Disturbed vitamin A metabolism in chronic liver disease and relevance for therapy
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Chapter 8

General Discussion

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The liver produces bile for efficient hepatic secretion of fat-soluble waste products, as well as effective absorption of fat-soluble nutrients in the intestine, including vitamin A. Bile acids are essential components in bile for this physiologically important process. Excess vitamin A is efficiently stored in the liver, from which it is released in a highly controlled manner in times of insufficient dietary intake. However, the interdependence between vitamin A and bile acids goes far beyond the simple requirement of bile acids for intestinal absorption of (pro-)vitamin A. Vitamin A metabolites collaborate with bile acids to control key physiological processes, like glucose and lipid metabolism as well as immune regulation. Moreover, they control their own (bile acid and vitamin A) homeostasis. The retinoic acid receptor (RAR), retinoid X receptor (RXR) and farnesoid X receptor (FXR) are the central players that control these processes [1–3].

The liver plays a central role in controlling vitamin A metabolism and chronic liver diseases, such as biliary atresia, primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), viral hepatitis, alcoholic liver disease (ALD), and non-alcoholic fatty liver disease (NAFLD and the progressive form non-alcoholic steatohepatitis [NASH]) are associated with impaired vitamin A homeostasis that may lead to vitamin A deficiency [1]. Animal studies have shown the beneficial effects of vitamin A derivatives, e.g., retinoic acids or pharmacological modulators of RARs and RXRs, in the treatment of chronic liver diseases. However, oral vitamin A supplements appear not very effective in restoring adequate serum vitamin A (retinol) levels in NAFLD patients, suggesting that vitamin A metabolism is intrinsically impaired. Relatively little is known, however, about changes in vitamin A metabolism in the liver diseases, including possible changes in storage (retinyl esters) vs. circulating (retinol, RA) forms and possible redirection of vitamin A pools within the liver (hepatocytes vs. hepatic stellate cells) and/or to extrahepatic tissues. Insight into these processes is needed to get a better view of the possible therapeutic application of vitamin A and/or its derivatives (retinoids acting on RARs and rexinoids acting on RXRs) in the liver diseases. Therefore in this thesis, we studied vitamin A metabolism in patients, as well as animal and cellular models of chronic liver diseases.

The key findings reported in this thesis are: 1) Quiescence hepatic stellate cells (qHSC) expresses hormone-sensitive lipase (HSL), which harbors retinyl ester hydrolyzing activity and contributes to hepatic vitamin A metabolism (chapter 3). 2) NAFLD is associated with perturbed vitamin A metabolism characterized by low hepatic retinol levels, while circulatory retinol and RBP4 levels increased in NAFLD. Moreover, retinyl
palmitate (being the most dominant retinyl ester) levels are clearly enhanced in NAFLD and accumulate in hepatocytes (rather than in HSC) (chapter 5). An important technical issue addressed in this chapter is that the accurate quantification of retinyl esters in fatty livers is highly dependent on the lipid extraction method used. 3) fatty liver that is associated with glycogen storage disease type 1a (GSD Ia) is also characterized by impaired vitamin A metabolism leading to increased serum retinol and RBP4 levels (chapter 6). 4) Manipulation of FXR activity in mice (either by gene deletion or pharmacological activation) effectively deplete hepatic vitamin A stores, in which hepatic FXR plays a key role (chapter 7).

A main finding of these studies is that metabolic diseases, as well as drug therapies to treat them, heavily affect vitamin A metabolism in the liver that is not reflected at all by the serum retinol levels. This may be good for providing peripheral tissues with necessary vitamin A, but may go at the expense of disease progression in the liver. Thus, manipulation of vitamin A metabolism may hold great promise in the treatment of chronic liver diseases, but requires more sophisticated approaches than simply (oral) vitamin A supplementation when serum retinol levels are low.

8.1. **Hormone-sensitive lipase (HSL) acts as a retinyl ester hydrolase in quiescence hepatic stellate cells (qHSC)**

Vitamin A homeostasis in the liver is a result of a fine-tuned balance between hepatic retinol esterification (storage) and retinyl ester hydrolysis (release). Lecithin retinol acyltransferase (LRAT) is the major enzyme involved in retinol esterification, while adipose triglyceride lipase (ATGL) and patatin-like phospholipase domain-containing protein 3 (PNPLA3) have been previously been identified as retinyl ester hydrolases (REHs). In chapter 3, we identified HSL as a 3rd REH in qHSC that contributes to hepatic vitamin A metabolism. HSL has a broad substrate-specificity and besides acting as a REH, it also converts cholesterol esters and triglycerides to free cholesterol and free fatty acids, respectively. We found that mRNA levels of Lipe, the gene encoding HSL, were actually higher qHSC as compared to hepatocytes, which may point to a broader role of qHSC-located HSL in hepatic lipid metabolism. Our study shows that HSL is mostly in its active phosphorylated state in qHSL, but pharmacological induction of HSL-phosphorylation further enhances retinyl ester hydrolysis in qHSC.
So far, limited data is available on the expression and function of HSL in the liver, while its retinyl ester hydrolase activity has been well-documented for adipose tissue [4]. HSL also hydrolyses cholesterol esters, triglycerides, mono- and di-acylglycerol and other lipids [5]. However, *in vitro* studies using recombinant HSL indicate that retinyl esters are the preferred substrate for HSL as compared to diacylglycerols, which was considered the best substrate for HSL until that report [4]. Earlier studies analyzed the putative presence of HSL in HSC, but were unable to confirm this. [6,7]. We cannot fully explain this apparent discrepancy at this point, other than that these earlier studies may have used (partly) activated HSC [6]. Moreover, *Lipe* expression was not detected in any type of liver cell [7], while it has been detected by many others in total liver tissue [5,8–10]. Our data suggest that HSL is not only dominantly present in qHSC, but also likely regulates retinyl ester hydrolysis along with lipases (ATGL and PNPLA3) to control the intracellular lipid turnover in HSC, especially in the early phase of HSC activation. However, the relative contribution of each lipase in retinyl esters hydrolysis in qHSC still needs to be determined.

Pharmacological inhibitors of HSL display great therapeutic potential in various metabolic complications, including dyslipidemia, elevated blood glucose levels and insulin resistance [11–13]. HSL null mice accumulate retinyl esters in adipose tissue [4], while the hepatic retinyl ester pool is comparable to WT control animal [6]. This may be explained by the fact that HSC expresses 3 different REH, ATGL, PNPLA3 and HSL, that can compensate for each other absence. Indeed, also ATGL-null mice do not have altered hepatic retinyl ester levels [6]. It may actually require a triple knock of *Pnpla2* (ATGL), *Pnpla3* and *Lipe* (HSL) to sufficiently eliminate REH activity in the liver. Cholesteryl esters accumulated in whole body HSL-null mice [5,14], but this phenotype was not replicated in hepatocyte-specific HSL-null mice [15]. This indicates the possible involvement of another cell type in the accumulation of cholesterol esters, which, taken our data in account, most likely are HSC. It is well-known that accumulation of cholesterol and retinyl esters in HSC also disturbs the intrinsic vitamin A metabolism. For example, *Lxraβ/-* mice accumulate cholesterol and retinyl esters and enhance intrinsic retinoic acid receptor signaling [16]. Primary HSC isolated from *Lxraβ/-* mice rapidly lose vitamin A stores and quickly acquire a more fibrogenic phenotype during culture activation. Moreover, *Lxraβ/-* mice show increased susceptibility to CCL4-induced liver injury as compared to control animals, and is –in part- the result of enhanced intrinsic RAR-signaling [16]. Inversely, our results with the
pharmacological activation of HSL indicate that enhanced hydrolysis of intracellular retinyl esters in HSC combined with reduced retinoic acid production (in thereby decreases intrinsic RAR-signaling) is associated with reduced the proliferation and activation of in vitro-cultured HSC. Collectively, these data support the notion that intrinsic retinoic acid metabolism directly impacts on HSC activation.

It needs to be noted that HSL is also expressed in other liver cells types, such as Kupffer cells, cholangiocytes and possibly other cells in the biliary tree (Chapter 3). Future studies need to address its role in these cell types in liver health and disease.

8.2. Redundancy of retinyl ester hydrolases in the liver/stellate cell (e.g. ATGL. PNPLA3 and HSL)

Together with our results presented in chapter 3, there are now three enzymes identified that are involved in retinyl ester hydrolysis in HSC, e.g. ATGL/PNPLA2, ADPN/PNPLA3, and HSL/LIPE. Their relative contribution to vitamin A metabolism, however, will strongly depend on the activation state of HSC, as we show that HSL/LIPE and ADPN/PNPLA3 expression is rapidly lost when qHSC transdifferentiate to aHSC, while ATGL/PNPLA2 levels remain the same. These gene-specific expression characteristics also explain earlier observations that pharmacological inhibition of ATGL leads to accumulation of retinyl esters in in vitro-cultured activated mouse HSC, while this was not observed with HSL inhibitors [6]. Assuming that the regulation of Pnpla2 and Lipe is the same in mouse and rat HSC, Lipe expression is extremely low in mouse aHSC and thus blocking its activity does not resort in a measurable effect on vitamin A metabolism in these cells. Whole body knock-out mice for either Pnpla2 or Lipe did not show altered levels of hepatic retinyl esters [6]. As these animals were not exposed to a fibrosis-inducing liver disease model, it is expected that all three different REHs are expressed in the quiescent HSC in the healthy livers, and can largely compensate for each other's absence. Interestingly, though, levels of retinyl esters in both human liver [17] and isolated primary HSC [18] are elevated in patients/cells homozygous for the PNPLA3 I148M allele. The enzyme activity of this variant of ADPN is markedly decreased. This observation may imply that ADPN/PNPLA3 may be the most dominant REH in the liver. Still, it cannot be excluded that the impaired function of ADPN may also affect the expression and/or activity of the other REHs, as for instance the absence of ATGL causes hyper-phosphorylation/activation of HSL [6]. Evidently, HSC harbor multiple and
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interconnected mechanisms to secure efficient hydrolysis of retinyl esters, most likely in order to maintain release of retinol to the circulation in times where dietary input of vitamin A is insufficient. This seems in contrast to the mechanism of hepatic vitamin A storage, is this (almost) completely depends on the enzyme LRAT [19]. Future studied using single, double and triple knock-out models of the different REHs need to establish the individual contribution of these enzymes in regulating hepatic vitamin A stores.

8.3. Impaired vitamin A metabolism is associated with NAFLD

NAFLD is the most prevalent chronic liver disease and its association with impaired vitamin A status is well-documented. In chapter 4, we reviewed the current knowledge about vitamin A metabolism in NAFLD, as well as the overlap with Type 2 diabetes (T2D) and metabolic syndrome (MetS). Most studies show that NAFLD is associated with vitamin A insufficiency or even vitamin A deficiency (VAD). This is primarily based on analysis of circulating retinol, where serum levels between 0.7 and 1.05 μmol/L are considered insufficient and below 0.7 μmol/L as deficient. Only a few anecdotal reports analyzed hepatic retinol and/or retinyl esters, which also seem reduced in fatty livers, but controversy exists on this topic.

This prompted us to analyze hepatic vitamin A metabolism in more detail in chapter 5. We indeed confirmed reduced retinol levels in fatty livers in mice. In sharp contrast, however, hepatic levels of retinyl palmitate (being the most dominant retinyl ester) were significantly increased. We noticed that the method of processing of the liver tissue for vitamin A quantification strongly influenced the ultimate levels of retinyl palmitate obtained, which may underlie the controversy between previous reports (discussed later). mRNA levels of retinoic acid-responsive genes were increased suggesting that production of retinoic acids is also enhanced in NAFLD. Interestingly, we found that vitamin A accumulated preferentially in hepatocytes in NAFLD (rather than in stellate cells), which may be an important additional factor contributing to disturbed vitamin A metabolism in NAFLD.

Limited data are available in which hepatic vitamin A storage (retinyl ester levels) were analyzed in NAFLD. However, three different studies by the same scientific group evaluated hepatic retinyl palmitate levels in NAFLD and reported strongly reduced retinyl esters levels in mice with NAFLD [20–22]. These contradicting results compared to our findings have long been a puzzling issue to us, as we consistently detected enhanced hepatic retinyl palmitate levels in the liver in different models of NAFLD mice.
Still, circulatory and hepatic retinol levels in these studies were in line with our findings. A critical evaluation of methods used in these and other previous studies [20,21], revealed that this may be caused by different protocols to extract lipophilic compounds out of the liver tissue (discussed in next section). The enhanced hepatic retinyl palmitate levels we detected in mouse NAFLD livers, were in line with studies that analyzed vitamin A autofluorescence in liver tissue of NAFLD patients and mice, which also indicated a redistribution of vitamin A pools to hepatocytes [23–25]. In addition to this, we also detected a hyperdynamic state of vitamin A metabolism in the liver of mice with NAFLD, similar to previous patient studies [26,27]. This hypermetabolic state was characterized by enhanced hepatic expression of the genes involved in vitamin A storage as well as conversion to retinoic acids.

In contrast to the reduced serum retinol levels, serum RBP4 levels are typically elevated in MetS patients and NAFLD with or without T2D [28–32]. In line, we also found elevated serum RBP4 levels in mice with NAFLD. Hepatocytes are the primary source of circulatory RBP4 [28], and secretion of hepatic RBP4 is strongly stimulated by the availability of retinol [33–35] as well as retinoic acids [34]. The combination of 1) reduced RBP4 protein levels in the liver, 2) unchanged Rbp4 mRNA levels in the liver and 3) increased mRNA levels of retinoic acid-responsive genes led us to speculate that enhanced RBP4 release from the liver may contribute to accumulation of RBP4 in the serum. However, impaired kidney function may also lead to elevated circulatory RBP4 levels [36–40] as this is the organ where RBP4 is cleared. Indeed, Western type high-fat diets have been shown to cause kidney injury as well [41–43], thus, it remains to be determined what the organ-specific (liver versus kidney) contributions are to the enhanced serum RBP4 levels in NAFLD. Unfortunately, we did not collect data on kidney function in our studies analyzing NAFLD mice. **Figure 1** shows our current working model to explain the biochemical effects we observed with respect to impaired vitamin A metabolism in NAFLD; the low hepatic retinol levels in NAFLD are likely a cumulative effect of elevated hepatic retinyl esters formation, excessive release of retinol and RBP4 from the liver, and enhanced retinol metabolism to retinoic acids.
**Figure 1. A schematic diagram presenting the effect NAFLD on vitamin A homeostasis.**

1) Retinyl esters increase and accumulate in the hepatocytes in NAFLD. 2) Elevated excretion of retinol and RBP4, increased circulatory levels in NAFLD mice, 3) but decreases hepatic levels of retinol in NAFLD. 4) Hyper-dynamic state of vitamin A metabolism increased the conversion of retinol metabolism into retinoic acid and 5) increased hepatic expression of retinoic acid-responsive genes, inflammatory markers and fibrotic markers in NAFLD. (↑)upregulated, (↓)downregulated. “Green” arrow marks increased flow and “red” arrow marks decreased flow. “Black” arrow marks normal flow.

### 8.4. Technical issues related to hepatic retinyl ester quantification.

Typically serum retinol levels are analyzed to assess the individuals vitamin A status. It is, however, well-known that serum retinol levels are maintained at a very stable level irrespective of the size of hepatic pool. Only when the hepatic pools are depleted, this will also be reflected in circulating retinol levels. Moreover, the systemic retinol levels are only a small fraction of the total body pool of vitamin A, which mostly consists of retinyl esters stored in the liver (≥80%) and adipose tissue (10-20%) [44–46]. Since it requires liver biopsies or resection material, hepatic vitamin A levels cannot easily be analyzed in NAFLD patients. Still, a few recent studies [20–22] analyzed both hepatic retinol and retinyl palmitate levels in human and mouse NAFLD and reported that hepatic levels of both retinol and retinyl palmitate were strongly reduced compared to healthy controls [20]. In chapter 5, we also detected the low hepatic retinol levels in NAFLD mice, but, in contrast found that hepatic retinyl palmitate levels were significantly increased. How to explain this apparent contradiction?
A critical and thorough comparison of the protocols used for retinol and retinyl palmitate quantification used by us and others revealed that there are different solvents in use to extract lipophilic compounds, like retinol and retinyl esters, from serum and/or liver tissue. We used \( n \)-hexane in our protocol, while others use acetonitrile-butanol for retinol and retinyl palmitate extraction from liver tissue and plasma. Remarkably, in an ono-to-one comparison of both methods using the same liver samples, we found that these solvents show very different capacities to extract retinyl palmitate from fatty liver tissue. Retinyl palmitate recovery was similar with both methods using healthy control, but in sharp contrast, recovery of retinyl palmitate from fatty liver tissue was significantly lower when using acetonitrile-butanol compared to \( n \)-hexane-based procedures. To complex matters, the recovery of retinol from control and fatty livers was not different between both procedures. This indicates that the extraction efficiency of retinyl esters by acetonitrile-butanol is compromised when a lot of fat is present in the (liver) tissue, while this is not the case for retinol. As these extraction procedures are likely optimized for retinol extraction (mostly from plasma or serum), such a differentiating effect on retinyl esters from fatty tissue has remained unnoticed so far. Evidently, this technical issue heavily affects the interpretation of the results reported so far. As we have performed the methods side-by-side in an attempt to exactly copy the procedures used by others (using the same quantities of tissue, extraction volumes and buffer compositions, including the use of anti-oxidants), we feel that using \( n \)-hexane as extraction solution gives the most reliable quantification of retinyl esters. Thus, we conclude that mouse NALFD livers are not prone to develop vitamin A deficiency, but rather show impaired vitamin A metabolism leading to retinyl ester accumulation, but a reduction in hepatic retinol levels. Importantly, the increased autofluorescence of vitamin A in NAFLD livers, as observed by us (Chapter 5) and others [23,25], further supports this interpretation.

8.5. Not vitamin A deficiency, but vitamin A metabolism should be targeted in NAFLD

Our data indicate that NAFLD is not associated with "plain" VAD, as hepatic storage of vitamin A in retinyl esters is even increased. Evidently, hepatic vitamin A metabolism is disturbed in NAFLD, with cell type-specific redistribution of vitamin A pools within the liver and reduced hepatic and serum retinol levels, possibly with increased serum RBP4. These effects may be a result of lipid accumulation in the liver, but may also
further contribute to disease progression. Accordingly, restoring proper vitamin A metabolism in the liver is a relevant therapeutic target for the treatment of NAFLD. Natural and synthetic ligands for RAR and RXR target cellular mechanisms that are impaired in NAFLD, including lipogenesis, lipolysis, fatty acid β-oxidation, adipogenesis, production of reactive oxygen species, cell proliferation and cell death (Chapter 4 [47]). However, this may require highly-selective therapeutic targeting of these pathways in NAFLD. Indiscriminate supplementation of vitamin A and/or its metabolites may lead to toxicity or adverse effects. A recent study emphasized the significance of a selective targeting approach in NAFLD and exemplified how adverse consequences in NAFLD may arise with alternative approaches are used to target the RAR-controlled transcription program. Selective targeting of RAR-β2 using the pharmacological ligand AC261066 improved hyperglycemia and hepatic steatosis in several animal models of NAFLD and type 2 diabetes, while targeting of RAR-α with AM80 exacerbated hyperglycemia and NAFLD in such animal models [21,22]. Moreover, the natural ligand for RARs, all-trans-retinoic acid; ATRA) stimulates the insulin secretion, while 9 cis-retinoic acid (9cRA), the ligand for RXR, suppressed insulin secretion from pancreatic β-cells [48]. These findings support the notion that vitamin A-responsive transcription factors are important therapeutic targets for NAFLD, but this requires highly selective modulation of specific pathways.

Still, manipulation of endogenous intrahepatic retinoic acid synthesis and metabolism may be another therapeutic target in NAFLD. Retinoic acid production from retinol is a 2 step process. First, retinol is converted into retinaldehyde by retinol dehydrogenases (RDH), followed by the production of retinoic acid by retinaldehyde dehydrogenase (RALDH) [1]. Inhibition of these enzymes suppresses the production of retinoic acids and subsequent RAR/RXR-signaling. Some studies have already identified possible targets relevant to the treatment of NAFLD. For example, heterozygous mice containing only one genomic copy of the RDH10 gene give rise to a moderate (~25%) reduction in hepatic ATRA levels, which was associated with a significant increase in hepatic steatosis and glucose intolerance in Western diet-fed mice, while these metabolic effects were corrected by pharmacological application of ATRA [49]. On the other hand, inhibition of Raldh1 has also beneficial effects in animal models of NAFLD, as it improved genetic- and diet-induced obesity, insulin resistance and energy dissipation [50]. This therapeutic effect was primarily assigned to the accumulation of retinyl aldehyde.
Our data indicate that sufficient vitamin is present in the fatty liver and that vitamin A supplementation, which could be indicated clinically due to the low serum retinol levels, may actually work counterproductive and cause vitamin A hepatotoxicity. Instead, supplementation or inhibition of the synthesis of particular metabolites may achieve more desirable therapeutic effects. For example, retinaldehyde supplementation or inhibition of retinaldehyde dehydrogenase show therapeutic potential in animal models of NAFLD [50]. Moreover, FXR ligands, like obeticholic acid (OCA), may reduce hepatic retinyl ester accumulation (Chapter 7) in NAFLD, and contribute to the therapeutic effect by normalizing vitamin A metabolism in the liver.

8.6. Genetic association of two novel genes (PNPLA3 and HSD17B13) of vitamin A metabolism with NAFLD

Interestingly, also genome-wide associations studies (GWAS) have clearly linked impaired vitamin A metabolism to NAFLD [26,51,52]. The first one is ADPN/PNPLA3. ADPN/PNPLA3 is a lipase that hydrolyses triglycerides into free fatty acids. A specific SNP was identified in this gene, PNPLA3 I148M, which is strongly associated with NAFLD and disease progression. The enzymatic activity of the PNPLA3 I148M variant is markedly reduced, leading to hepatic steatosis [18,53]. However, PNPLA3 also contains high REH activity and is highly expressed both in hepatocytes and HSC. As described before, PNPLA3 I148M carriers show increased levels of retinyl ester in the liver, in conjunction with reduced serum retinol levels [17]. So far, it is unknown how much the impaired function of PNPLA3 I148M in vitamin A metabolism contributes to the disease phenotype, as its role in triglyceride and retinyl ester hydrolysis go hand-in-hand. More recently, several SNPs in the gene encoding 17β-Hydroxysteroid dehydrogenase type 13 (HSD17B13) were found associated with specific histological features of NAFLD such as increased steatosis, but reduced inflammation, hepatocyte ballooning and cirrhosis. HSD17B13 proteins associates with lipid droplets in hepatocytes and harbors retinol dehydrogenase (RDH) activity. Overexpression of HSD17B13 markedly increased hepatic lipid accumulation [54]. Hepatic expression of both PNPLA3 and HSD17B13 is strongly increased in NAFLD [51,55].

Collectively we can conclude that genetic, biochemical and pharmaceutical approaches all point to a clear relationship between impaired vitamin A metabolism and NAFLD. However, despite all the compelling evidence, no clinical studies or trials
are ongoing to evaluate the therapeutic effect of selective targeting of vitamin A metabolism in NAFLD [56].

8.7. Perturbed vitamin A metabolism is also associated with GSD Ia

Patients with glycogen storage disease Ia (GSD Ia) are at risk to develop a fatty liver. Given our observations in chapter 5, we got interested in studying vitamin A metabolism in this patient group as well, as fatty liver is a common pathology in GSD Ia. Accordingly, in chapter 6 we analyzed whether GSD Ia patients and the liver-specific glucose-6-phosphatase knockout mouse (L-G6pc-/-) are at risk for impaired vitamin A metabolism. To our surprise, we found that serum retinol and RBP4 levels were significantly enhanced in GSD Ia patients and L-G6pc-/- mice, whereas hepatic retinol levels were significantly reduced. No change in retinyl ester levels was observed. These results indicate that GSD Ia is a rare example of a disease characterized by chronically elevated circulating retinol levels (a condition we termed “metabolic hypervitaminosis A”), which may contribute to various disease symptoms, including osteoporosis and hepatic steatosis.

GSD Ia and NAFLD share a number of common pathologies including hyperlipidemia, hyperinsulinemia and hepatic steatosis. With respect to vitamin A metabolism, both conditions are characterized by strongly reduced retinol levels in the liver, in conjunction with enhanced serum RBP levels. Other than that, many features were clearly different. Hepatic retinyl ester levels were clearly increased in the NAFLD animal models, while they were not affected –and even showed a trend towards reduction, in L-G6pc-/- mice. Mechanistically, this may be related to the fact that LRAT levels, the enzyme responsible for retinol esterification, was strongly increased in NAFLD mice, while they were not changed (at protein level) in L-G6pc-/- mice. A schematic representation of vitamin A metabolism in GSD Ia is presented in Figure 2.
Figure 2: A schematic diagram presenting the effect GSD Ia on vitamin A homeostasis.
1) Retinol-RBP4-TTR release from liver enhances in GSD Ia and 2) cause metabolic circulatory hypervitaminosis A and 3) reduced the tissue levels of retinol. 4) Retinoic acid conversion reduced while 5) hydrolysis of retinyl esters (vitamin A storage) was enhanced likely compensate the reduced hepatic retinol and retinoic acid levels. 6) No significant effect on retinyl esters synthesis was observed. (↑) upregulated, (↓) downregulated. “Green” arrow marks increased flow and “red” arrow marks decreased flow and “Black” arrow marks normal flow.

Interestingly, mice fat a high-fat diet (without the addition of cholesterol), also did not further accumulate retinyl esters in the liver. This may suggest that particularly the addition of cholesterol to the diet promotes hepatic retinyl ester accumulation. Indeed, HFC-diets lead to a significant increase in concentrations of total and free cholesterol in the liver features that are absent in the livers of L-G6pc-/- mice. Another clear difference between fatty livers in the NAFLD mice and the L-G6pc-/- mice is the differential expression of Cyp26a1, encoding an enzyme that catabolizes retinoic acids [57]. Cyp26a1 transcription is under the strict control of retinoic acids [58], so may serve as an indirect measure of these metabolites in the liver. Cyp26a1 mRNA levels are evidently elevated in NAFLD mice, while the opposite (strongly decreased) was observed in L-G6pc-/- mice. This indicated that the hepatic production of retinoic acids is enhanced in NAFLD, while this is impaired in L-G6pc-/- mice. So far, the mechanisms involved remain unclear. Interestingly, livers from NAFLD mice and L-G6pc-/- mice also showed opposite features of markers of inflammation and fibrosis. Where livers from HFC fed mice showed clear signs of inflammation and fibrosis, all such markers were suppressed in livers of the L-G6pc-/- mice. Future research should determine whether this might be caused by the differential effects on hepatic retinoic acid production in these two mouse models. Alternatively, such effects may also be
caused by the differences in hepatic cholesterol accumulation in these mouse models, as described above.

Thus, much remains to be studied to delineate the molecular mechanism that leads to impaired vitamin A metabolism in GSD Ia patients. Still, the ultimate effect seems to be that this disease is associated with chronically elevated circulating retinol levels. The absolute increase in serum retinol level appears relatively small, but the fact that this is a chronic condition may mean that it does contribute to symptoms, like osteoporosis, skin problems, and steatosis. These symptoms are now typically linked to insufficient vitamin D levels in these patients. Unfortunately, there is no easy way to reduce serum retinol levels. In clinical management of GSD Ia, it is at least important to be extra careful with vitamin A-containing supplements.

8.8. **Farnesoid X receptor (FXR) and bile acids regulate vitamin A storage**

Finally, in chapter 7, we analyzed the role of FXR and FXR agonists in vitamin A metabolism. As eluded to in chapter 2, vitamin A metabolites are well-known to (co)regulate bile acid metabolism. In contrast, nothing was known about the regulatory role of FXR(agonists) on vitamin A metabolism. Moreover, FXR is an important therapeutic target in chronic liver diseases, including NAFLD, making such information even more relevant. Our primary observation was that FXR null mice contain very low levels of vitamin A (both retinyl esters and retinol) in the liver, suggesting a crucial role for this transcription factor in hepatic vitamin A storage and metabolism. Liver-specific deletion revealed a similar phenotype, whereas intestine-specific FXR null mice had normal hepatic vitamin A levels. In contrast to what then might be expected, treatment of mice with the FXR ligands obeticholic acid (OCA) or cholic acid (CA) also lead to depletion of hepatic retinyl esters. So far, we are unable to trace these effects to transcriptional effects of OCA-activated FXR. Instead, OCA showed pronounced post-transcriptional effects in the liver, in particular on retinyl hydrolases, e.g. ATGL, PNPLA3 and HSL. Further studies are needed to delineate the mechanisms that are involved in the FXR-mediated effects on vitamin A metabolism.

FXR controls highly diverse metabolic processes, varying from bile acid, triglyceride, cholesterol, glucose to amino acid metabolism [59–62]. In addition, it regulates autophagy, inflammation and extracellular matrix production [63–65]. Our study was the first in which the putative role of FXR in hepatic vitamin A metabolism is analyzed (chapter 7). FXR-null mice have previously been shown to develop mild steatohepatitis, particularly accumulating triglycerides, which may progress to NASH.
even when fed a chow diet [66–70]. Dietary vitamin A uptake is accompanied by the intestinal fat absorption and any impairment in the intestinal absorption of dietary fat also impairs the uptake of dietary vitamin A [10,11]. Intestinal FXR is pivotal for the absorption of fats and regulates lipid homeostasis. Intestinal-specific FXR-deletion in mice suppresses diet-induced obesity [12], while hepatic FXR-deficiency increases diet-induced lipid accumulation in the liver independent to FGF-15 signaling [13]. In sharp contrast to the enhanced hepatic lipid content, our data show that the hepatic vitamin A pool (retinyl palmitate and retinol) was strongly reduced (>90%) in total FXR-null mice as compared to the age and sex-matched controls. Serum retinol levels were not decreased in these animals. Moreover, intestine-specific FXR-null mice had normal vitamin A reserves as compared to the control mice. So, normal circulatory retinol level, in the presence of extremely low vitamin A reserves, once again reveal that little hepatic reserves (5% in FXR-null mice) are sufficient to maintain normal circulatory retinol levels and do not mirror what might be happening with vitamin A metabolism in the liver.

The analysis of hepatic vitamin A levels in OCA-fed mice (3 weeks) surprised us with a sharp reduction in retinyl ester (> 60% down) and retinol (>5-fold up) levels in the liver. At present, we are unable to explain the underlying mechanism even after intensive transcriptional and post-translational analysis of regulatory pathways in the liver. Although we detected increased mRNA levels of hepatic lipases (Pnpla3 and Lipe), the corresponding protein levels were sharply reduced. One hypothesis still to be tested is whether this effect may possibly develop from the gut. OCA is a high-affinity ligand for FXR, but may impair bile acid-driven vitamin A absorption in the intestine and thereby prevent efficient hepatic storage. As a consequence, vitamin A would be mobilized from the liver to provide peripheral tissues with this essential vitamin. Hepatic retinyl ester and retinol levels rapidly build up in early life, so “the reduction” caused by 3-week OCA treatment in ~10 week old mice may be caused by a block in retinyl ester storage, enhanced retinyl ester hydrolysis or a combination of both. A detailed analysis of vitamin A metabolites in the intestine and circulation is required to shed light on this issue, samples that were unfortunately not available for this study.

Importantly, our data suggest that patients under OCA or bile acid therapy may be at risk to develop hepatic vitamin A deficiency, which may compromise metabolic, immunologic and regenerative control in the liver. This is particularly relevant for
patients with chronic liver disease, as they already show impaired vitamin A metabolism. However, this could go either way: enhanced metabolism of vitamin A could actually be part of the therapeutic effect in NAFLD as it helps to mobilize hepatic retinol. In contrast, OCA treatment may further deplete hepatic vitamin A stores in other chronic liver diseases that are associated with true hepatic and circulatory VAD, such as PBC and PSC.

8.9. Conclusion and future perspective

The liver is the central organ controlling bile acid and vitamin A metabolism. It stores vitamin A in hepatic stellate cells that are capable of maintaining stable blood retinol levels for months to years even when dietary intake is insufficient. This is a very important function of the liver as vitamin A metabolites are required for many different physiological processes, including growth, vision, immunity and tissue differentiation/regeneration. Vitamin A metabolism is impaired in chronic liver diseases, but we need to redefine our clinical view that low serum retinol levels are a sign of systemic and hepatic vitamin A deficiency. Hepatic retinol levels rare indeed typically reduced in fatty liver, but hepatic vitamin A storage in retinyl esters may be actually increased in these conditions. So, dietary vitamin A supplementation is unlikely to provide any therapeutic effect in NAFLD. In contrast, it may even aggravate disease progression. Specific retinoic acids have already shown promising therapeutic effects in animal models of NASH and cholestatic liver disease and this needs further exploration. Not only as a single therapy but maybe also combined with FXR agonists, as they also affect hepatic vitamin A metabolism.

With respect to vitamin A metabolism in the liver, many fundamental questions remain unanswered: 1) How and in what form is vitamin A transported between hepatocytes, HSC and the circulation? 2) What is the relative contribution of different retinyl ester hydrolases (HSL, ATGL, PNPLA3) to the release of retinol form HSC? 3) Does impaired vitamin A metabolism actually contribute to the progression of liver diseases? 4) How do glucose and/or glycogen interact with vitamin A metabolism? 5) Is the therapeutic effect of FXR agonists, like OCA, linked to its effect on vitamin A metabolism? In conclusion, there is still a lot to learn about vitamin A metabolism in the liver in healthy- and pathological conditions, which will hopefully reveal novel therapeutic targets for the treatment of chronic liver disorders, and in particular to prevent the development of advanced hepatic pathologies, such as fibrosis, cirrhosis and hepatocellular carcinoma.
REFERENCES


Chapter 8


Discussion


develop increased energy expenditure, improved glucose control and liver damage resembling NASH. PloS One. 8 (2013) e64721. doi:10.1371/journal.pone.0064721.
