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Intestinal function in cholestasis and essential fatty acid deficiency

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CHAPTER 6

Essential fatty acid deficiency in mice: milder fat malabsorption and a more hydrophobic bile salt composition

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In preparation

ABSTRACT

Cholestatic liver disease is frequently accompanied by fat malabsorption and essential fatty acid (EFA) deficiency. EFA deficiency in mice disturbs fat malabsorption via unidentified mechanisms, presumably at the level of the intestinal mucosa. The farnesoid X receptor (FXR) is involved in the regulation of bile salt homeostasis in the enterohepatic circulation. We addressed the role of FXR in fat absorption and bile salt homeostasis in EFA deficient mice.

Fxr$^{-/-}$ and *Fxr*$^{+/+}$ (control) mice were fed an EFA deficient diet for 8 weeks, after which fat absorption was determined by 72 h fat balance. Bile production and intestinal mRNA expression of proteins relevant for bile salt homeostasis and lipid homeostasis were analyzed.

EFA deficient diet induced a similar degree of EFA deficiency in *Fxr*$^{-/-}$ and *Fxr*$^{+/+}$ mice (triene/tetraene ratio: 0.14 (median, range 0.09-0.32) vs. 0.13 (0.07-0.24), resp. Fat absorption, however, was significantly better preserved in *Fxr*$^{-/-}$ mice (78±4% of amount ingested) compared with controls (70±4%, $P<0.05$). Correspondingly, *Fxr*$^{-/-}$ mice gained more body weight during the experimental period compared with controls (+14±7 vs. +7±5% of initial body weight, respectively; $P<0.05$). Bile flow and biliary secretion rates of bile salts, cholesterol and phospholipids were similar in *Fxr*$^{-/-}$ and control mice. The composition of the biliary bile salt pool was altered in *Fxr*$^{-/-}$ mice, however, characterized by increased hydrophobicity (cholic acid-muricholic acid ratio: 1.5 (0.93-4.21) *vs.* 1.0 (0.34-1.12), $P<0.05$). *Fxr*$^{-/-}$ mice had a higher fecal bile salt loss (+60%, $P<0.01$), coinciding with a lower intestinal mRNA expression of bile salt transporter Asbt in the terminal ileum.

We conclude that inactivation of FXR ameliorates the fat malabsorption and improves growth of EFA deficient mice, probably by increasing the hydrophobicity of the bile salt pool.

INTRODUCTION

Cholestasis is defined as a decreased flow of bile and its consituents into the small intestine. Biliary bile salts aid in the absorption of lipids and lipid-soluble vitamins from the intestine. Consequently, cholestatic liver disease is frequently accompanied by fat malabsorption and essential fatty acid (EFA) deficiency, eventually leading to malnutrition. Malnutrition seriously worsens the prognosis and treatment outcome in cholestatic children [1-3].

The fatty acids linoleic (LA; C18:2n-6) and linolenic (ALA; C18:3n-3) acid cannot be synthesized *de novo*. These so-called essential fatty acids therefore need to be acquired from external sources, usually the diet. After absorption, linoleic LA and ALA can be converted into long chain polyunsaturated fatty acids (LCPUFAs) such as arachidonic (AA; C20:4n-6), eicopentaenoic (EPA; C20:5n-3) and docosahexaenoic (DHA; C22:6n-3) acid. Deficiency of EFAs and LCPUFAs has been associated with obesity, hypertension, diabetes mellitus, schizophrenia, Alzheimer's disease and cancer [4,5].

Not only cholestasis can induce fat malabsorption, EFA deficiency in itself also causes fat malabsorption in rats and mice [5]. The mechanism by which EFA deficiency, in the absence of cholestasis, decreases fat absorption is incompletely understood. EFA deficiency-induced fat malabsorption in rats has been ascribed to decreased bile formation, impaired triglyceride re-esterification and impaired chylomicron formation [6,7]. EFA deficiency in mice, however, is accompanied by increased bile formation [8].

The farnesoid X receptor (FXR) has been implicated in the regulation of bile salt and lipid metabolism [9]. Fxr-deficient mice display increased bile flow and bile salt pool size, and a more hydrophobic bile salt composition due to an increased contribution of cholic acid (CA) [8,10]. Interestingly, we previously demonstrated that EFA-deficiency in (wild-type) mice has similar phenotypic characteristics [8]. Based on this similarity, we addressed whether FXR inactivation would affect the phenotype of EFA deficiency in mice. We hypothesized that EFA deficiency in Fxr-null mice would ameliorate fat malabsorption, compared with control mice, by (further) increasing bile flow, bile salt pool size and relative CA contribution.

MATERIAL AND METHODS

Animals and housing

We used Fxr knockout mice that were originally described by Kok *et al.* [10]. Male homozygous (*Fxr*$^{-/-}$) and wild-type (*Fxr*$^{+/+}$) mice (C57BL/6J-129/OlaHsd; 25-35 g) were bred at the animal facility of the University of Groningen. Mice were housed in a light- and temperature-controlled facility. Food and water were available *ad libitum*. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, Netherlands.

Experimental diet

We used high-fat EFA-deficient (16 wt% and 34 energy% fat) to induce EFA deficiency in mice. The diet was custom synthesized by Arie Bloks BV (Woerden, the Netherlands, diet code: #4141.08). The EFA-deficient diet contained 70 mol% palmitic acid (C16:0), 19 mol% stearic acid (C18:0), 9 mol% oleic acid (C18:1n-9) and 2 mol% linoleic acid (C18:2n-6). Fatty acid contents of the diets were analyzed by extracting, hydrolyzing and methylating total dietary fatty acids as described by Muskiet *et al.* and subsequent separation and

quantification of fatty acid methyl esters was performed by gas chromatograhy as described previously [8,11].

Experimental procedures

Mice were fed standard laboratory chow containing 6 weight% fat from weaning, and switched to an EFA deficient high-fat (16 weight%) diet at 8 wk of age. At the end of an 8 wk-period on EFA deficient diet, feces were collected over 72 hours to measure fat balance. Bile production was determined by bile collection for 30 min via cannulation of the gallbladder. After bile collection, mice were sacrificed by obtaining a large blood sample via cardiac puncture. Erythrocyte EFA status was assessed by the triene/tetraene ratio, obtained from gaschromatography of fatty acid methyl esters [12]. The small intestine was excised, flushed with ice-cold PBS and was divided into 3 pieces of equal length. Material was harvested for gene expression from the middle of each piece and the distal end of the third piece, representing the proximal, medial, distal and terminal ileal segment of in the intestine.

Analytical methods

The erythrocyte triene/tetraene ratio was determined as described by Werner *et al.* [12]. Fat absorption was determined by quantification of fatty acid ingestion and fecal excretion over a 72 h period, using gas chromatography [13]. Bile salt composition of bile and fecal excretion of bile salts were determined [14]. Plasma and biliary cholesterol and phospholipids, and plasma triglyceride concentrations were determined by routine laboratory techniques.

RNA isolation and measurement of mRNA levels by real-time PCR (Taqman)

mRNA expression levels in proximal, medial, distal and terminal ileal part of the small intestine were measured by real-time PCR, as described previously [15]. PCR results were normalized to β-actin mRNA levels. The sequences of the primers and probes are listed in Table 1.

Table 1. Primer and probe sequences

Gene	GenBank	Forward Primer	Reversed Primer	TaqMan® probe
β-actin	NM_007393	AGC CAT GTA CGT AGC CAT CCA	TCT CCG GAG TCC ATC ACA ATG	TGT CCC TGT ATG CCT CTG GTC GTA CCA C
Fat	BC010262	GAT CGG AAC TGT GGG CTC AT	GGT TCC TTC TTC AAG GAC AAC TTC	AGA ATG CCT CCA AAC ACA GCC AGG AC
Fatp-4	NM_011989	CCA GAC AAG GGT TTT ACA GAT AAG CT	ACC TGC TGT GCA CCA CAA TG	CGG GCA CCA CGG GGC TAC CC
Ifabp	NM_007980	GAG TTG AGG CCA AGC GAT TCT	GAG CCT GGC ATT AGC ATG ATG	CTC TTC AGC GTT GCT CCA GGC TCT GAG
Dgat-1	NM_010046.2	GGT GCC CTG ACA GAG CAG AT	CAG TAA GGC CAC AGC TGC TG	CTG CTG CTA CAT GTG GTT AAC CTG GCC A
Dgat-2	NM_026384.2	GGG TCC AGA AGA AGT TCC AGA AG	CCC AGG TGT CAG AGG AGA AGA G	CCC CTG CAT CTT CCA TGG CCG
Mttp	NM_008642	CAA GCT CAC GTA CTC CAC TGA AG	TCA TCA TCA CCA TCA GGA TTC CT	ACC GCA AGA CAG CGT GGG CTA CA
Fxr-α	U09417	CTT TCT GAA AGC TTA TTT GGT ATG CTA A	AGT ACG ATT CCA AAT CCA GAT TCT G	AAC ACG CGG CAG GCC CTC TG
Fxr-β	AK002513	GTG AAG CCA GCT AAA GGT ATG CTA A	AGT ACG ATT CCA AAT CCA GAT TCT G	AAC ACG CGG CAG GCC CTC TG
Fgf-15	NM_008003	GCC ATC AAG GAC GTC AGC A	CTT CCT CCG AGT AGC GAA TCA G	CGC TCA TGC AGA GGT ACC GCA CG
Asbt	NM_011388	ACC ACT TGC TCC ACA CTG CTT	CCC GAG TCA ACC CAC ATC TT	CCC TTG GAA TGA TGC CTC TTT GCC TC
Ibabp	NM_008375	CCC CAA CTA TCA CCA GAC TTC G	ACA TCC CCG ATG GTG GAG AT	TCC ACC AAC TTG TCA CCC ACG ACC T

Statistical analysis

Results are provided in means ± SD or median and range for the indicated number of mice per group. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we analyzed the results with the two-tailed Student's t-test for normally distributed data or with the Mann-Whitney *U*-test for data that were not normally distributed. Variance among data was determined using Levene's test for equality of variances. $P<0.05$ was considered significant.

RESULTS

EFA deficiency in *Fxr$^{-/-}$* mice: higher weight gain and fat absorption

EFA deficiency was induced in *Fxr*$^{-/-}$ and *Fxr*$^{+/+}$ mice to a similar extent as indicated by the triene/tetraene ratio measured in RBC (median (range): 0.14 (0.09-0.32) vs. 0.13 (0.07-0.24), resp., NS). After 8 weeks on an EFA deficient diet, *Fxr*$^{-/-}$ mice had gained more weight compared to *Fxr*$^{+/+}$ mice (+14±7 vs. +7±5% of initial body weight, $P<0.05$). Fat ingestion was similar in *Fxr*$^{-/-}$ and *Fxr*$^{+/+}$ mice, while fecal fat excretion was lower in *Fxr*$^{-/-}$ mice (197±56 vs. 268±53 µmol/day, $P<0.05$; Fig 1). Absorption coefficients of total fatty acids, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9) and linoleic acid (C18:2n-6) were higher in *Fxr*$^{-/-}$ mice compared to *Fxr*$^{+/+}$ mice (all $P<0.05$; Fig 1).We quantified the mRNA expression of proteins that have been implicated in fatty acid transport, including fatty acid translocase (Fat), fatty acid transport protein 4 (Fatp-4), and cytosolic intestinal fatty acid binding protein 1 (Ifabp-1). mRNA expression of *Fat*, *Ifabp-1* and *Fatp-4* was not changed in *Fxr*$^{-/-}$ mice. *Fat* and *Fatp-4* expression was significantly higher in proximal and medial part compared to the distal part of the small intestine in both groups ($P<0.01$), similar to previous reports [16-18]. *Ifabp-1* expression was higher in the medial part compared to the proximal and distal part of the small intestine in the *Fxr*$^{-/-}$ mice ($P<0.01$; Fig 1).

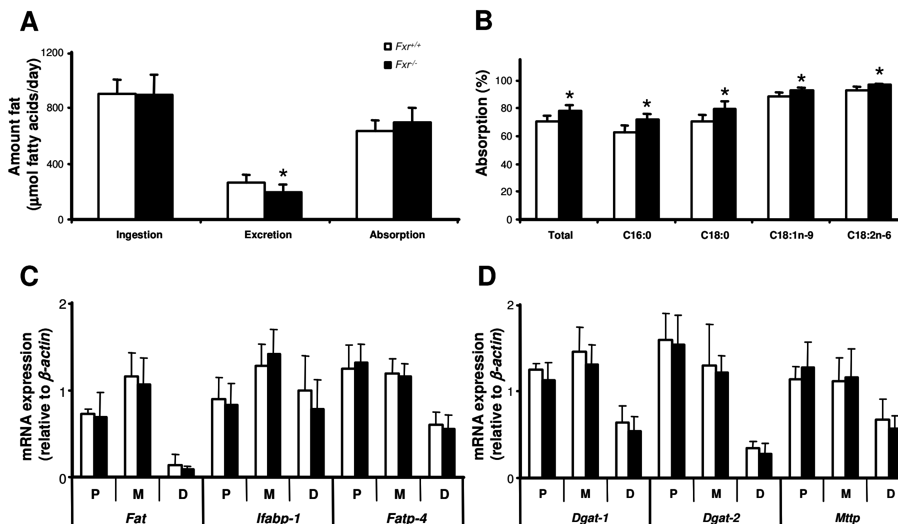

Figure 1. (A) Fat ingestion, fecal excretion and net absorption measured over a 72h period in EFA deficient Fxr$^{+/+}$ (white bars) and Fxr$^{-/-}$ mice (black bars). (B) Absorption percentages of total fatty acids and of the major fatty acids: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9) and linoleic acid (C18:2n-6). (C) mRNA expression of *Fat*, *Fatp-4* and *Ifabp*, normalized to *β-actin* levels, in the proximal (P), medial (M) and distal (D) part of the small intestine of EFA deficient *Fxr*$^{+/+}$ (white bars) and *Fxr*$^{-/-}$ mice (black bars), measured by quantitative PCR. (D) mRNA expression of *Dgat-1*, *Dgat-2* and *Mttp*, normalized to *β-actin* levels, in the proximal (P), medial (M) and distal (D) part of the small intestine of EFA deficient *Fxr*$^{+/+}$ (white bars) and *Fxr*$^{-/-}$ mice (black bars), measured by quantitative PCR. *Fxr*$^{-/-}$ mice; n=7 and *Fxr*$^{+/+}$ mice; n=5. Results are expressed as means ± SD. *$P<0.05$.

The enzymes involved in re-esterification of triglycerides in the enterocyte include acyl-CoA:diacylglycerol acyltransferase (DGAT) 1 and 2, catalyzing the esterification of diacylglycerol to triglycerides. Microsomal triglyceride transfer protein (Mttp) is essential for the proper assembly of cholesterol, triglycerides, phospholipids and apolipoprotein B into chylomicrons [19,20]. Expression of *Dgat-1*, *Dgat-2* and *Mttp* showed higher expression in the proximal and medial part compared to the distal part (*P*<0.01; Fig 1), similar to previous reports [21,22]. Inactivation of FXR did not alter this expression pattern.

EFA deficiency in *Fxr*$^{-/-}$ mice increases plasma cholesterol and phospholipids concentration

Plasma triglycerides were slightly, but not significantly higher in EFA deficient *Fxr*$^{-/-}$ mice, compared with controls (0.6±0.2 vs. 0.4±0.0 mmol/L, NS). Plasma concentrations of cholesterol (+31%, *P*<0.01) and phospholipids (+38%, *P*<0.05) were increased in EFA deficient in *Fxr*$^{-/-}$ compared to EFA deficient control mice (Fig 2).

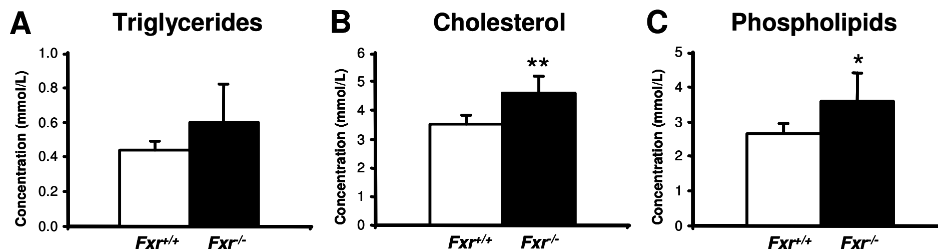

Figure 2. Plasma concentrations of (A) triglycerides, (B) cholesterol, and (C) phospholipids in EFA deficient *Fxr*$^{+/+}$ (white bars, n=5) and *Fxr*$^{-/-}$ mice (black bars, n=7). Results are expressed as means ± SD. **P*<0.05 and ***P*<0.01.

Bile salt homeostasis and enterohepatic circulation

Figure 3 shows that bile production parameters were similar in EFA deficient *Fxr*$^{-/-}$ and control mice. Bile flow was also similar in *Fxr*$^{-/-}$ and control mice (5.5±1.1 vs. 6.5±1.5 µL/min/100 g body weight, respectively, as was the biliary bile salt output (258±104 vs. 239±117 nmol/min/100 g body weight, respectively). *Fxr*$^{-/-}$ mice fed a regular (not EFA-deficient) diet and wild type EFA deficient mice both have an increased contribution of CA to total bile salt composition [8,10]. Figure 4 shows the result of the combination, EFA deficient *Fxr*$^{-/-}$ mice; the contribution of CA to total bile salts was higher in EFA deficient *Fxr*$^{-/-}$ mice, at the expense of β-muricholic acid (β-MA) and α-muricholic acid (α-MA), resulting in a increased cholic acid-muricholic acids ratio, compared with controls [median 1.5, (range 0.93-4.21) vs. 1.0 (0.34-1.12), *P*<0.05].

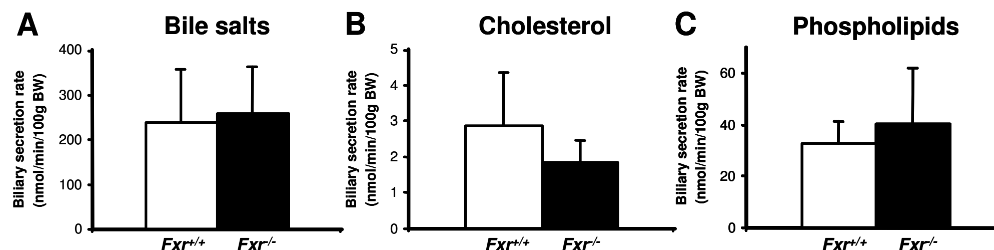

Figure 3. Biliary secretion rates of (A) bile salts, (B) cholesterol, and (C) phospholipids in EFA deficient *Fxr*$^{+/+}$ (white bars, n=5) and *Fxr*$^{-/-}$ mice (black bars, n=7). Results are expressed as means ± SD.

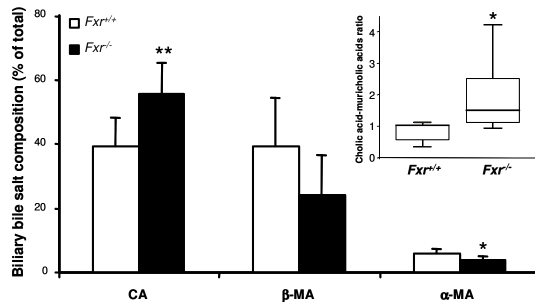

Figure 4. Biliary bile salt composition in EFA deficient $Fxr^{+/+}$ (white bars, n=5) and $Fxr^{-/-}$ mice (black bars, n=7). Expressed are cholic acid (CA), β-muricholic acid (β-MA) and α-muricholic acid (α-MA) as a percentage of the total bile salt concentration. Inset: cholic acid-muricholic acids ratio. Results are expressed as means ± SD. *P<0.05 and **P<0.01.

Fecal bile salt excretion is increased in EFA deficient $Fxr^{-/-}$ mice

EFA-deficiency increased fecal bile salt excretion in $Fxr^{-/-}$ mice (3.6±0.6 vs. 2.3±0.4 µmol/day, P<0.01), coinciding with decreased Asbt mRNA expression (-30%; P<0.05). Fxr deficiency decreased the expression of the FXR target genes *Fgf-15* and *Ibabp* in EFA deficient mice, but this reached statistical significance only in the latter (Fig 5).

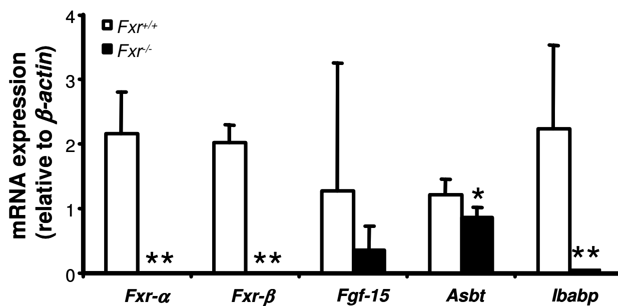

Figure 5. mRNA expression of Fxr and relevant Fxr target genes in the terminal ileum in EFA deficient $Fxr^{+/+}$ (white bars, n=5) and $Fxr^{-/-}$ mice (black bars, n=7). (A) mRNA levels were determined of *Fxr-α*, *Fxr-β*, *Fgf-15*, *Asbt* and *Ibabp*, all normalized to *β-actin*, using quantitative PCR. Results are expressed as means ± SD. *P<0.05 and **P<0.01.

DISCUSSION

We addressed to what extent the gastrointestinal phenotype, i.e. fat malabsorption and bile salt homeostasis, of EFA deficiency was influenced by FXR inactivation in mice. Our data show that inactivation of FXR renders mice more resistant to EFA deficiency induced fat malabsorption and increases their weight gain, possibly by the production of bile with a more hydrophobic bile salt composition.

For our study, we used the $Fxr^{-/-}$ mice originally described by Kok *et al.* [10]. The complete abolishment of *Fxr-α* and *Fxr-β* expression (Fig 5) in the $Fxr^{-/-}$ mice confirmed that the mice did not express the Fxr gene. Kok *et al.* demonstrated that chow-fed $Fxr^{-/-}$ mice had an increased bile salt pool size and an increased fecal bile salt loss [10]. This observation was explained by the abolishment of the negative feedback regulation that FXR exerts on hepatic bile salt synthesis under physiological conditions. Sinal *et al.,* however, reported a decreased bile salt pool and fecal bile salt loss in another $Fxr^{-/-}$ mouse model [23]. It is tempting to speculate that the difference between the two mouse models is due to the fact that the $Fxr^{-/-}$ mice used in Sinal's study still have a DNA-binding domain which could affect the expression FXR target genes.

Similar to FXR inactivation, EFA deficiency in mice increases bile salt pool size, bile flow and biliary output rate of bile salts [8,10]. Based on this information, we expected that EFA

deficiency would further increase bile flow, biliary bile salt output and pool size in *Fxr*$^{-/-}$ mice, compared with EFA deficient control mice. However, bile flow, biliary bile salt output and the bile salt pool size were similar in EFA deficient *Fxr*$^{-/-}$ and control mice. This observation suggests that either FXR inactivation or EFA deficiency maximally induces biliary bile salt output and pool size.

The fat absorption coefficient was higher in EFA deficient *Fxr*$^{-/-}$ mice compared to control mice, associated with a higher body weight gain in the former. Previous studies in EFA deficient wild type mice indicated that the fat malabsorption involves the mucosal phase of fat absorption, i.e, fatty acid translocation, triglyceride esterification and/or chylomicron formation [8]. We did not find indications for different mRNA expression levels of Fat, Ifabp-1, and Fatp-4, or of Dgat-1, Dgat-2 and Mttp in EFA deficient *Fxr*$^{-/-}$ and control mice. These results do not support the theoretical possibility that fatty acid transport across intestinal membranes, triglyceride re-esterification or chylomicron assembly account for the relatively preserved fat absorption in EFA deficient *Fxr*$^{-/-}$ mice. Our data do show, however, that inactivation of FXR was associated with an increase in cholic acid-muricholic acids ratio during EFA deficiency in mice. The increase in the cholic acid-muricholic acids ratio renders the bile salt pool more hydrophobic. The cholic acid-muricholic acids ratio (hydrophobicity of the bile salt pool) in bile has been positively associated with the capacity to absorb cholesterol in rats [24,25]. In analogy to these data on cholesterol absorption, we speculate that the higher cholic acid-muricholic acids ratio in EFA deficient *Fxr*$^{-/-}$ mice enhances the absorption of fatty acids and monoglycerides, possibly by facilitating their transfer across the unstirred water layer and thus their translocation across the apical membrane of the enterocytes. A higher cholic acid-muricholic acids ratio is found in EFA deficiency in mice (+63%), as well as in *Fxr*$^{-/-}$ mice (+77%), compared to the control situations. The increase in the cholic acid-muricholic acids ratio was not higher in EFA deficient *Fxr*$^{-/-}$ mice (+53%) compared to the increase in EFA deficient mice (+63%) or *Fxr*$^{-/-}$ mice (+77%), suggesting that FXR inactivation or EFA deficiency maximally increase the hydrophobicity of the bile salt pool [8,10]. In EFA deficient mice fat absorption was lower than in non EFA deficient mice, despite the high cholic acid-muricholic acids ratio in the former [8]. These observation suggests 1. that fat absorption would have been even more affected if the bile salt pool composition was not changed upon EFA deficiency; 2. that other factors than bile salt pool composition seem to contribute to EFA deficient fat malabsorption.

The mechanism underlying EFA deficiency-induced or FXR deficiency-induced increased hydrophobicity of the bile salt pool is still unclear. As discussed previously by Kok *et al.*, it is counterintuitive that Cyp7a1 expression is increased and Cyp8b1 expression remains similar in *Fxr*$^{-/-}$ mice. Cyp8b1 catalyzes the 12α-hydroxylation of intermediates of the classic pathway of bile salt biosynthesis, leading to cholic acid biosynthesis and therefore appears to be important in determining the ratio between the ratio of cholic acid to chenodeoxycholic acid metabolites, the muricholic acids [26,27].

In conclusion, EFA deficiency in *Fxr*$^{-/-}$ mice causes milder fat malabsorption and a higher weight gain compared to *Fxr*$^{+/+}$ mice, probably related to the production of bile with more hydrophobic bile salts. These results support the theoretical concept that antagonists of FXR and/or increasing the hydrophobicity of the bile salt pool could ameliorate the phenotype of EFA deficiency in patients.

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