary
The Interrelationship Between Bile Acid and Vitamin A Homeostasis

ataresia and gallstone disease, which dysregulate the flow of bile to the intestine, may lead to malabsorption of fat-soluble vitamins and associated clinical symptoms of hypovitaminosis.

The primary focus of this thesis is the fat-soluble vitamin A, but also the other fat-soluble vitamins will be briefly discussed.

2.1. Vitamin A

In 1930, Moore proved that the yellow plant pigment beta-carotene is converted to colourless retinol in the mammalian body (87). In 1931, Karrer deduced the molecular structure of retinol and beta-carotene (85, 86) (Figure 1.3A-B). Nowadays, the term vitamin A is used as the generic descriptor for retinoids exhibiting the biological activity of retinol. Retinoids are a class of compounds consisting of four isoprene (2-methyl-1,3-butadiene) units joined in a head-to-tail manner. (86).

Vitamin A-derivatives fulfil numerous important functions in the mammalian body, including roles in vision, maintenance of epithelial surfaces, immune competence, reproduction and embryonic growth and development (88). Dietary sources of vitamin A are provitamin A carotenoids (mainly β-carotene, from plant sources), preformed vitamin A (retinyl esters from animal sources) and precursors of retinol (88). The recommended daily intake of vitamin A is approximately 700 and 900 µg for adult women and men, respectively. Dietary intake of solely β-carotene may be inadequate to maintain normal levels of vitamin A, retinyl esters should therefore be considered an essential component of a healthy diet (89). Approximately 80% of the total body pool of vitamin A is stored in the liver as retinyl esters (90).

Some of vitamin A’s numerous biological functions are related to its antioxidant properties. Especially carotenoids, such as lycopene and β-carotene, are potent antioxidants (91, 92), but also retinol, retinyl esters and retinoic acid (RA) have been shown to prevent lipid peroxidation in vitro (93, 94). Another well-known function of vitamin A is its role in the visual cycle by photoisomerization. Rhodopsin, the visual pigment of the rod photoreceptor cell, contains 11-cis retinal as its light-sensitive cofactor. Light activation is achieved by 11-cis to all-trans isomerisation, followed by the release of all-trans retinaldehyde (95).

However, most of the biological functions of vitamin A involve the activation of ligand-dependent transcription factors. This hormonal function of vitamin A gained tremendous scientific interest with the discovery of two vitamin A receptors that are members of the nuclear receptor superfamily (96). All-trans retinoic acid (atRA) and 9-cis retinoic acid (9cRA) (Figure 1.3C-D) are the two biologically active isomers of RA that regulate gene transcription via the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). These nuclear receptors will be discussed in section 4.
Chapter 1

β-carotene

retinol

all-trans retinoic acid

9-cis retinoic acid

calcitriol

phylloquinone

menaquinones

α-tocopherol

Figure 1.3. Fat soluble vitamins A, D, E and K

Beta- (β-) carotene (A) is the primary source of vitamin A in plant-derived foods, which can be cleaved into two molecules of retinol (B). Retinol and its esters are the main transport and storage forms of vitamin A. The 2 predominant biologically active forms of vitamin A are all-trans and 9-cis retinoic acid (C and D). The biologically active compound of vitamin D is calcitriol (1,25-dihydroxycholecalciferol or 1,25-dihydroxyvitamin D₃) (E). Alpha- (α-) tocopherol (F) is the most abundant form of vitamin E in nature, it also has the highest biological activity. Phylloquinones (G) are the primary dietary source of vitamin K, while menaquinones (H) are produced by bacteria in the gastro-intestinal tract.

2.2. Vitamin D, Important Micronutrient But Not Really a Vitamin

Vitamin D was discovered as an essential nutrient to prevent rickets, e.g. softening of bones leading to skeletal deformations in children and an increased risk of fractures later in life. Vitamin D deficiency can result in osteoporosis (decreased bone mineral density) in adults, causing osteomalacia (softening of bone), muscle weakness and increased risk of fractures (97). Vitamin D is required for optimal absorption of dietary calcium and phosphate and plays an essential role in calcium homeostasis.
and bone metabolism. UV-light irradiation of the skin was found to prevent rickets as this stimulates formation of vitamin D$_3$ in the skin. The ability to produce sufficient amounts of vitamin D$_3$ with adequate sunlight exposure indicates that vitamin D is actually not a vitamin (98). The name “vitamin D” refers to a group of steroids that exhibit the biological activity of cholecalciferol. The two physiologically relevant forms are vitamin D$_2$ (ergocalciferol) and vitamin D$_3$ (cholecalciferol). Very few foods contain significant amounts of vitamin D. D$_2$ is plant derived and is present in mushrooms that are exposed to UV-light. The animal-derived D$_3$ is present in, amongst others, fish oil, egg yolk and liver. (97, 99).

In the liver, vitamin D$_3$ is metabolized to 25-hydroxycholecalciferol (calcidiol, 25-(OH)D), which is subsequently metabolized by the kidney into the biologically active steroid hormone 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] (Figure 1.3E) (97). Vitamin D is stored in adipose tissues as calcidiol. Hypovitaminosis D may develop through a combination of inadequate vitamin D intake and insufficient exposure to sunlight in otherwise healthy people (97, 100). In addition, vitamin D deficiency may develop as a result of multiple disease conditions, including impaired synthesis in the skin, impaired intestinal absorption as well as acquired and heritable disorders of vitamin D metabolism and responsiveness.

2.3. Vitamin E, Solely Antioxidant or More

In 1922, vitamin E was first described as an essential nutrient for reproduction in rats. Vitamin E is the generic description for α-, β-, γ-, and δ- tocopherols and the corresponding four tocotrienols. Alpha-tocopherol is the most abundant form in nature, which has the highest biological activity and reverses symptoms of vitamin E deficiency in humans (Figure 1.3F) (101-103). Nuts, seeds, and vegetable oils are among the richest sources of alpha-tocopherol and significant amounts are present in green leafy vegetables. Tocopherol is a potent antioxidant that prevents lipid peroxidation of polyunsaturated fatty acids in cellular membranes. In addition to its antioxidant activities, vitamin E is reported to be involved in cell signalling, immunity, gene expression and metabolic processes (104). However, others believe that all vitamin E actions arise from its antioxidant protection of polyunsaturated fatty acids in the plasma membrane, and that vitamin E is nothing more than an antioxidant (105). The liver plays a central role in α-tocopherol homeostasis. Vitamin E travels the body as a component of lipoproteins that are taken up (chylomicrons, low density lipoprotein (LDL) and high density lipoprotein (HDL)) and excreted (very low density lipoprotein (VLDL)) by the liver. One third of the body content of vitamin E is stored in the liver, mainly in hepatocytes (106).

2.4. Vitamin K

Vitamin K refers to a group of chemically similar compounds called naphthoquinones. The naturally occurring forms of vitamin K are K$_1$- (phylloquinone) and the K$_2$-compounds (menaquinones) that contain unsaturated side chains of varying length (Figure 1.3G-H). Phytonadione is found in plants and is the primary
dietary source of vitamin K for humans. Menaquinones are produced by bacteria in the gastrointestinal tract and contribute to the intake of vitamin K (107-109). Vitamin K is required for normal blood clotting. In fact, the name vitamin K is derived from “Koagulations-Vitamin” in German and Scandinavian languages (110). It acts as a cofactor for γ-glutamyl carboxylase. This enzyme is responsible for post-translational modification of specific glutamate residues in proteins to γ-carboxyglutamate. The majority of γ-carboxylated proteins function in blood coagulation, while others play a role in calcium homeostasis and normal bone metabolism (111, 112).

Green vegetables, such as Brussels sprouts, spinach, cauliflower and broccoli, as well as certain vegetable oils, including soybean oil, rapeseed oil and olive oil, are rich dietary sources of vitamin K. Animal-derived foods contain limited amounts of vitamin K (109).

Vitamin K deficiency occurs when intake is inadequate or when the intestinal flora is altered. Clinical symptoms of vitamin K deficiency include clotting disorders (e.g. vitamin K deficiency bleeding (VKDB)) and increased risk of bone fractures. Toxicity of excess vitamin K has not been reported (108, 113).

2.5. Liver Disease and Occurrence of Fat-soluble Vitamin Deficiency

Fat-soluble vitamin deficiency is observed in patients suffering from liver diseases of various etiologies. A recent study reported deficiencies of vitamin A (29%), vitamin D (71%), vitamin E (6%) and up to 68% showed signs of subclinical vitamin K deficiency, in a group of 31 patients suffering from cholestatic liver disease (114). Similarly, a prevalence of fat-soluble vitamin A, D, E and K deficiency is reported to be 33.5%, 13.2%, 1.9% and 7.8%, respectively, in a group of over 150 PBC patients (115). In PSC patients a prevalence of vitamin A, D and E deficiency was found of respectively 40%, 14% and 2%. In pre-transplant patients prevalence was increased to 82%, 57% and 43%, respectively (116, 117). A recent study by De Paula et al. reports 60% of patients (35 out of 58) suffering from liver cirrhosis have lowered plasma retinol levels, 23 of these patients were diagnosed as vitamin A deficient (VAD) (118). More than half (54.3% out of n = 140) of patients suffering from hepatitis C virus-related chronic liver disease were VAD (119). The vast majority (85%) of patients (n = 147) with alcoholic liver cirrhosis have a compromised vitamin D status, 37% of these patients were vitamin D deficient (VDD). In the same study, 47% of PBC patients (n = 58), had lowered vitamin D serum levels, 18% of these patients were VDD (120). Sokol et al. reported that 17% of PBC patients (7 out of 42) suffered from vitamin E deficiency. In multiple studies, patients suffering from PBC, biliary atresia and autoimmune hepatitis presented lower serum levels of vitamins A, D, E and K when compared to controls, without reaching deficiency (120-123). The prevalence of fat-soluble vitamin deficiencies varies between studies because of differences in disease etiology, group size, patient age and the definition of “deficiency”. Collectively, these studies identify vitamin A deficiency as the most frequent deficiency of all fat-soluble vitamins in (cholestatic) liver diseases.
2.6. Supplementation of Fat-soluble Vitamins and Liver Disease
Guidelines for supplementation of fat-soluble vitamins to patients suffering from liver disease differ per country. In literature, fat-soluble vitamin supplementation is recommended in patients suffering from cholestatic liver disease, especially in neonates and infants, as stores of fat-soluble vitamins are low at birth (124, 125). Vitamin A supplementation is recommended as a single 50,000 IU dose every 15 days for adults and every month for children. However, no clear guidelines exist for vitamin A supplementation for patients with liver disease in The Netherlands. Administration of vitamin E, 100-200-400 IU/day, is recommended for all patients with cholestasis and neurological symptoms of unknown etiology. Vitamin K malabsorption is rapidly corrected by subcutaneous administering of vitamin K, 10 mg/day for 3 days, followed by long-term oral supplements of vitamin K, 5-10 mg/day or 10 mg/month subcutaneously (126, 127).

When fat-soluble vitamin supplements are given attention should be paid to the route of administering fat-soluble vitamin supplements. Oral supplementation of fat-soluble vitamins to (obstructive) cholestatic patients may not be as efficient as subcutaneous supplementation, since fat absorption is disturbed in these patients because of limited bile flow to the intestine.

3. Vitamin A Metabolism

3.1. Ileal Vitamin A Absorption Requires Bile Salts
Various forms of -precursors of- vitamin A are entering the digestive tract depending on the composition of the diet. Plant carotenoids are thought to be absorbed into the intestinal epithelium by passive diffusion after being incorporated into micelles that mainly consist of bile salts and dietary fats. However, recent studies in Caco-2 monolayers suggest a facilitated uptake mechanism of carotenoids that involves scavenger receptor class B member 1 (SR-BI) (128, 129). Retinyl esters are first converted to retinol within the intestinal lumen, followed by uptake in the enterocyte (87). Unesterified retinol enters the cell by diffusion, but the efflux of retinol may be partially facilitated by the ABC transporter ABCA1 (128). The pancreatic triglyceride lipase (PTL) and the intestinal brush border membrane enzyme phospholipase B are responsible for the hydrolysis of retinyl esters. The enzymatic activity of PTL depends on the presence of bile. Lipases, such as PTL, act on a water-lipid interface and bile aids in the formation of such interfaces. The emulsifying properties of bile salts stabilize the interface and bile phospholipids are part of the interface (130). Administration of bile salt sequestering agents to humans lowers the total carotenoid levels in serum (131), while administration of the bile salt TCA enhanced vitamin A absorption in rats (132), further underscoring the role of bile salts in vitamin A absorption.

3.2. Vitamin A Transport
Beta-carotene can be converted to retinoids inside the enterocyte (87). Symmetric cleavage of one molecule β-carotene by beta-carotene 15,15’-monooxygenase 1
Chapter 1

(BCMO1) yields two molecules of retinal (133). Alternatively, via a series of asymmetric cleavages one molecule of β-carotene can be converted to one molecule of retinal. Subsequently, retinal is reduced in a reversible process to retinol. Several enzymes are capable of catalysing this conversion, including members of the short- and medium-chain alcohol dehydrogenase/reductase superfamily that will be discussed later. The intestinal reductase activity, however, remains to be identified (134).

Free retinol is bound by the cellular retinol-binding protein type II (CRBP-II/RBP2). Most of the retinol absorbed by the enterocyte is re-esterified to saturated long-chain fatty acids, mainly palmitic acid. Binding of retinol to CRBP-II facilitates the esterification of retinol by lecithin:retinol acyl tranferase (LRAT). Uncleaved carotenoids and newly-synthesized retinyl esters, but not the ingested retinyl esters, are incorporated into chylomicrons (CMs) and secreted into lymph (128, 135, 136).

Chylomicrons are very heterogeneously-sized particles that consist of a core of triglycerides and cholesterol esters and a monolayer of phospholipids, cholesterol and proteins. CMs are excreted by the enterocyte in the postprandial state after fat ingestion. CM excretion is impaired in the absence of bile salts (137). Two clinical manifestations of impaired CM assembly are abetalipoproteinemia and CM retention disease. Patients suffer from neurologic disorders and visual impairment. In a mouse model for CM retention disease it was observed that the absorption of fat, vitamin A and E was severely impaired and the mice showed significantly reduced growth rates (135), underscoring the importance of CM in the efficient absorption of fat and fat-soluble vitamins, such as vitamin A.

Although most retinoids leave the enterocyte as retinyl esters, also a considerable amount of free retinol is released into the portal circulation (130).

3.3 Vitamin A Storage and Distribution

Chylomicrons distribute nutrients to the various tissues and the chylomicron remnants are subsequently cleared by the hepatocyte. Most of the retinyl esters are still present in these remnants. Within the hepatocyte, retinyl esters are again hydrolysed to retinol. The trafficking of vitamin A throughout the body is depicted in Figure 1.4. Free hepatic retinol is now either used directly for distribution to tissues or stored in the liver. For distribution throughout the organism, retinol associates with RBP4, followed by secretion into the blood circulation. Approximately 95 % of plasma retinol-RBP4 is complexed with transthyretin (TTR) in a 1:1 ratio. This interaction reduces glomerular filtration of retinol (88, 138). Extrahepatic tissues take up retinol by means of the RBP4-receptor “Stimulated by Retinoic Acid gene 6 homolog” (STRA6). The retinol-RBP4-TTR complex dissociates at the receptor and free retinol is taken up by the cell. Free RBP4 in the circulation is catabolized in the kidney tubules. The second direction is transport of retinol to, and storage in, the hepatic stellate cell. Here, free retinol is esterified with long chain fatty acids, again predominantly palmitate, for storage in cytosolic lipid droplets. Retinol esters make up 30-50% of the lipid content of the lipid droplets in stellate cells (90).
The Interrelationship Between Bile Acid and Vitamin A Homeostasis

Prior to absorption, dietary retinyl esters (RE) are hydrolysed to retinol (ROH) in the intestinal lumen, while carotenoids are absorbed and converted intracellularly to retinol by the enterocyte. In the enterocyte, retinol reacts with fatty acids to form retinyl esters, which are incorporated into chylomicrons (CM). Via the intestinal lymph chylomicrons reach the general circulation. Chylomicron remnants (CMR), which still contain most of the retinyl esters, are formed within the blood capillaries. These are cleared by the liver parenchymal cells and to some extend by extrahepatic tissues. In the hepatocytes, retinyl esters are hydrolysed to retinol, which then binds to cellular retinol binding protein 1 (CRBP1/RBP1). The retinol-CRBP1 complex is secreted and transported into hepatic stellate cells. Retinol entering the stellate cell is either stored in lipid droplets as retinol palmitate or bound by plasma retinol-binding protein 4 (RBP4) and secreted into blood. In the bloodstream most retinol-RBP4 is reversibly complexed with transthyretin (TTR). Uncomplexed retinol is taken up by cells via a receptor mediated mechanism. Free RBP4 in the circulation is catabolized in the kidney tubules. Based on Senoo (2004) (85) and Blomhoff et al. (2006) (83).

Although the mechanism for retinol storage in, and mobilization from, stellate cells has not been fully elucidated, the factor shuttling retinol between different liver cell types is the cellular retinol-binding protein (CRBP-I/RBP1). The involvement and recycling of RBP4 (139) has been ruled out, since mice lacking Rbp4 accumulate excess of vitamin A in the liver. While still able to store vitamin A, these mice were unable to mobilise it to plasma. Moreover, extrahepatic expression of RBP4 does not restore vitamin A mobilisation in these animals, indicating that circulating RBP4 is not re-used by the liver. STRA6 is not expressed in the liver, ruling out a role of this receptor in hepatic storage of retinoids (140, 141).

Under vitamin A sufficient conditions, most of the retinyl esters absorbed from the chylomicron remnants are transferred as retinol to the stellate cells, where over 80%
of the body supply of vitamin A is stored (88). Smaller amounts are also stored in lipid droplets in the hepatocytes and in stellate cells residing in extrahepatic organs and tissues, such as kidneys, digestive tract, adrenal gland, lung, testis, uterus, bone marrow and thymus (90, 142-144). Extrahepatic storage sites of retinyl esters may provide a local supply of vitamin A to tissues with a high demand, such as the retina. The importance of extrahepatic vitamin A pools is demonstrated by the observation that storage of retinyl esters in retinal pigment epithelial cells is prerequisite for normal visual function (144). The stores of vitamin A are sufficient to maintain a steady physiological concentration of 1-2 µM retinol in plasma, despite strong fluctuation in daily intake of vitamin A (144).

3.4. Vitamin A Metabolism

The three main circulating metabolites of vitamin A in the body differ in their oxidation state: the hydroxyl/alcohol form (retinol), the aldehyde form (retinal) and the carboxylic form (retinoic acid, RA) (96) (Figure 1.5). As described above, vitamin A is distributed throughout the body as retinol. It is generally believed that conversion of retinol into the biologically active retinal and retinoic acid occurs locally (88, 145). Retinol is converted into retinoic acid in a two-step process (reviewed in (145, 146)). Three enzyme families are capable of catalysing the first step in which retinol is reversibly converted into retinal: the cytosolic medium-chain alcohol dehydrogenases (ADH), the (membrane-associated) short chain dehydrogenases (SDR) and the cytochrome P450s (CYPs) (88, 147-149). In the second step, retinal is converted into RA in an irreversible reaction. Multiple enzymes contain the retinaldehyde dehydrogenase (RALDH) activity. Best characterized are the mouse and human RALDH1, RALDH2 and RALDH3 that have been shown to be involved in RA synthesis in vivo (145). Of these three enzymes, only RALDH1 prefers cis-retinaldehyde over all-trans retinal with the highest activity towards 9-cis retinal (150). Also various enzymes belonging to the human cytochrome P-450 family have been shown to be capable of metabolizing retinals to retinoic acids. These include CYP1A1, CYP1A2 and CYP3A4, of which CYP1A2 shows the highest activity for 9-cis retinal (151). The expression levels of the metabolic enzymes involved in RA synthesis may affect the ratio between atRA and the cis-RAs, since these enzymes differ in their substrate specificity. In vivo isomerization between atRA and 9cRA has been described and may contribute in fine-tuning individual retinoic acid levels (152).

Although produced locally, RA may act everywhere in the organism. The circulation contains low levels of atRA (0.2 to 0.7 % of plasma retinol). The plasma atRA contributes to variable extend to the tissue pool of RA, depending on the tissue/organ. In liver and brain, the retinoic acid pool originates primarily from the circulation rather than from local synthesis (153). RA concentrations in tissue are very low, on average 3-15 µg per kilogram (88). Therefore, its contribution to dietary intake of vitamin A is negligible. Still, it has been shown that animals grow normally on retinoic acid as the sole source of vitamin A-derivatives indicating that RA is the main bioactive derivative of vitamin A (142).
Total RA levels are regulated by an interplay between RA synthesizing and catabolising enzymes. Retinoic acid is metabolized in a two-step process. In the first step, phase I enzymes of the CYP450 superfamily catabolise the different isomers of RA. One of these enzymes is retinoic acid-inactivating cytochrome P450 (P450RAI/CYP26). This metabolizes atRA, but does not appreciably metabolize cis-RA isomers (9-cis and 13-cis). AtRA induces expression of CYP26, thereby controlling its own catabolism (154, 155). While numerous CYP enzymes have been identified that catabolize atRA and/or 13-cis RA, only a few 9cRA-metabolising enzymes have been identified. Of the CYPs that are dominantly expressed in the adult human liver, CYP2C9, -2C8 and -3A4 are the major ones involved in 9-cis RA metabolism. The efficiency of these CYPs to metabolize 9cRA is higher than that for atRA, which may explain why the concentrations of 9cRA in vivo are low. In the second step, phase II enzymes facilitate the conjugation of phase I metabolites. All trans-RA and its phase I metabolites 4-oxo-RA, 5,6-epoxy-RA, and 4-OH-RA were found to be glucuronidated by the human glucuronyl transferase UGT2B7 (147, 156).

4. Nuclear receptors/metabolic sensors in control of bile salt homeostasis

In the past decade, bile salts have been shown to be key signalling molecules in various biological pathways, in addition to their long-known fat-emulsifying actions. With the discovery of the bile acid-activated Farnesoid X Receptor (FXR) and its
transcriptional regulation of specific target genes, it has become clear how bile acids control their own synthesis and transport. FXR belongs to the superfamily of nuclear receptors (NRs). Many metabolic processes, including glucose, lipid and cholesterol/bile acid metabolism are regulated by NRs. These are ligand-activated transcription factors. Ligands to these NRs are often small lipophilic molecules including hormones, metabolites and food-derived compounds.

4.1 Nuclear Receptor Superfamily

At present, 49 members of the nuclear receptor (NR) superfamily have been identified (reviewed by Francis et al. (157)). The superfamily of NRs is divided into seven subfamilies (NR0 to NR6) based on sequence homology. The nomenclature of nuclear receptors has been standardized per subfamily (reviewed by Germain et al. (158)). An in-depth overview of the seven subclasses and their members is beyond the scope of this review (see review by Aranda et al. (159)). The relevant subfamilies for this thesis will be briefly discussed. Members of the NR1 thyroid hormone receptor-like subfamily include the thyroid hormone receptor (TR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR), liver X receptor (LXR), vitamin D receptor (VDR), pregnane X receptor (PXR) and FXR. The NR2 subfamily, or retinoid X receptor-like nuclear receptors, include hepatocyte nuclear factor-4 (HNF-4) and retinoid X receptor-alpha (RXRa). In general, members of the NR1 subfamily need to form a heterodimer with RXRa to become transcriptionally active (160, 161). In several cases, NRs were identified before their ligands were known. These receptors were therefore designated “orphan receptors”. As their natural and synthetic ligands became known, some of these orphan receptors have been “adopted” (162). Two nuclear receptors that remain orphan to date are the liver receptor homolog-1 (LRH-1) belonging to the NR5 subfamily of steroidogenic factor-like receptors and the small heterodimer partner (SHP), belonging to the NR0 family of miscellaneous NRs.

NRs have a modular structure consisting of multiple functional domains. A typical nuclear receptor consists of a variable N-terminal region (region A/B), a conserved DNA-binding domain (DBD) (region C), a linker (region D), and a conserved E region that contains the ligand binding domain (LBD). Some receptors contain also a C-terminal region (F) with unknown function. Isoforms (alpha, beta, gamma) originate from homologous genes and isoforms (alpha 1, alpha 2) of nuclear receptors are generated via alternative translation initiation sites and/or alternative mRNA splicing. The ligand-independent transcriptional activation domain (AF-1) is contained within the A/B region, and the ligand-dependent transactivation domain (AF-2) is located within the C-terminal portion of the LBD (159).

Nuclear receptors regulate gene transcription by binding to specific DNA sequences, so-called hormone responsive elements (HREs), in the promoter region of specific genes. NR/RXRa heterodimers bind to a variety of tandem repeats of the hexamers AGGTCA or AGTTCA typically spaced by 1 or more base pairs (163, 164). The orientation of these hexamers, or so-called half-sites, may vary giving rise to a direct
repeat (DR) (AGGTCAnAGGTCA), a palindromic everted repeat (ER) (TGACCTnAGGTCA) or a palindromic inverted repeat (IR) (AGGTCAnTGACCT) (Figure 1.6). It should be noted that these HREs are consensus sequences and that the actual genomic HREs may slightly differ from the consensus.

Gene regulation by NRs is, however, far more complex than just receptor binding to a responsive element in a promoter. Competition between agonists and antagonists, RXRα availability, heterodimerization efficiency, cofactor recruitment and NR protein modification, together determine the ultimate transcriptional efficiency (165).

Figure 1.6. NR1 class nuclear receptors interact as a dimer with RXRα with hormone response element
RXRα is the obligate heterodimer partner of most members of the NR1 thyroid hormone receptor like subfamily, including the thyroid hormone receptor (TR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR), liver X receptor (LXR) pregnane X receptor (PXR), farnesoid X receptor (FXR) and vitamin D receptor (VDR). The NR/RXRα heterodimer interacts with so called hormone responsive elements (HREs), in the promoter region of certain genes. These HREs typically consists of tandem repeat of the hexamers AGGTCA or AGTTCA spaced by 1 or more bases, giving rise to a direct repeat (DR), a palindromic everted repeat (ER) or a palindromic inverted repeat (IR).


4.2. Vitamin A Receptors
RXR and RAR, both activated by vitamin A metabolites, were among the first nuclear receptors that were characterized.

4.2.A. Retinoid X Receptor
The retinoid X receptor (RXR) was identified by Mangelsdorf et al. in 1990 (167). Three isotypes of RXR exist, -alpha (RXRα/NR2B1), -beta (RXRβ/NR2B2) and -gamma (RXRγ/NR2B3). For each isotype two isoforms have been identified: RXRα (α1 and α2), RXRβ (β1 and β2), and RXRγ (γ1 and γ2) (168). RXRα is highly expressed in liver and to a lesser extend in lung, muscle, kidney and spleen. RXRγ is expressed in spleen, adrenal gland, heart, intestine and kidney. RXRβ is rather ubiquitously expressed, but relatively low in liver and intestine (169).

The natural ligand of RXR is the vitamin A-derivative 9cRA (169-173). Physiological levels of 9cRA have proven difficult to detect in mammalian tissue (153, 174-176), but have been reported (177, 178). This has triggered some scepticism to whether 9cRA is the main endogenous ligand of RXR (179). Other ligands that have been
shown to modulate RXR activity include poly unsaturated fatty acids (PUFAs), such as docosahexaenoic acid, arachidonic acid and oleic acid (180, 181). Even though 9cRA may not be the only endogenous ligand for RXR, various studies showed that vitamin A signalling (or lack of vitamin A signalling) via RXR affects expression of NR/RXR target genes, supporting that vitamin A-derivatives are physiological ligands for RXR (Chapters 2 and 3 of this thesis). Homodimeric RXR interacts with DR-1 sequences (182). More importantly though, RXR is the obligate heterodimer partner of most nuclear receptors belonging to the NR1 subfamily. As such, RXR is a key factor in a multitude of metabolic processes, including bile salt homeostasis.

4.2.B. Retinoic Acid Receptor
As for RXR, also three isotypes of the retinoic acid receptor (RAR) exist, -alpha (RARα/NR1B1), -beta (RARβ/NR1B2) and -gamma (RARγ/NR1B3) (183). Each isotype has several isoforms, of which the expression has been extensively studied in mouse embryonic development. RARα is ubiquitously expressed, while RARβ and RARγ show a more tissue- and cell type-specific distribution. RARβ is present in the liver capsule as well as in the epithelium and outer mesenchyme of the intestine. RARγ is largely absent from the gastrointestinal tract, with the exception of the squamous epithelium of the stomach (184).
RAR forms heterodimers with RXR (185). RAR/RXR typically interacts with retinoic acid response elements (RAREs) consisting of a direct repeat of AGGTCA interspaced by 5 nucleotides (DR-5) (186). The main natural ligand of RAR is atRA. RARs also bind 9cRA, but with lower affinity than atRA (187, 188). Target genes of RAR are numerous and include genes involved in vitamin A metabolism, such as RBP4 and RBP2 (189, 190).
RAR/RXR target genes involved in bile acid homeostasis include \( \text{Ntcp} \) (191, 192) and \( \text{ASBT} \) (84). Also rat \( \text{Cyp7a1} \) is reported to be an RAR/RXR target gene (193). Furthermore, RAR/RXR represses \( \text{MRP3} \) transcription (194).

4.3. Bile Acid Receptors
Three members of the nuclear receptor family have been identified as bile acid sensors. FXR is regarded the primary bile acid receptor, regulating transcription of key genes involved in bile acid synthesis and transport. The other two bile acid sensing NRs are PXR and VDR. These are mainly involved in the adaptive response to prevent bile acid hepatotoxicity. In addition, CAR is also activated by increased levels of bile salts. Although CAR is not a bile acid sensor by itself, bile salts and bilirubin induces nuclear translocation of CAR, thereby indirectly activating this NR. Like PXR and VDR, CAR is involved in the detoxification of bile salts. These receptors are extensively reviewed by Moore et al. 2006 (195). An additional bile acid receptor is the G protein-coupled receptor TGR5. Unlike the intracellular NRs, TGR5 is a cell surface receptor, responding to extracellular bile acids (196, 197). TGR5 signalling is involved in energy expenditure (198) and does not seem to play a role in bile
acid synthesis. Therefore, functions of TGR5 will be not discussed in this chapter. An overview of bile acid receptor-mediated gene expression of genes involved in bile salt synthesis, transport and metabolism is depicted in Figure 1.7.

**Figure 1.7. Bile sensors promote bile acid transport and detoxification**

Bile acid-activated nuclear receptors promote transport, conjugation and detoxification of bile salts. While FXR primarily dictates the cellular efflux of bile salt, PXR and VDR mainly regulate the detoxification of bile salts. Dotted lines represent VDR/PXR signalling in general, meaning not restricted to bile salt-induced signalling.

### 4.3.A. Farnesoid X Receptor Is the Mammalian Bile Salt Sensor

The farnesoid X receptor (FXR/NR1H4) was originally identified in 1995 as a retinoid receptor-interacting protein (RIP14, (199)) and found to be activated by farnesol (200), retinoic acid and TTNPB (4-[E-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid) (201). FXR was named after its first identified ligand, although a direct interaction with any of the above compounds was never established.

A few years later, it was established that bile acids are highly potent physiological activators of FXR. The strongest activating bile acid is CDCA (202-204). With the discovery of its endogenous ligands FXR was renamed bile acid receptor (BAR) (202), but the name FXR is still commonly used today. Other endogenous ligands of FXR include androsterone (205), PUFAs (206) and oxysterol 22(R)-hydroxycholesterol (207). Synthetic ligands include GW4064 (208) and 6-Ethyl-CDCA (6-ECDCA) (209). Plant-derived guggulsterone can function as an FXR agonist (210), but also as an FXR antagonist (211).

FXR is profoundly expressed in tissues that are exposed to bile salts, such as liver and intestine, but also in kidney and adrenal glands (200, 212). Four isoforms of FXR have been identified in rodents and humans, designated Fxrα1, Fxrα2, Fxrβ1, and Fxrβ2.
(213). To complicate matters, a homolog of NR1H4 was identified, NR1H5, which was named FXRβ. FXRβ encodes a functional receptor for lanosterol in rodents and other mammals but is a pseudogene in humans (214, 215). The conserved FXRa (generally termed FXR/NR1H4) gene encodes four protein products. Because of differential translation initiation sites, FXRa3 (formerly “Fxrβ1”) and FXRa4 (formerly “Fxrβ2”) differ at their N-terminus. As a result of differential transcript splicing, FXRa1 (“Fxrα1”) and FXRa3 contain a four amino acid-insert in the hinge region that is not present in FXRa2 (“Fxrα2”) and FXRa4 (195). The FXR isoforms are differentially expressed. In adult humans, FXRa1/2 mRNA is predominant in liver and adrenal gland. Expression of FXRa3/4 mRNA is most abundant in colon, duodenum, and kidney. FXRa3/4 mRNA levels are generally lower than that of FXRa1/2 (213).

For most transcriptional targets, FXR needs to form a heterodimer with RXRa to be transcriptionally active (201). The typical FXR responsive element (FXRE) is an inverted repeat spaced by one base pair (IR-1), with the consensus sequence AG-GTCAnTGACCT (200). FXREs have been identified in the promoter regions of genes involved in bile acid homeostasis, including BSEP (216, 217), OSTα/β (218, 219), IBABP (204, 220), BAAT/BAT (221), bile acid-CoA synthetase (BACS) (221), OATP1B3 (222), SHP (223-225) and within the second intron of fibroblast growth factor 19 (FGF19) in humans (226) and its rodent ortholog Fgf15 (227). Alternative FXR binding sequences were detected in the promoter elements of MRP2 (an everted repeat spaced by 8 base pairs; ER-8) (228), sulfotransferase 2A1 (SULT2A1/STD) (an inverted repeat without spacing; IR-0) (229), UDP glucuronosyltransferase 2B4 (UGT2B4) and the insulin-responsive glucose transporter 4 (GLUT4) (both a hexamer half-site) (230, 231). In the latter two, FXR induces transcription in the absence of RXRa. FXR-mediated transcriptional suppression has been described for apolipoprotein A-I (Apo-AI) and C-III (Apo-CIII) (232, 233). An overview of FXR target genes is given in Table 1.1.

FXR appears to be a multipurpose nuclear receptor (234), as it regulates expression of numerous target genes beyond bile acid synthesis and transport. Target genes of FXR are involved in processes such as blood clotting (fibrinogen (FBG) (235, 236) and complement factor 3 (C3) (227)), inflammation/adhesion (kininogen (KNG) (237) and intercellular adhesion molecule 1 (ICAM-1/CD54) (238)) and lipid and cholesterol homeostasis (fatty acid synthase (FAS) (239), phospholipid transfer protein (PLTP) (240) and apolipoprotein C-II (Apo-CII) (241)). Furthermore, FXR has been associated with liver regeneration and regrowth, (242) with glucose metabolism (reviewed in (243, 244)) and differentiation of bone marrow stromal cells into osteoblasts (245).

Studies with Fxr−/− mice revealed the central role of FXR in bile salt homeostasis. Wild type mice tolerate CA-feeding by reducing bile acid synthesis (Cyp7a1) and hepatic bile salt import (Ntcp), while simultaneously increasing bile salt excretion via BSEP. This regulation is strongly impaired in Fxr−/− mice that maintain bile acid synthesis and import upon CA-feeding with consistently low Bsep levels. As a consequence, CA-fed Fxr−/− mice develop significant bile acid-induced liver damage (79).
The farnesoid X receptor (FXR) is the primary mammalian bile acid receptor. By interacting with FXR, bile salts promote their own transport (e.g. bile salt export pump (BSEP)) and metabolism/clearance (cytochrome P450 3A4 (CYP3A4)). Via a negative feedback loop which involves the FXR target genes SHP and FGF19, bile acids suppress the synthesis of de novo bile acids. However, FXR signalling is not limited to bile salt synthesis and transport. FXR target genes are also involved in processes such as blood clotting (fibrinogen (FBG) and complement factor 3 (C3)), inflammation/adhesion (kininogen (KNG) and intercellular adhesion molecule 1 (ICAM-1/CD54)) and lipid and cholesterol homeostasis (fatty acid synthase (FAS), phospholipid transfer protein (PLTP) and apolipoprotein C-II (Apo-C2)) and glucose metabolism (insulin-responsive glucose transporter type 4 (GLUT4)).
4.3.B. Pregnane X Receptor

The pregnane X receptor (PXR/NR1I2), also called the steroid and xenobiotic receptor (SXR), is highly expressed in liver and intestine. PXR is activated by xenobiotic compounds, steroids and bile acids, including LCA, CDCA and DCA (266-271). The PXR/RXRα heterodimer binds to so-called xenobiotic response elements (XREs) present in the promoter region of genes encoding drug metabolizing enzymes and drug transport proteins. These XREs consist of specific repeats of AG(G/T)TCA oriented as either a direct repeat spaced by three to five nucleotides (DR-3, DR-4 or DR-5) or as an everted repeat spaced by six or eight nucleotides (ER-6 and ER-8) (272). PXR is a master regulator of drugs and xenobiotics metabolism (273) and is involved in the protective response to bile salt hepatotoxicity (270). PXR/RXRα induces transcription of CYP3A4/Cyp3a11 (267) and SULT2A1 (274) that respectively catalyse hydroxylation and sulfoconjugation of compounds such as drugs, xenobiotics and bile salts. Hydroxylation and sulfoconjugation makes these compounds more hydrophilic (polar, water-soluble), less toxic and more amenable for urinary excretion (82). Some of the membrane-embedded transport proteins that excrete these detoxified compounds are regulated by PXR and also transport (metabolites of) bile salts, including MRP3 (275), MRP2 (228) and MDR1 (273, 276). Expression of the organic anion transporter OATP1B1, which imports bile salts, is also regulated by PXR (272). LCA-activated PXR was shown to induce expression of murine Cyp3a11 and Atp1a4 (269).

Besides bile salt transport, PXR activity influences bile acid synthesis. Studies with Pxr−/− mice showed that PXR represses Cyp7a1 expression (269), which include both SHP- and FGF15-dependent mechanisms. The human SHP promoter contains a PXR/RXR binding site and LCA-activated PXR was shown to induce FGF19 promoter activity (277). Both SHP and FGF19 (278, 279) repress CYP7A1 expression (discussed in section 5.3). To make matters more complex, PXR expression itself is regulated by FXR (263). This may further potentiate the role of PXR to bile acid signalling. Thus, bile acid signalling through PXR likely contributes to the adaptive response to prevent bile acid toxicity.

4.3.C. Vitamin D Receptor

In addition to its main natural ligand 1,25-dihydroxy vitamin D₃ (1,25-(OH)₂D₃), the vitamin D receptor (VDR; NR1I1) is also activated to the same extend by LCA and 3-keto-LCA (280, 281). VDR regulates calcium homeostasis and bone metabolism (reviewed by St-Arnaud (282)). The VDR/RXR heterodimer binds to vitamin D response elements (VDREs). The typical VDRE consists of a direct hexameric (GGTC-CA, AGGTCA, or GGGTGA) repeat spaced by 3 nucleotides (DR-3), although the number of spacing nucleotides may vary (283). Less frequent, everted repeats have also been found to act as VDREs (reviewed in (284)).

With regard to bile acid metabolism, LCA-activated VDR was shown to induce expression of CYP3A4 (280, 281, 285), MDR1 (286) and MRP3 (287), while Cyp7a1 expression is repressed by LCA-activated VDR (286). In addition, VDR signalling
induces SULT2A1 (288, 289) expression and was reported to inhibit the CDCA-dependent transcription of the FXR target genes SHP, IBABP and BSEP (290). VDR thus actively participates in the detoxification and clearance of bile acids, fine-tunes expression of FXR target genes and inhibits bile acid biosynthesis.

4.3.D. Constitutive Androstane Receptor
The constitutive androstane receptor (CAR; NR1I3) is highly expressed in liver and, as the name suggests, is a constitutive active receptor (291, 292). Transcriptional activity of CAR has different modes of action (review by Tzameli et al. (293)). Although CAR does not require a ligand to become transcriptionally active (294), ligands have been identified that modulate its constitutive activity. CAR plays a role in bilirubin clearance as is apparent from studies with Car−/− mice challenged with a single dose of bilirubin. Bilirubin itself was shown to activate CAR. However, bilirubin does not seem to be a direct agonist of CAR (295), but rather promotes its nuclear translocation. In a similar fashion certain bile acids are able to indirectly activate CAR by inducing nuclear translocation (296). The CAR/RXR heterodimer interacts with RAREs (291). CAR protects against bile acid toxicity in CA-fed Fxr and Pxr double knockout mice. Pretreatment with CAR activators resulted in decreased bile acid and bilirubin serum levels, through upregulation of hepatic genes involved in bile acid and/or bilirubin metabolism and excretion (297). Similar results were obtained comparing LCA-fed Car−/− and WT mice (298). Target genes of CAR involved in bile acid detoxification and clearance include cytosolic sulfotransferases (SULTs) (299), 3’-phosphoadenosine 5’-phosphosulfate synthetase 2 (Pappss2) (299), Cyp3a11 (300), Mrp3 and Mrp4 (reviewed in (14)).

4.3.E. Additional Nuclear Receptors Involved in Bile Acid Metabolism
Two orphan NRs have a prominent role in the transcriptional regulation of bile acid homeostasis. The liver receptor homolog-1 (LRH-1) induces transcription of various genes involved in bile acid transport and synthesis, while the small heterodimer partner (SHP) is involved in a negative feedback loop, counteracting the activity of LRH-1 and various other NR/RXR heterodimers. The interplay between LRH-1 and SHP is one of the main mechanisms that fine-tunes bile salt synthesis and transport.

LRH-1 induces bile salt synthesis and transport
The liver receptor homolog-1 (LRH-1/NR5A2), also known as fetoprotein transcription factor (FTF), is expressed in liver, intestine, pancreas, ovary and adrenal glands (301-303). It has also been named CYP7A promoter-binding factor, as it is well-known as a positive regulator of expression of hepatic Cyp7A1 (301). Unlike most NRs, LRH-1 does not form a heterodimer with RXR, but instead binds DNA as a monomer to an extended nuclear receptor half-site YCAAGGYCR (where Y is any pyrimidine and R is any purine) (302, 304).
No endogenous (or synthetic) ligand has been identified to date that is associated with the constitutive activity of LRH-1. Although in silico modelling studies pre-
dict that phospholipids may directly bind to LRH-1 (reviewed by Forman (305)), the biological relevance of this putative interaction remains elusive. Murine LRH-1 was shown to exist as a stable monomer in the absence of a ligand, suggesting that it might not need a ligand at all to be transcriptionally active (306).

LRH-1 responsive elements (LRE) have been identified in the promoter regions of Cyp7a1, Cyp8b1, ASBT, MRP3, BSEP, OSTα/OSTβ, ABCG5/ABCG8 and SHP (84, 301, 307-313). LRH-1 facilitates the LXRα-mediated expression of rodent Cyp7A1 (225, 314) and Fas (315) in vitro. Hepatic Lrh-1−/− mice showed that Cyp8b1, but not Cyp7a1 transcription critically depends on LRH-1 in vivo (316, 317). Also murine hepatic and intestinal Shp transcription depends largely on LRH-1 (316, 317).

Transactivation by LRH-1 seems to be required for maximal induction of FXR target genes in response to bile salts, as was shown for BSEP (311) and MRP3. For the latter, two adjacent LREs were identified within its promoter. Mutating either of these responsive elements resulted in loss of CDCA-induced MRP3 promoter activity (318). Additionally, a recent study demonstrated that LRH-1 recruits FXR to the promoter of genes involved in lipid metabolism (319).

Bile acid-induced transcription of LRH-1/Lrh-1 is observed in human hepatic and intestinal cell lines and in rat liver (224, 310, 318, 320). However, involvement of FXR in LRH-1 expression has not been established. Interestingly, bile acid-regulated expression of LRH-1 was not observed in mice (225). Taken together, current knowledge establishes LRH-1 as a general positive regulator of bile acid and cholesterol homeostasis and in some species bile salts may increase LRH-1 expression.

Small Heterodimer Partner Is a Repressor of Bile Salt Homeostasis

The small heterodimer partner (SHP/NR0B2) is an atypical member of the NR-family, as it lacks a DNA-binding domain and a natural ligand has not been identified to date. However, recent evidence suggests that SHP activity can be modulated in a ligand-dependent fashion, albeit by synthetic compounds (e.g. 4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3-Cl-AHPC)) (321). SHP is expressed in liver, small intestine, adrenal gland and spleen (322). SHP is a general negative transcription factor of signalling pathways involving nuclear receptors and plays an important role in the negative feedback on bile salt synthesis and transport. However, functions of SHP extend far beyond bile salt homeostasis, including control of viral replication (323), tumor suppression (324), sexual maturation (325), obesity and (type 2) diabetes (326, 327).

Transcription of SHP/Shp is induced by FXR (223-225) and LXRα (in humans) (328). As a consequence, Shp transcription is low in Fxr−/− mice and CA-feeding does not alter Shp transcription in these animals (79). SHP represses gene transcription by directly interacting with other NRs, cofactors or chromatin-modifying enzymes (322, 329). An important SHP-target involved in positive regulation of bile acid synthesis and transport is LRH-1. The role of SHP in the negative feedback of bile salt homeostasis will be discussed in section 5.
Liver X Receptor

Two isoforms of the liver X receptor (LXR) are described; LXRα (NR1H3) and LXRβ (NR1H2) (330). LXRα is profoundly expressed in liver, intestine, kidney, adipose tissue and spleen (330, 331), while LXRβ is ubiquitously expressed (332). The typical LXR/RXR responsive element (LXRE) is a hexanucleotide sequence (AGGTCA) separated by four nucleotides (DR-4) (330, 331). LXRs are activated by cholesterol-derivatives and intermediates of cholesterol metabolism, including the oxysterols 22(R)-hydroxycholesterol, and 24(S)-hydroxycholesterol, but also 6-α-hydroxy bile acids (333-336). LXR signalling controls cholesterol, fatty acid and carbohydrate metabolism (reviewed in (337, 338)).

Lxrα−/− mice suffer from impaired cholesterol and bile acid metabolism (339), indicating the interconnected regulation of bile acid, cholesterol, and lipid metabolism (162, 302, 340). Target genes of LXR that are involved in bile acid and cholesterol metabolism include Cyp7a1 (334, 339), ABCG5 and ABCG8 (341), SHP (328) and LRH-1 (342). Notably, expression of rodent, but not human CYP7A1, is regulated via LXR (328). LXR and FXR share, or compete for, responsive elements in the promoter regions of hIBABP and mOstα/β (343, 344).

FXR signalling has been suggested to affect cholesterol transport via ABCG5 and ABCG8 (345). CA feeding of mice resulted in increased expression of hepatic Lxra, but FXR does not seem to control Lxr expression directly (346). LXR signalling thus influences bile acid synthesis and co-regulates transcription of certain FXR-target genes involved in bile salt transport.

4.4. Permissive Versus Nonpermissive Functioning of RXR/9cRA Within the NR/RXR-Heterodimer

In NR/RXR heterodimers, 2 different ligands play a role in modulating the transcriptional activity of the protein complex. The presence of 9cRA as the RXR ligand may have different effects on the NR/RXR activity depending on the specific NR and/or target gene studied and have been subclassified as permissive and nonpermissive NR/RXR complexes (159, 347). In permissive NR/RXR heterodimers, RXR is both a structural and functional component allowing for 9cRA signalling through such a NR/RXR heterodimer. Ligands of RXR and its NR partner can independently and synergistically activate gene transcription. This has been described for PPARs (348, 349), LXR (330) and also FXR (220, 240).

In nonpermissive NHR/RXR heterodimers RXR is merely a structural component of the heterodimer required for DNA-binding, but not necessarily acting as a receptor. Moreover, 9cRA may even repress expression of the target genes (347). RXR is nonpermissive in heterodimers with RAR (350), TR (351) and VDR (352, 353). However, a given NR/RXR combination is not strictly permissive or nonpermissive, as this also depends on the specific target gene. For instance, RXR is a permissive partner to TR in prolactin gene regulation (354). While RXR/CAR heterodimers seem to be neither strictly permissive nor nonpermissive, as the contribution of RXR/9cRA depends on the experimental approach. Tzameli et al. therefore argue that classifying a particular NR/RXR complex as either permissive or nonpermissive is an oversimplification (355).
The RXR/FXR heterodimer has been described as permissive, based on the regulation of IBABP and PLTP (220, 240). We and others have shown that RXRα acts as a nonpermissive partner to FXR in the regulation of human and mouse BSEP (Chapter 2, Kassam et al. (356)).

5. Feedback regulation on bile salt synthesis and transport
Increased levels of circulating bile salts repress de novo synthesis of bile acids, hepatic and intestinal bile salt uptake and promote bile salt export, detoxification and clearance. Both FXR-dependent and -independent mechanisms have been identified in this process. A schematic overview of this feedback regulation on synthesis and transport is depicted in Figures 1.8 and 1.9.

5.1. The FXR-SHP(-LRH-1) Cascade
Bile acid-activated FXR/RXRα enhances the expression of SHP (223, 224), which in turn represses the expression of hepatic CYP7A1 (223, 224), CYP8B1 (357), Ntcp (191) and ileal ASBT (84, 358), thereby reducing biosynthesis and cellular import of bile salts.

Many genes that are transcriptionally repressed by SHP are LRH-1 target genes. Interaction of SHP with LRH-1 prevents LRH-1-dependent gene transcription. SHP interacts with the AF-2 domain of LRH-1 and competes with co-activators for binding to this domain. Once bound to its target receptor and recruited to DNA, the inherent repression function of SHP further contributes to the overall inhibitory effect (359, 360).

SHP also represses the LRH-1-enhanced LXRα-dependent transcription of rodent Cyp7a1 (223, 225). Cyp7a1 and Cyp8b1 levels are only slightly elevated in Shp−/− mice compared to WT animals (346, 361-363) under normal conditions. However, these animals are more susceptible to bile duct- ligation-induced cholestasis, as Cyp7a1 (and Cyp8b1) transcription is not efficiently repressed in the absence of SHP (363).

Transcriptional regulation of rodent Cyp7a1 differs from human CYP7A1 as the LXRE is not conserved in the human CYP7A1 promoter (364, 365). Activated LXRα represses CYP7A1 transcription in primary human hepatocytes (328). Moreover, human CYP7A1 is repressed by bile acids and this mechanism may still include SHP, as CYP7A1 is repressed by SHP in primary human hepatocytes (328) as well. Thus, SHP is of key importance in the adaptive response to increasing hepatic bile acid concentrations.

Expression of hepatic and ileal bile salt import proteins is also repressed in response to rising bile salt concentrations. Expression of rat Ntcp is regulated by hepatocyte nuclear factor 1 (HNF1) (366, 367), hepatocyte nuclear factor 4 alpha (HNF4α) (368) and RAR/RXR (192). SHP represses transcription of rat Ntcp by interfering with RAR/RXR-dependent transcription (191) and by lowering the HNF4α-dependent transcription of Hnf1α (369). Not much is known about the bile acid-dependent repression mechanism on human NTCP expression.
Human and mouse, but not rat ASBT expression is repressed by bile acids. This species difference is explained by the absence of an LRE in the rat Asbt promoter. Moreover, LRH-1 seems not to be expressed in rat ileal epithelial cells (84, 309). ASBT expression in Fxr−/− mice is unresponsive to bile acid feeding (309). Both LRH-1 and RAR/RXR responsive elements have been identified in the human ASBT promoter region. Mutating either of these two sequences was found to reduce ASBT promoter activity, but only the RARE appeared to be required for bile acid-dependent inhibi-
on of ABST expression (84). Expression of both human NTCP and ASBT is strongly increased by the nuclear receptor glucocorticoid receptor (GR/NR3C1). ASBT/NTCP activation by GR can be inhibited by SHP (370, 371). Inhibition of BSEP, OST α/β and MRP3 expression via LRH-1 and SHP does not seem to contribute to the cytoprotective mechanisms activated by increasing intracellular bile acid levels, as it prevents the clearance of bile acids.

5.2. Questioning the Role of LRH-1 In Negative Feedback Regulation
Interestingly, studies with liver- and intestine-specific Lrh-1 deficient mice showed that LRH-1 is not required for the negative feedback regulation of bile acid synthesis, irrespective of its established role in bile acid homeostasis (316, 317). Cyp7a1 and Cyp8b1 transcription is higher in Lrh-1+/− heterozygote mice than in WT mice, which is accompanied by an increased bile salt concentration in bile. Overexpression of LRH-1 in mice repressed both Cyp7a1 and Cyp8b1 transcription, suggesting that LRH-1 might also act as a repressor of bile acid biosynthesis (372). Shp−/− mice have slightly elevated Cyp7a1, Cyp8b1 and Bsep mRNA levels (346, 361). The increase in Bsep is probably the result of the increased bile acid pool size in these animals (346). However, lack of SHP may also increase Bsep expression by lack of inhibition of LRH-1, like observed for Cyp7a1 and Cyp8b1 (311). Together, these studies indicate that both the basal levels of the constitutive active LRH-1 and its interaction with SHP determine its effect on bile acid homeostasis.

5.3. Redundant Pathways of Bile Acid Synthesis Repression
In contrast to what would be expected from the feedback control mechanisms described above, Cyp7a1 expression is strongly increased in bile duct-ligated rodents, despite high concentrations of bile salts in the liver and blood. Feeding these bile duct-ligated mice the FXR ligand GW4064 decreased hepatic Cyp7a1 expression (279). Also CA feeding of Shp−/− mice repressed Cyp7a1 and Cyp8b1 transcription (361, 362). These finding stipulate the existence of FXR-SHP-independent feedback mechanisms to repress bile acid biosynthesis.

5.3.A. FXR - FGF19 - JNK Mediated Repression of CYP7A1 Expression
An alternative mode of CYP7A1 repression is provided by FGF19 (Fgf15 in rodents) (226). Bile acids from the intestinal lumen activate ileal FXR/RXRα and increase expression of Fgf15. Ileal FGF15 is released in the blood and signals to the liver via the hepatic fibroblast growth factor receptor 4 (FGFR4), which leads to phosphorylation of JNK (226, 279). Activated JNK represses CYP7A1 expression (226, 346, 373). Consequently, both Fgf15−/− and Fgfr4−/− mice display higher Cyp7a1 mRNA expression than WT mice and an inability to repress Cyp7a1 in response to feeding of FXR ligands. Asbt−/− mice have lower Fgf15 transcription and higher Cyp7a1 and Cyp8b1 transcription levels than WT mice (374), indicating that reabsorbed bile acids in the ileum determine the rate of de novo bile acid biosynthesis.
The Interrelationship Between Bile Acid and Vitamin A Homeostasis

Figure 1.9. Bile acid sensors engage in negative feedback on bile acid biosynthesis and cellular import

Bile salt-activated FXR suppresses bile acid synthesis (CYP7A1) via SHP and FGF19. Inhibition of CYP7A1 via FGF19 involves the phosphorylation of JNK. SHP also limits cellular uptake of bile salts by inhibiting NTCP and ASBT expression. In addition to FXR signalling, bile acid signalling via PXR and VDR also contribute to the negative feedback regulation on bile acid synthesis. PXR signalling promotes expression of FGF19 and SHP. Furthermore, interaction between the ligand-activated PXR and SHP proteins enhances inhibition of CYP7A1 (dotted line). Moreover, PXR is an FXR-target gene itself. Bile acid signalling via VDR inhibits CYP7A1 by competing with other transcription factors at the CYP7A1 promoter. FXR-independent CYP7A1 repression mechanisms may converge the FXR-dependent mechanism on JNK phosphorylation.

Additionally, FGF15/FGF19 was recently shown to inhibit ASBT expression (375), expanding FXR-FGF15/FGF19 signalling to bile acid transport. Thus, the FXR-SHP and the FXR-FGF19 signalling cascades are both involved in feedback mechanisms that control bile salt synthesis and transport.

5.3.B. SHP and FGF19 Pathways Overlap

SHP-independent mechanisms of CYP7A1 repression seem to dominate over SHP dependent mechanisms, as Cyp7a1 repression prevails in the absence of Shp (362), but not in the absence of Fgf15 (279). However, SHP does not seem to be fully dispensable in the negative feedback loop of bile acid biosynthesis. Activation of either FXR or RXR by feeding mice synthetic ligands GW4064 or LG100268, respectively, was found to repress Cyp7a1 transcription, but this was dependent on SHP (346,
However, CA-feeding reduced Cyp7a1 (and Cyp8b1) mRNA levels in Shp\textsuperscript{+/−} mice, but this seems to depend on hepatoxicity rather than on FXR signalling (361). Moreover, the FGF15- and SHP-dependent repression of Cyp7a1 overlap, since SHP significantly enhances the FGF15 mediated repression of Cyp7a1 mRNA (279).

5.3.C. FXR Independent Repression
The negative feedback loop on bile acid biosynthesis also includes FXR-independent mechanisms. LCA-activated PXR (269) or VDR (376) represses Cyp7a1 gene expression. This mode of repression may involve FGF19/FGF15, as it has been shown that the FGF19 promoter is activated by LCA-activated PXR in intestinal cells (277). Alternatively, LCA-activated PXR is suggested to bind to the bile acid response element (BARE)-I in the CYP7A1 promoter and inhibits CYP7A1 transcription by both SHP-dependent and -independent pathways. PXR strongly interacts which SHP in a ligand-dependent manner (377). FXR-SHP-independent bile acid-induced expression of inflammatory cytokines (e.g., tumor necrosis factor alpha (TNFα) and interleukin beta (IL-1β)) by Kupffer cells may mediate CYP7A1 repression as well (378, 379). Ito and co-workers reported that mice lacking the membrane protein beta-klotho (KLB) have increased Cyp7a1 and Cyp8b1 mRNA expression, while bile acid-dependent repression of these genes was still largely intact. Recently, KLB has been shown to modulate FGF19/FGF15 signalling via FGFR4 (380, 381). This links the observation that KLB inhibits ASBT expression, to the repression of ABST by FGF19/FGF15 (375).

5.3.D. FXR Dependent and Independent Repression of CYP7A1 Converges at JNK
Bile acid signalling via FGF15/19, TNFα, IL-1β and protein kinase C (PKC) (377, 382) all activate JNK. Activated JNK in turn activates jun proto-oncogene (JUN), which inhibits the HNF4α-dependent transcription of CYP7A1 (383), and induces Shp expression (373). Additionally, phosphorylation of FXR by PKC promotes its transcriptional activity (384). This way, signalling via both FXR dependent and independent pathways may converge and amplify each other in the effort to repress CYP7A1 expression.
6. Aim and Outline of this Thesis

Vitamin A and bile acid homeostasis are interrelated at various levels. Bile salts are required for hydrolysis of retinyl esters in the intestinal lumen and the absorption of vitamin A into the intestinal epithelium. Consequently, patients suffering from obstructive cholestasis suffer from malabsorption of vitamin A. Synthesis of bile acids takes place in the liver, which also serves as the primary site of vitamin A storage in the mammalian body. Liver disease may progress to liver fibrosis and affect the hepatic stellate cells that contain the vitamin A in intracellular lipid droplets. During fibrosis, stellate cells become activated and differentiate to proliferative and contractile myofibroblasts that overproduce extracellular matrix proteins leading to the characteristic scar tissue formation. In this process they lose their retinoid content. Thus cholestasis effectively promotes depletion of vitamin A from the organism by emptying the stores and preventing uptake.

Bile acids and vitamin A-derivatives like 9-cis retinoic acid (9cRA) co-regulate bile acid synthesis in the liver and transport between the liver and intestine. They act mainly by activating transcription factors of the nuclear receptor family, in particular the farnesoid X receptor (FXR) and the retinoid X receptor-alpha (RXRα). FXR is activated by bile acids and RXRα by 9cRA and they act together as a heterodimer in regulating expression of genes involved in bile acid synthesis and transport.

The role of bile acid-activated FXR in regulating bile acid homeostasis has been well established. However, the role of its heterodimer partner RXR remains largely illusory. As many chronic liver diseases may develop to vitamin A deficiency, the putative role of vitamin A in bile salt homeostasis is therefore highly relevant.

In this thesis, we aim to determine the role of vitamin A in the FXR/RXRα-mediated regulation of bile acid homeostasis both in vitro and in vivo models.

In chapter 2, we performed in vitro and in vivo experiments to analyse the effect of vitamin A on the bile acid-induced expression of the transporter that is responsible for secretion of bile salts from the liver, the bile salt export pump (BSEP).

In chapter 3, we analysed the effect of vitamin A on the bile acid-induced expression of the small heterodimer partner (SHP), which is a transcriptional repressor of several genes in bile acid synthesis and transport. Even though expression of both BSEP and SHP is induced by FXR, we observed opposite effects of 9cRA on their expression, repressing BSEP and super-inducing SHP. Chapter 2 and 3 include a detailed analysis of the molecular mechanisms that cause this opposite effect of 9cRA on BSEP and SHP expression.

Given the fact that vitamin A-derivatives indeed directly affect expression of genes involved in bile salt biosynthesis and transport, we analysed in chapter 4 whether vitamin A deficiency in laboratory animals leads to changes in bile salt homeostasis. Finally, in chapter 5 we analysed whether the combination of vitamin A deficiency and obstructive cholestasis in laboratory animals leads to uncontrolled bile salt regulation and putatively to aggravation of liver damage.

In chapter 6 an integrated discussion of all our data is presented.
Chapter 1

References


44
The interrelationship between bile acid and vitamin A homeostasis


42. Dubin IN, Johnson FB. Chronic idiopathic jaundice with unidentified pigment in liver cells; a new clinicopathologic entity with a report of 12 cases. Medicine (Baltimore) 1954 Sep;33(3):155-197.
The Interrelationship Between Bile Acid and Vitamin A Homeostasis


Chapter 1


The Interrelationship Between Bile Acid and Vitamin A Homeostasis


The Interrelationship Between Bile Acid and Vitamin A Homeostasis


188. Repa JJ, Hanson KK, Clagett-Dame M. All-trans-retinol is a ligand for the retinoic acid receptors. Proc Natl Acad Sci U S A 1993 Aug 1;90(15):7293-7297.


The Interrelationship Between Bile Acid and Vitamin A Homeostasis


207. Deng R, Yang D, Yang J, Yan B. Oxysterol 22(R)-hydroxycholesterol induces the expression of the bile salt export pump through nuclear receptor farsenoid X receptor but not liver X receptor. J Pharmacol Exp Ther 2006 Apr;317(1):317-325.


The Interrelationship Between Bile Acid and Vitamin A Homeostasis


Chapter 1


The Interrelationship Between Bile Acid and Vitamin A Homeostasis


Chapter 1


318. Chong HK, Biesinger J, Seo YK, Xie X, Osborne TF. Genome-wide analysis of hepatic LRH-1 reveals a promoter binding preference and suggests a role in regulating genes of lipid metabolism in concert with FXR. BMC Genomics 2012;13:51.
The Interrelationship Between Bile Acid and Vitamin A Homeostasis


Chapter 1


348. Issemann I, Prince RA, Tugwood JD, Green S. The retinoid X receptor enhances the function of the peroxisome proliferator activated receptor. Biochimie 1993;75(3-4):251-256.


