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## Intestinal function in cholestasis and essential fatty acid deficiency

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# **CHAPTER 7**

## **General discussion**

## GENERAL DISCUSSION

Cholestatic liver disease covers a wide range of conditions characterized by defective bile formation associated with reduced bile salt transport from the liver into the intestinal lumen. Physiological consequences include retention of bile salts and other bile constituents in the hepatocytes, limited availability of bile salts in the intestinal lumen and elevated plasma bile salt levels. These consequences eventually lead to liver injury, lipid malabsorption, pruritus, jaundice and potentially peripheral tissue injury. Cholestatic patients, especially children, frequently develop failure to thrive which consists of failure to grow and nutritional deficiencies, including those of fat-soluble vitamins and essential fatty acids (EFA). A malnutritional state strongly affects prognosis of cholestatic patients. Identification of nutritional deficiencies in cholestasis and understanding of the pathophysiology underlying these deficiencies will help to improve the prognosis of cholestatic children by allowing optimization of their nutritional status. We therefore aimed to elucidate the effects of cholestasis and EFA deficiency on intestinal function, with emphasis on digestion and absorption of fats and carbohydrates.

### Fat digestion and absorption in cholestatic conditions

Fat absorption is significantly impaired during cholestasis and EFA deficiency. In cholestatic disorders, EFA deficiency is induced by fat malabsorption. However, EFA deficiency itself is also able to induce fat malabsorption. In this way, cholestatic children can enter a 'vicious circle' of fat malabsorption inducing EFA deficiency and vice versa. Hence, unraveling the mechanisms behind cholestasis-induced fat malabsorption and EFA deficiency-induced fat malabsorption are of great importance.

Fat malabsorption in cholestasis is obviously to a great part due to limited availability of bile salts in the intestinal lumen, resulting in impaired fat solubilization. In chapter 3 we set out to address intestinal function in cholestasis. Our data indicate that fat absorption is impaired in cholestatic as well as in bile-deficient rats. Fat malabsorption in cholestatic and bile-deficient animals is at least for a large part caused by insufficient micellar solubilization due to limited availability of bile salts and phospholipids in the intestinal lumen. Interestingly, bile-deficient rats that do not show systemic accumulation of bile salts as seen in cholestasis, but also lack luminal bile salts, had milder fat malabsorption than cholestatic rats. Bile-deficient rats appear to compensate by ingesting more fat. Cholestatic and bile-deficient rats share the intestinal phenotype, but differ in the accumulation of bile salts in hepatocytes, cholangiocytes and circulation. We speculate that in absence of accumulation of bile salts, compensation mechanisms exist to counteract fat malabsorption.

The mechanism behind EFA deficiency induced fat malabsorption is less clear. EFA deficiency in rats has been ascribed to decreased bile flow and biliary secretion of bile salts and phospholipids, leading to impaired fat solubilization<sup>1,2</sup>. In addition, intracellular events such as triglyceride re-esterification and chylomicron formation were found to be impaired<sup>1,3</sup>. Fat digestion and fatty acid uptake by the enterocytes, however, were not affected<sup>1,3</sup>. In mice, data in literature about EFA deficiency-induced fat malabsorption only relates to bile formation. In contrast to rats, EFA deficiency in mice is not due to impaired bile formation. Rather, EFA deficiency in mice increases bile flow and biliary secretion of bile salts and phospholipids<sup>4</sup>. Cholestasis is associated with accumulation of bile salts in hepatocytes and cholangiocytes. Bile salts are natural ligands for the nuclear receptor FXR, which is involved in bile salt homeostasis and lipid homeostasis. Since bile salt homeostasis and lipid homeostasis are also altered during EFA deficiency<sup>4</sup>, we wondered whether FXR is

involved in EFA deficiency-induced fat malabsorption. The FXR deficient and EFA deficient conditions separately have been associated with increased bile flow and bile salt pool size<sup>4,5</sup>. EFA deficient *Fxr*<sup>-/-</sup> mice had similar bile flow and similar bile salt pool sizes compared to EFA deficient control mice, suggesting that the bile flow and biliary bile salt output is maximally induced during EFA deficiency or FXR inactivation. Concluding, the amelioration of fat malabsorption in EFA deficient *Fxr*<sup>-/-</sup> mice compared to EFA deficient control mice is not due to altered bile flow.

In chapter 6, we suggested that altered composition of the bile salt pool might underly the improvement of fat malabsorption in EFA deficient *Fxr*<sup>-/-</sup> mice. The cholic acid to muricholic acids ratio was increased in EFA deficient *Fxr*<sup>-/-</sup> mice compared to EFA deficient mice, rendering the bile salt pool more hydrophobic. In rodents, the majority of the bile salt are conjugated with taurine. The order of taurine-conjugated bile salts ranging from relatively hydrophilic to relatively hydrophobic is:  $\alpha$ -muricholic acid -  $\beta$ -muricholic acid – ursodeoxycholic acid – cholic acid – chenodeoxycholic acid. Increased hydrophobicity of the bile salt pool has been associated with increased absorption of cholesterol<sup>6-9</sup>, probably due to enhanced solubilization of cholesterol and/or facilitated dissociation at the unstirred water layer. Increased solubilization and/or dissociation could also account for increased uptake of fatty acids, leading to amelioration of fat malabsorption as seen in EFA deficient *Fxr*<sup>-/-</sup> mice compared to EFA deficient control mice. This phenomenon was also observed in the separate deficiencies, EFA deficiency and FXR deficiency<sup>4,5</sup>. We can speculate about the mechanism underlying the increased hydrophobicity of the bile salt pool in absence of FXR and EFAs. The key enzyme regulating the hydrophobicity of the bile salt pool is sterol 12 $\alpha$ -hydroxylase CYP8B1. CYP8B1 is required for the conversion of cholesterol to the primary bile salt cholic acid. The rate-controlling enzyme involved in bile salt biosynthesis CYP7A1 is required for synthesis of both cholic acid and chenodeoxycholic acid. In rodents, the majority of chenodeoxycholic acid is converted to  $\alpha$ -muricholic acid, which is subsequently converted to  $\beta$ -muricholic acid. Thus the CYP8B1 activity level relative to the CYP7A1 activity level determines the hydrophobicity of the bile salt pool. Kok *et al.* demonstrated that FXR inactivation in mice was associated with increased mRNA expression of *Cyp7a1*, while mRNA expression of *Cyp8b1* was similar in *Fxr*<sup>-/-</sup> mice compared and control mice. The ratio of *Cyp7a1* to *Cyp8b1* expression, however, was increased, suggesting a shift of the balance towards chenodeoxycholic acid and muricholic acids synthesis rather than cholic acid<sup>5</sup>. We speculate that *Cyp8b1* may not be involved in regulation of bile salt pool hydrophobicity.

In cholestatic animals, in contrast, the cholic acid to muricholic acid ratio is decreased, leading to increased hydrophilicity of the bile salt pool (unpublished observations). Concomitantly, CYP8B1 expression has been shown to be repressed in cholestatic rats<sup>10</sup>. Thus, the hydrophobicity of the bile salt pool is decreased in cholestatic animals to counteract hydrophobic bile salt-induced cytotoxicity and the hydrophobicity of the bile salt pool is increased in EFA deficient animals to counteract fat malabsorption.

Interestingly, FXR inactivation and inactivation of its target gene SHP have been associated with protection against bile salt-induced liver injury<sup>11,12</sup>. Thus, removal of FXR or its ligands, bile salts, results in an improvement in fat absorption and protection against bile salt-induced liver injury. Since bile salts and FXR are involved in many other physiological functions, such as energy homeostasis and glucose homeostasis<sup>13</sup>, antagonism of FXR would likely lead to many side effects. However, if we can unravel which mechanisms underly the amelioration of fat malabsorption and hepatoprotection in EFA deficient *Fxr*<sup>-/-</sup> mice compared to EFA

deficient control mice and bile-deficient rats compared to cholestatic rats, we might have a target to intervene clinically.

### **Carbohydrate digestion and absorption in cholestatic conditions**

Defects in bile production, as observed in cholestasis, limit the availability of bile salts in the intestinal lumen for the facilitation of lipid-soluble nutrient absorption. Another aspect of cholestasis is a strongly increased plasma bile salt level, due to regurgitation of bile salts into the circulation from the hepatocytes<sup>14-16</sup>. We wondered whether this high plasma bile salt level could affect intestinal epithelial cells through processes such as proliferation, differentiation or apoptosis, eventually leading to altered intestinal function. There have been numerous reports demonstrating that bile salts can induce proliferation, differentiation or apoptosis *in vitro*, depending on their hydrophobicity, conjugative state, concentration, cell signaling pathways and cell type involved<sup>17-32</sup>.

As cholestatic liver disease is frequently accompanied by nutritional defects<sup>16</sup>, we aimed to explore intestinal digestion and absorption of carbohydrates in cholestatic conditions. *In vivo* data about intestinal function during cholestasis, specifically carbohydrate digestion or absorption is scarce. Jejunal absorption of glucose was found unaffected in cholestatic rats<sup>33</sup>. Also bile-deficiency alone, i.e. without cholestasis did not impair sucrase enzyme activity in rats<sup>34</sup>, suggesting that intestinal nutrient absorption is maintained in animal models of intestinal bile deficiency. We addressed whether carbohydrate digestion was affected in cholestatic rats (chapter 3). Interestingly, carbohydrate digestion was maintained in cholestatic rats, but impaired in EFA-deficient mice (chapter 5). This discrepancy can be explained by the fact that the cholestatic rats were subjected to the carbohydrate digestion and absorption test 1 week after bile duct ligation, which is too short to develop EFA deficiency. Concluding from chapter 3, 4 and 5 it is highly likely that lactose digestion is impaired in cholestatic disorders that are accompanied by EFA deficiency.

Enterocytes are protected during cholestasis, both in *in vitro* (chapter 4) and *in vivo* (chapter 3). In chapter 4 we propose that nutrient absorption is not affected in cholestatic conditions, because nutrient absorption and bile salt re-absorption are localized in different intestinal segments. The jejunum is the major site of nutrient absorption, while bile salt re-absorption is restricted to the terminal ileum<sup>35</sup>. We demonstrated that reduced sucrase activity was associated with increased expression of intestinal bile salt transporters (ASBT). Concluding, intestinal function, i.e. sucrase activity, is impaired when high amounts of bile salt are transported into the enterocytes. In accordance with this theory, Lee *et al.* showed that mRNA and activity levels of sucrase were strongly reduced in the terminal ileum compared to the duodenum, jejunum and proximal ileum<sup>36</sup>. That the separation of nutrient absorption and bile salt re-absorption in the intestine is highly important and tightly regulated was also demonstrated by Bosse *et al.* The authors identified the transcription factor Gata-4 as the major determinant of jejunal-ileal identities in mice. Synthesis of a transcriptionally inactive Gata-4 mutant in the mouse jejunum resulted in an attenuation of expression of genes involved in nutrient absorption and an induction of genes involved in bile salt re-absorption<sup>35</sup>.

Snipes *et al.* noted pathological changes in the intestines of EFA deficient rats, i.e. restricted surface area due to villi shortening and a lack of cellular differentiation<sup>37</sup>. This effect has been ascribed to the decreased EFA content in membrane phospholipids<sup>1,3</sup>. Our study with EFA deficient mice also pinpointed the defect in lactose digestion to the jejunum, though not accompanied by villi shortening (chapter 5). Interestingly, a significant difference in the EFA

linoleic acid (LA) concentration was observed between jejunal membrane phospholipids of EFA deficient and control mice. Cell membrane fluidity is determined by its lipid composition. Incorporation of saturated fatty acids and cholesterol in the membrane will render the membrane more rigid, while incorporation of unsaturated fatty acids will make it more fluid. The number of receptors and their affinity to their respective ligands is considered to depend on the fluidity of the cell membrane<sup>38</sup>. A reduction in plasma membrane fluidity has also been associated with decreased luminal membrane permeability to macromolecules<sup>39</sup>. Moreover, decreased incorporation of EFA-derived fatty acids in membrane phospholipids has been associated with altered activity of hydrolytic enzymes in the jejunum<sup>40</sup>. This is in accordance with the observation that lactose digestion was impaired in EFA deficient mice. In contrast to lactose digestion, glucose absorption was maintained in EFA deficient mice. The altered composition of membrane fatty acids may only affect activity of hydrolytic enzymes such as lactase that are attached to the membrane<sup>41</sup>, while the membrane-spanning glucose transporters are unaffected. However, it should be realized that not only the activity of lactase was decreased in EFA deficient jejunum, but also its mRNA expression. This observation indicates that the effects of EFA deficiency cannot completely be explained by alterations of the cell membrane fluidity.

#### **Concluding remarks and perspectives**

In conclusion, cholestasis as such does not lead to impaired intestinal carbohydrate digestion. When accompanied by EFA deficiency, however, cholestasis in rats is also associated with impaired lactose digestion. Thus, our initial conclusion (chapter 3) that carbohydrate addition to the diet of cholestatic patients should be valuable for optimization of their nutritional status has to be fine-tuned. Addition of monosaccharides to the diet of cholestatic patients is not beneficial, since they have a high glycemic index. However, by adding small amounts of disaccharides more frequently per day, we may be able to maximally utilize the limited carbohydrate digestion capacity.

In order to assess carbohydrate digestion and absorption in an animal model we adapted an existing test based on stable isotope dilution technique<sup>42,43</sup>. The blood spot technique, developed by our group, made it possible to test these parameters in mice<sup>44</sup>. Combination of both techniques makes it possible to test digestion and absorption of various nutritional compounds using small volumes of blood, and can thus be utilized for small animals and neonates. The development and adaptation of this technique to specific nutrients, organisms and disorders, will accelerate the identification of specific nutritional deficiencies in cholestatic children

Regarding the role of FXR in therapies for EFA deficiency, FXR has been implicated in so many regulatory functions that antagonizing FXR solely for the purpose of enhancing fat absorption is not applicable. Perhaps, if we identify the mechanism behind the FXR inactivation-induced amelioration of fat malabsorption, we can aim to develop gene-selective or organ-selective ligands to specifically target the gene or organ responsible.

In this thesis we gained more insight in the pathophysiology of cholestasis-induced failure to thrive. On this account, we can aim to further optimize the nutritional status of children with cholestatic liver disease.

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