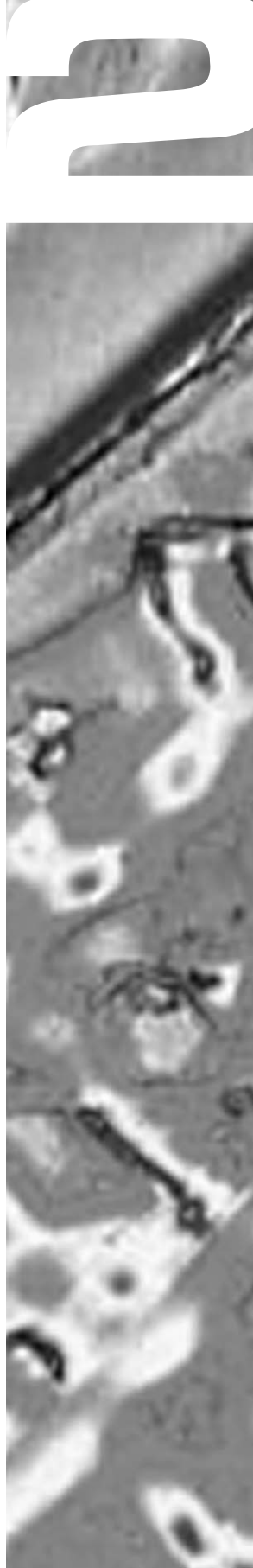


# Fat malabsorption in essential fatty acid-deficient mice is not due to impaired bile formation

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## ABSTRACT

**Background:** Essential fatty acid (EFA) deficiency induces fat malabsorption, but the pathophysiological mechanism hereof is unknown. Bile salts and EFA-rich biliary phospholipids affect dietary fat solubilization and chylomicron formation, respectively. We investigated whether altered biliary bile salt and/or phospholipid secretion mediate EFA deficiency-induced fat malabsorption in mice.

**Methods:** FVB mice received EFA-sufficient (EFAS) or EFA-deficient (EFAD) chow for eight weeks. Subsequently, fat absorption, bile flow and bile composition were determined. Identical dietary experiments were performed in *Mdr2*<sup>-/-</sup> mice, secreting phospholipid-free bile.

**Results:** After eight weeks, EFAD chow-fed wildtype and *Mdr2*<sup>-/-</sup> mice were markedly EFA-deficient (plasma triene (C20:3n-9) / tetraene (C20:4n-6) ratio >0.2). Fat absorption decreased (70.1±4.2% vs. 99.1±0.3%, p<0.001) but bile flow and biliary bile salt secretion increased in EFA-deficient mice compared to EFA-sufficient controls (4.87±0.36 vs. 2.87±0.29  $\mu$ l/min/100g bodyweight, p<0.001; 252±30 vs. 145±20 nmol/min/100g bodyweight, p<0.001). Bile salt composition was similar in EFAS and EFAD chow-fed mice. Similar to EFA-deficient wildtype mice, EFA-deficient *Mdr2*<sup>-/-</sup> mice developed fat malabsorption associated with twofold increased bile flow and bile salt secretion.

**Conclusion:** Fat malabsorption in EFA-deficient mice is not due to impaired biliary bile salt or phospholipid secretion. We hypothesize that EFA deficiency affects intracellular processing of dietary fat by enterocytes.

## INTRODUCTION

Essential fatty acid (EFA) deficiency has been associated with several pathological consequences in humans and in experimental animals, including fat malabsorption<sup>(1-5)</sup>. The pathophysiological mechanisms underlying EFA deficiency-induced fat malabsorption have not been elucidated. To address this issue, the consecutive intraluminal and intracellular steps in the absorption process have been investigated in control and EFA-deficient rats, i.e., lipolysis of dietary triglycerides by lipases, solubilization of lipolytic products by bile components, uptake by the enterocyte and intracellular re-esterification to triglycerides and, finally, chylomicron formation and secretion into lymph<sup>(3,4)</sup>. Available data from rat models of EFA deficiency have indicated that neither fat digestion nor fatty acid uptake by the enterocyte is responsible for the fat malabsorption encountered in EFA deficiency<sup>(3,4)</sup>. In rats, EFA deficiency resulted in significantly decreased bile flow and biliary secretion of bile salts, phospholipids (PL) and cholesterol<sup>(3,6)</sup>. Acyl chain analysis of biliary PL from EFA-deficient rats revealed that biliary PL contents of linoleic acid (C18:2n-6) and arachidonic acid (C20:4n-6) were decreased to 10% and 26% of control values, respectively, and were replaced by oleic acid (C18:1n-9)<sup>(4)</sup>. In addition to alterations in bile, intracellular events such as fatty acid re-esterification and chylomicron production were severely impaired<sup>(3,4)</sup>. It thus seems that EFA deficiency in rats affects bile formation and/or intracellular processing of fat by the enterocyte, resulting in fat malabsorption. In a previous study we demonstrated the role of biliary phospholipids in intestinal chylomicron formation, using *Mdr2*<sup>-/-</sup> mice (gene symbol: ABC-B4) which are unable to secrete phospholipids into bile<sup>(7,8)</sup>. We reasoned that this animal model could be important to further delineate the pathophysiological mechanism of EFA deficiency-induced fat malabsorption.

If altered biliary phospholipid secretion were involved in EFA deficiency-associated fat malabsorption, one would expect fat absorption in *Mdr2*<sup>-/-</sup> mice to be relatively unaffected by changes in EFA status. If, however, the pathophysiological mechanism would not involve alterations in biliary phospholipid secretion, EFA deficiency would be expected to equally affect the process in *Mdr2*<sup>-/-</sup> and in wildtype mice. In the present study we investigated the role of bile formation in general and of biliary phospholipid secretion in particular, in the pathophysiology of EFA deficiency-associated fat malabsorption. We first developed and characterized a murine model for EFA deficiency with respect to fat absorption and bile formation. Then, we compared fat absorption and bile formation in EFA-deficient and EFA-sufficient *Mdr2*<sup>-/-</sup> mice.

## MATERIALS AND METHODS

### Animals

Mice homozygous for disruption of the multidrug resistance gene-2 (*Mdr2*<sup>-/-</sup>) and wildtype (*Mdr2*<sup>+/+</sup>) mice with a free virus breed (FVB) background were obtained from the breeding colony at the Central Animal Facility, Academic Medical Center, Amsterdam, the Netherlands<sup>(9)</sup>. All mice were 2-4 months old and weighed 25-30 g. Mice were housed in a light controlled (lights on 6 AM - 6 PM) and temperature controlled (21°C) facility and allowed tap water and chow (Hope Farms B.V. Woerden, the Netherlands) *ad libitum*. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, the Netherlands.

### Experimental procedures

#### *Fat absorption and bile secretion in EFA-deficient mice*

FVB mice (n=14) were fed standard low-fat chow (6 weight% fat, 14 energy% fat) for standardization for one month, after which they were anesthetized with halothane and a baseline blood sample was obtained by tail bleeding for determination of EFA status. Blood was collected in micro-hematocrit tubes containing heparin, and plasma was separated by centrifugation at 9000 rpm for 10 min (Eppendorf centrifuge, Eppendorf, Germany) and stored at -20°C until analysis. Subsequently, mice were randomly assigned to either an EFA-sufficient (EFAS) or an EFA-deficient (EFAD) diet. The EFAS and EFAD diets were isocaloric and contained 16 weight% fat. The EFAS chow contained 20 energy%, 34 energy% and 46 energy% from protein, fat and carbohydrate, respectively, and had the following fatty acid profile: 32 mol% palmitic acid (C16:0), 6 mol% stearic acid (C18:0), 32 mol% oleic acid (C18:1n-9), and 30 mol% linoleic acid (C18:2n-6) (custom synthesis, Hope Farms BV, Woerden, the Netherlands). The EFAD diet had identical energy percentages derived from protein, fat and carbohydrate, and had the following fatty acid composition: 41 mol% C16:0, 48 mol% C18:0, 8 mol% C18:1n-9, and 3 mol% C18:2n-6) (custom synthesis, Hope Farms BV, Woerden, the Netherlands). At 2-weekly intervals, blood samples were taken in the manner described above. After 4 and 8 weeks of experimental diet, a 72h fecal fat balance was performed involving quantitative feces collection and determination of chow intake. At 8 weeks, mice were anaesthetized by intraperitoneal injection of Hypnorm (fentanyl/fluanisone) and diazepam, and gallbladders were cannulated for collection of bile for 1h as described previously<sup>(10)</sup>. At the end of bile collection, a large blood sample (0.6-1.0 ml) was obtained by heart puncture.

**Hepatic expression of Cyp7A and Cyp27**

A separate group of male FVB mice was fed EFA-containing or EFA-deficient chow for 8 weeks (n=6 per dietary group). After 8 weeks, animals were anaesthetized with halothane and blood was collected by cardiac puncture for lipid analysis. Livers were removed and liver samples were immediately frozen in liquid nitrogen, and stored at -80°C for determination of mRNA levels of two key enzymes in bile salt biosynthesis, cholesterol 7- $\alpha$ -hydroxylase (Cyp7A) and sterol 27-hydroxylase (Cyp27).

**Plasma accumulation of [<sup>3</sup>H]-triolein and [<sup>14</sup>C]-oleic acid after Triton WR-1339**

In a separate experiment, male FVB mice fed EFAS or EFAD chow for 8 weeks (n=5 per group) were injected i.v. with 12.5 mg Triton WR-1339 (12.5 mg/100  $\mu$ L PBS) to block lipolysis of lipoproteins in the circulation. Then an intragastric fat bolus was administered containing 200  $\mu$ L olive oil, in which 10  $\mu$ Ci [<sup>3</sup>H]-triolein (glycerol tri-[9,10(n)-[<sup>3</sup>H]]-oleate) (Amersham, Buckinghamshire, UK) and 2  $\mu$ Ci [<sup>14</sup>C]-oleic acid (NEN Laboratories, Boston, MA) were dispersed. Before (t=0) and at 1, 2, 3 and 4 hours after label administration, blood samples (75  $\mu$ L) were taken by tail bleeding. [<sup>3</sup>H] and [<sup>14</sup>C] in plasma (25  $\mu$ L) were measured by scintillation counting

**Plasma appearance of retinyl-palmitate after retinol administration**

In addition to the absorption of fatty acids during EFA deficiency, the plasma appearance of the less polar lipid molecule retinol (vitamin A) was investigated. Retinol (5000 IU) in an olive oil bolus (100  $\mu$ L per mouse) was administered intragastrically to EFA-deficient mice and control mice (n=7 per group) and blood samples were taken at 0, 2 and 4 hours after bolus administration.

**Fat absorption and bile secretion in EFA-deficient *Mdr2*<sup>-/-</sup> mice**

*Mdr2*<sup>-/-</sup> mice (n=14) were fed low-fat chow (6 weight% fat, 14 energy% fat) for standardization for one month. Baseline blood samples were taken for determination of plasma EFA status, after which mice were randomly assigned to either the EFA-containing or the EFA-deficient diet. The diets were identical to those described above. After eight weeks of feeding the respective diets, blood samples were obtained by tail bleeding under halothane anesthesia. As in the wildtype mice, fat absorption was measured by means of a 72h fecal fat balance. Retinol in a bolus of olive oil was administered intragastrically to EFA-deficient *Mdr2*<sup>-/-</sup> mice (n=7) and their EFA-sufficient controls (n=7). Blood samples were taken at 0, 2 and 4 hours after bolus administration. Gallbladders were cannulated and bile was collected for 1h as described above<sup>(10)</sup>. At the end of bile collection, mice were sacrificed after obtaining a large blood sample (0.6-1.0 ml) by heart puncture.

## Analytical techniques

Fatty acid status was analyzed by extracting, hydrolyzing and methylating total plasma lipids, liver homogenates and biliary lipids according to the method described by Lepage and Roy<sup>(11)</sup>. To account for losses during lipid extraction, heptadecanoic acid (C17:0) was added to all samples as an internal standard prior to extraction and methylation procedures, and BHT was added as an antioxidant. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard gas chromatograph model 6890, equipped with a 50mx0.2mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA) and a FID detector. The injector and detector were set at 260°C and 250°C, respectively. The oven temperature was programmed from an initial temperature of 160°C to a final temperature of 290°C in 3 temperature steps (160°C held 2 min; 160-240°C, ramp 2°C/min, held 1 min; 240-290°C, ramp 10°C/min, held 10 min). Helium was used as a carrier gas with a constant flow rate of 0.5 ml per minute. Individual fatty acid methyl esters were quantified by relating the areas of their chromatogram peaks to that of the internal standard heptadecanoic acid (C17:0).

Plasma lipid levels (cholesterol, HDL-cholesterol, triglycerides, free fatty acids and phospholipids) were measured using commercially available kits (Roche Diagnostics, Mannheim resp. WAKO Chemicals Neuss, Germany) according to the instructions provided. Cholesterol, cholesterol ester and triglycerides in liver tissue were determined after Bligh and Dyer lipid extraction<sup>(12)</sup> and biliary bile salt composition was measured as described previously. Total protein concentrations of liver homogenates were determined according to the method described by Lowry *et al.*<sup>(13)</sup>.

Total RNA was isolated from liver tissue using Trizol Reagent (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions. Single stranded cDNA was synthesized from 4.5 g RNA and subsequently subjected to quantitative real-time detection RT-PCR<sup>(14;15)</sup>. The following primers and probes were used: for Cyp7A (accession number: L23754): 5'-CAG GGA GAT GCT CTG TGT TCA-3' (forward primer), 5'-AGG CAT ACA TCC CTT CCG TGA-3' (reverse primer), 5'-TGC AAA ACC TCC AAT CTG TCA TGA GAC CTC C-3' (probe). For Cyp27 (M73231, M62401): 5'-GCC TTG CAC AAG GAA GTG ACT-3' (forward primer), 5'-CGC AGG GTC TCC TTA ATC ACA-3' (reverse primer), 5'-CCC TTC GGG AAG GTG CCC CAG-3' (probe), and for  $\beta$ -actin (M12481): 5'-AGC CAT GTA CGT AGC CAT CCA-3' (forward primer) 5'-TCT CCG GAG TCC ATC ACA ATG-3' (reverse primer) 5'-TGT CCC TGT ATG CCT CTG GTC GTA CCA C-3' (probe). For each real-time PCR, 4  $\mu$ L cDNA was used in a final volume of 20  $\mu$ L, containing 500 nmol/L of forward and reverse primers and 200

nmol/L of probe, 250 nmol/L  $MgCl_2$ , 10 nmol/L deoxy-ribonucleoside triphosphate mix, 5  $\mu$ L Real-time PCR buffer (10x), and 1.25 U Hot GoldStar (Eurogentec). Real-time detection PCR was performed on the ABI PRISM 7700 (PE Applied Biosystems) initialized by 10 minutes at 95°C to denature the complementary DNA followed by 40 PCR cycles each at 95°C for 15 seconds and 60°C for 1 minute.

Feces and chow pellets were freeze-dried and then mechanically homogenized. From aliquots of each, lipids were extracted, hydrolyzed and methylated<sup>(11)</sup>. Resulting fatty acid methyl esters were analyzed by gas chromatography for their fatty acid content as described previously<sup>(16)</sup>. Fatty acids were quantified using heptadecanoic acid (C17:0) as internal standard.

Retinyl palmitate concentrations in plasma were determined after two extractions with hexane<sup>(17)</sup>. Retinyl acetate was added to plasma samples as an internal standard before lipid extraction. Samples were resuspended in ethanol and analyzed by reverse-phase HPLC using a 150 x 4,6 mm Symmetry RP18 column (Waters Corp., Milford, MA, USA)<sup>(18)</sup>. Peak area of retinyl palmitate was normalized to that of retinyl acetate. At each time point, concentrations were expressed as  $\mu$ mol retinyl palmitate per L plasma.

## Calculations

### ***Fatty acid status in plasma and in bile***

Relative concentrations (mol%) of plasma, liver and biliary phospholipid fatty acids were calculated using the summed areas of major fatty acid peaks (palmitic, stearic, oleic, linoleic acids) and then expressing the area of each individual fatty acid as a percentage of this amount. EFA deficiency was determined by calculating the triene/tetraene ratio (C20:3n-9/C20:4n-6) in plasma of mice. A ratio of >0.2 was considered deficient<sup>(19)</sup>.

### ***Fatty acid absorption using 72h balance techniques***

Absorption of major dietary fatty acids (palmitic, stearic, oleic, linoleic acids) was determined by subtracting the amount of each individual fatty acid excreted in feces in 72h, from the amount of this dietary fatty acid ingested in 72h (net fat absorption). This quantity was subsequently expressed as a percentage of the amount of fatty acid ingested in 72h (coefficient of fat absorption; 100-fold the ratio between (amount ingested - amount recovered in feces) and the amount ingested). Net amount of fat absorption was calculated by subtracting the fecal loss of the four major fatty acids from the amount ingested.

## Statistics

All results are presented as means  $\pm$  S.D. for the number of animals indicated. Data were statistically analyzed using Student's two-tailed t test. The level of significance for all statistical analysis was set at  $p < 0.05$ . Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

## RESULTS

Body weight and chow ingestion in EFA-deficient and EFA-sufficient wildtype mice  
Body weight was monitored at the start of experimental feeding, and then every two weeks for eight weeks. No differences in basal or final weight were found for wildtype mice fed EFAS or EFAD chow (basal:  $24.4 \pm 2.0$  and  $24.6 \pm 1.3$  gram; final:  $23.3 \pm 1.0$  and  $21.9 \pm 2.0$  gram, respectively). Chow intake, measured after four and eight weeks of experimental diet feeding, was similar in both dietary groups (data not shown).

### Essential fatty acid status

#### *Triene/tetraene ratio in plasma and in liver*

The classical biochemical parameter describing EFA status, the triene/tetraene ratio (C20:3n-9/C20:4n-6), was measured in plasma at baseline and every two weeks for eight weeks, and in liver after eight weeks of feeding EFA-deficient chow. Baseline plasma triene/tetraene ratio was  $0.02 \pm 0.00$ , which is well below the cutoff value for EFA deficiency (0.20). Already after two weeks on EFA-deficient diet, plasma triene/tetraene ratios reached the cutoff-value for EFA deficiency, i.e.,  $0.19 \pm 0.08$  for EFAD chow-fed mice vs.  $0.01 \pm 0.00$  for controls. After eight weeks on EFAD chow, mice had a pronounced EFA deficiency with triene/tetraene ratios of  $0.66 \pm 0.05$  for plasma and  $0.56 \pm 0.09$  for liver, in contrast to control mice ( $0.01 \pm 0.00$  for plasma,  $p < 0.001$  and  $0.02 \pm 0.00$  for liver,  $p < 0.001$ ) (data not shown)

#### *Characterization of EFA deficiency in mice*

Since EFA deficiency has not been characterized before in a murine model, plasma and liver lipids were measured. No differences were found in cholesterol, HDL-cholesterol, free fatty acid or phospholipid concentrations in plasma between EFAS and EFAD diet-fed mice (Table 1). Plasma triglyceride concentrations were decreased in EFA-deficient mice compared with mice fed EFA-containing chow ( $p < 0.001$ ). Liver fat analysis revealed a significant fat accumulation (triglyceride, unesterified cholesterol, cholesterol ester) in EFA-deficient mice compared with their EFA-sufficient counterparts (Table 2).



	EFAS	EFAD
Cholesterol	2.04 ± 0.23	2.21 ± 0.12
HDL-cholesterol	1.44 ± 0.16	1.41 ± 0.08
Triglyceride	0.48 ± 0.07	0.27 ± 0.05 *
Free fatty acids	0.58 ± 0.10	0.60 ± 0.04
Phospholipids	2.84 ± 0.44	3.11 ± 0.17

**Table 1:** Plasma lipid concentrations in mice fed EFA-containing (EFAS) or EFA-deficient (EFAD) chow for 8 weeks. Concentrations are in mM, \* p<0.001; n=7 mice / group.

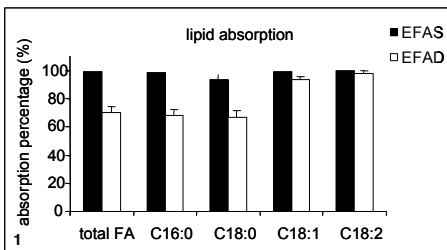
	EFAS	EFA D
Triglyceride	151.9 ± 21.8	219.9 ± 27.8 *
Total cholesterol	34.9 ± 2.7	75.6 ± 5.3 *
Cholesterol esters	8.0 ± 2.0	35.8 ± 9.4 *

**Table 2:** Hepatic lipid concentrations in mice fed EFA-containing or EFA-deficient chow for 8 weeks. Concentrations are given in nmol/mg protein; \* p<0.001; n=7 mice per group.

## Fat absorption

### Fecal fatty acid balance

The fecal fat balance revealed a decreased absorption of total dietary fat in EFAD mice compared with their EFAS controls (p<0.01) (Figure 1). The absorption coefficient for EFAD mice was 70.1±1.6%, compared with absorption coefficients above 95% for mice fed EFA-containing chow. Individual fatty acid balances for palmitic, stearic, oleic and linoleic acids were calculated (Figure 1). In EFAD mice, absorption of saturated fatty acids (palmitic and stearic acids) was more affected than absorption of unsaturated fatty acids (oleic and linoleic acids).



**Figure 1.** Fat absorption coefficients of total dietary fat, and separately of major dietary fatty acids (C16:0, C18:0, C18:1n-9, C18:2n-6) in mice fed EFAS and EFAD chow for 8 weeks. Feces were collected after a 72h period in which chow intake was monitored by weighing chow containers. Absorption was calculated by subtracting fecal excretion of these fatty acids after 72h from their dietary intake in 72h and then multiplying the result by 100, as detailed in the Materials and Methods section. Data represent means ± SD of 7 mice per group. For all lipid classes p<0.01

### Bile secretion and composition

Table 3 shows that bile flow and biliary secretion of bile salt, cholesterol and phospholipid during a 1h period immediately after interruption of the enterohepatic circulation were higher in EFA-deficient mice compared with controls (for each parameter, p<0.001). Theoretically, alterations in bile salt hydrophobicity could contribute to fat malabsorption in EFA deficiency. However, bile salt composition appeared to be similar between both dietary groups (Table 4).

	EFAS	EFAD
Bile flow <sup>1</sup>	2.87 ± 0.71	4.87 ± 0.87 *
Bile salts <sup>2</sup>	145 ± 48	353 ± 74 *
Cholesterol <sup>2</sup>	2.23 ± 0.47	4.96 ± 0.79 *
Phospholipids <sup>2</sup>	30.11 ± 7.72	55.13 ± 9.23 *

**Table 3:** Bile flow and biliary secretion rates in mice fed EFA-sufficient or EFA-deficient chow for 8 weeks. Biliary output rates are given in <sup>1</sup>  $\mu\text{l}/\text{min}/100\text{g}$  body weight or <sup>2</sup>  $\text{nmol}/\text{min}/100\text{g}$  body weight. \*  $p < 0.001$ ;  $n = 5-7$  mice per group

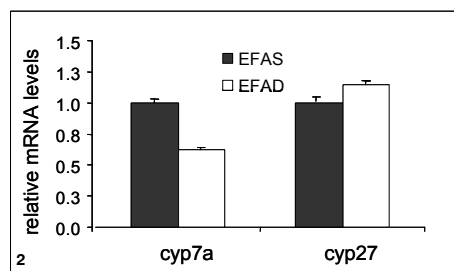
	EFAS	EFAD
Cholate	49.0 ± 3.2	58.9 ± 4.5 *
Beta-muricholate	39.0 ± 1.3	28.1 ± 4.6 *
Omega-muricholate	6.5 ± 2.7	7.4 ± 1.5
Alpha-muricholate	2.7 ± 0.5	2.5 ± 0.5
Deoxycholate	1.6 ± 0.6	2.1 ± 0.9
Chenodeoxycholate	1.1 ± 0.7	1.0 ± 0.4

**Table 4:** Biliary bile salt composition in mice fed EFA-containing or EFA-deficient chow for 8 weeks. >90% of all bile salts represented. Minor metabolites (<1% of total area) have been excluded. Values are expressed as a percentage of the total amount. Data represent means ± SD, \*  $p < 0.01$ ;  $n = 5-7$  mice per group.

EFA deficiency-associated changes in acyl chain composition of biliary PL were analyzed by gas chromatography. Relative concentrations (mol%) of C16:1n-7, C18:1n-9 and C18:1n-7 were higher ( $p < 0.001$ ) and concentrations of C16:0, C18:2n-6, C18:0 and C20:4n-6 were lower ( $p < 0.05$ ) in bile of EFA-deficient mice compared to mice fed EFA-containing chow (data not shown).

### Cyp7A and Cyp27 mRNA levels

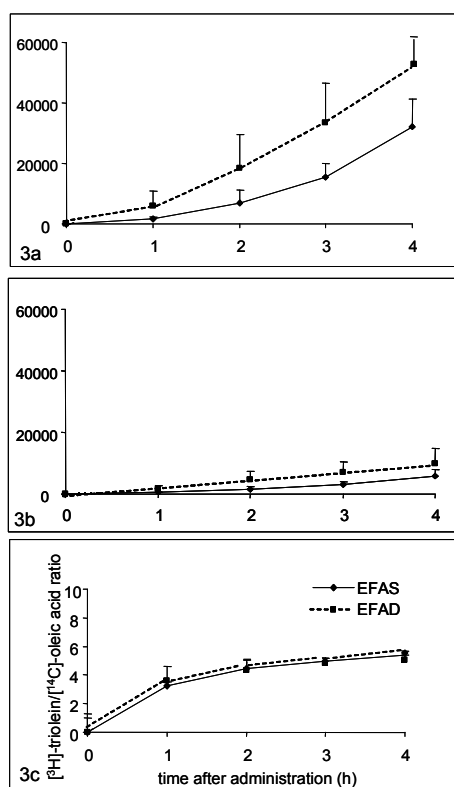
To determine whether the increased bile salt secretion in EFA-deficient mice might be due to increased hepatic bile salt synthesis, hepatic mRNA levels of Cyp7A and Cyp27 were measured by quantitative real-time RT-PCR, using  $\beta$ -actin mRNA as a housekeeping signal. No significant differences in hepatic mRNA levels of Cyp7A and Cyp27 were observed between mice fed EFAS and EFAD chow ( $1.00 \pm 0.64$  vs.  $0.63 \pm 0.29$  for Cyp7A and  $1.00 \pm 0.22$  vs.  $1.14 \pm 0.19$  for Cyp27) (Figure 2). Values represent the ratio of specific hepatic mRNA levels of Cyp7A and Cyp27 to the hepatic mRNA level of  $\beta$ -actin, normalized to the EFA-sufficient control group.



**Figure 2.** Hepatic mRNA levels of Cyp7A and Cyp27 in mice fed EFAS or EFAD chow for 8 weeks determined by quantitative real-time RT-PCR. Values represent the ratio of specific hepatic mRNA levels of Cyp7A and Cyp27 to that of  $\beta$ -actin, normalized to the EFA-sufficient control group. Data represent means ± SD of 6 mice per group. No significant differences were found between groups.

### Plasma accumulation of [ $^3\text{H}$ ]-triolein and [ $^{14}\text{C}$ ]-oleic acid after Triton WR-1339 administration

Figure 3a and 3b show the time course of plasma [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] radioactivity after intragastric administration of [ $^3\text{H}$ ]-triolein and [ $^{14}\text{C}$ ]-oleic acid. EFA-deficient mice had a slightly increased plasma concentration of both radioactive labels during the studied time frame, reaching a significant difference at 3 hours after bolus administration. If EFA deficiency would differentially affect lipolysis and fatty acid uptake, an altered [ $^3\text{H}$ ]/[ $^{14}\text{C}$ ] ratio in plasma would be expected. However, when expressed as the ratio between plasma [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ] (figure 3c), no significant difference was found between EFA-deficient and EFA-sufficient mice. Both for EFA-containing and EFA-deficient chow-fed mice, thin layer chromatography revealed that [ $^3\text{H}$ ] as well as [ $^{14}\text{C}$ ] radioactivity was predominantly (>95%) present in the triglyceride fraction.

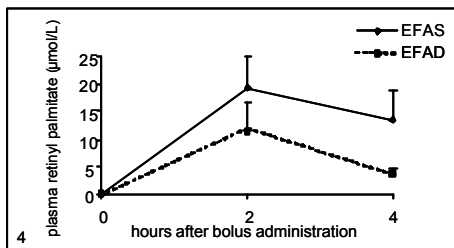


**Figure 3.** Plasma appearance of [ $^3\text{H}$ ]-triolein (**3a**) and [ $^{14}\text{C}$ ]-oleic acid (**3b**) in mice after an intragastric load of olive oil containing these fats and after intravenous injection of Triton WR-1339 at time point zero. Mice were fed EFAS (solid line) or EFAD (dotted line) chow for 8 weeks. Blood samples were taken hourly for 4 hours and fats were extracted from plasma following extraction. Thin layer chromatography was performed to isolate the triglyceride fraction for determination of radioactivity by scintillation counting. Scanning of the thin layer plates indicated that essentially all radioactivity (95%) was in the triglyceride fraction. Data represent means  $\pm$  SD of 5 mice per group (dps per milliliter plasma). Statistical significance was reached for [ $^3\text{H}$ ]-triolein and [ $^{14}\text{C}$ ]-oleic acid at 3h after administration ( $p < 0.05$ ). (**3c**) The ratio of [ $^3\text{H}$ ]/[ $^{14}\text{C}$ ] in plasma of in mice fed EFAS (solid line) or EFAD (dotted line) chow for 8 weeks after an intragastric load of olive oil containing [ $^3\text{H}$ ]-triolein and [ $^{14}\text{C}$ ]-oleic acid and after intravenous injection of Triton WR-1339 at time point zero. The [ $^3\text{H}$ ]/[ $^{14}\text{C}$ ] in the infusate was  $4.43 \pm 0.05$ . Data represent means  $\pm$  SD of 5 mice per group. No significant differences were found between groups at any time point.

### Plasma appearance of retinyl-palmitate after retinol administration

The absorption of dietary oleic acid, a polar lipid (class III, soluble amphiphile)<sup>(20)</sup> was only mildly impaired in EFA-deficient mice (Figure 1). In a separate experiment we

investigated plasma appearance of retinol (vitamin A), a less polar lipid (class I, insoluble non-swelling amphiphile), after its intragastric administration. Plasma concentrations of retinyl palmitate were significantly lower in EFA-deficient mice compared to controls at 2 and 4 hours after retinol administration (Figure 4).



**Figure 4:** Time course of retinyl palmitate concentration in plasma of mice fed EFAS (solid line) or EFAD chow (dotted line) for 8 weeks after intragastric administration of 5000 IU retinol at time zero. Data represent means  $\pm$  SD of 7 mice per group.  $p < 0.05$  at 2 and 4 hours after bolus administration.

### **Body weight and chow ingestion in EFA-deficient and EFA-sufficient *Mdr2*<sup>-/-</sup> mice**

Basal body weights of *Mdr2*<sup>-/-</sup> mice entering the EFAS and EFAD group were similar (26.1 $\pm$ 1.6 vs. 25.4 $\pm$ 1.5, respectively). However, body weights of *Mdr2*<sup>-/-</sup> mice fed EFAD chow gradually decreased compared to *Mdr2*<sup>-/-</sup> mice fed EFAS chow, and by eight weeks this resulted in a significantly lower body weight for EFAD- compared to EFAS chow-fed *Mdr2*<sup>-/-</sup> mice (20.2 $\pm$ 1.0 g vs. 25.3 $\pm$ 1.1g, respectively;  $p < 0.01$ ). No significant difference in chow intake was observed between the two dietary groups after four or eight weeks on either diet (data not shown).

## **Essential fatty acid status**

### **Triene/tetraene ratio in plasma and in liver**

The baseline triene/tetraene ratios (C20:3n-9/C20:4n-6) in plasma and liver were significantly higher in *Mdr2*<sup>-/-</sup> compared to *Mdr2*<sup>+/+</sup> mice (0.035 $\pm$ 0.003 vs. 0.018 $\pm$ 0.001,  $p < 0.001$ ), but were still well below the cutoff value for EFA deficiency (0.2). *Mdr2*<sup>-/-</sup> mice fed EFAD chow for 8 weeks had developed EFA deficiency according to triene/tetraene ratios in plasma and in liver (0.46 $\pm$ 0.03 for plasma and 0.38 $\pm$ 0.04 for liver), in contrast to the EFA-sufficient *Mdr2*<sup>-/-</sup> controls (0.01 $\pm$ 0.01 for plasma,  $p < 0.001$  and 0.02 $\pm$ 0.00 for liver,  $p < 0.01$ ).

### **Characterization of EFA deficiency in *Mdr2*<sup>-/-</sup> mice**

Plasma concentrations of cholesterol and phospholipid were increased, whereas the plasma triglyceride level was decreased in EFA-deficient *Mdr2*<sup>-/-</sup> mice compared to their EFA-sufficient controls ( $p < 0.05$ ) (Table 5). Similar to the situation in wildtype mice, a pronounced hepatic fat accumulation characterized by increased levels of triglyceride, unesterified and esterified cholesterol, was observed in EFA-deficient *Mdr2*<sup>-/-</sup> mice (Table 6).

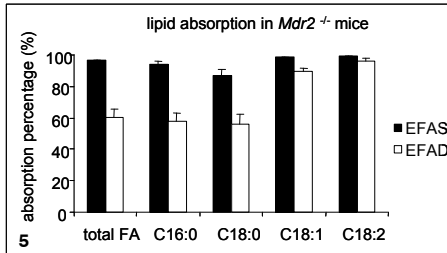
	EFAS	EFAD
Cholesterol	1.39 ± 0.14	1.82 ± 0.21 *
HDL-cholesterol	1.04 ± 0.12	1.30 ± 0.19 *
Triglyceride	0.46 ± 0.09	0.34 ± 0.12 *
Free fatty acids	0.61 ± 0.08	0.66 ± 0.11
Phospholipids	2.18 ± 0.18	2.82 ± 0.24 *

**Table 5:** Plasma lipid concentrations in *Mdr2*<sup>-/-</sup> mice fed EFA-containing or EFA-deficient chow for 8 weeks. Concentrations are in mM, \* p<0.05; n=7 mice / group.

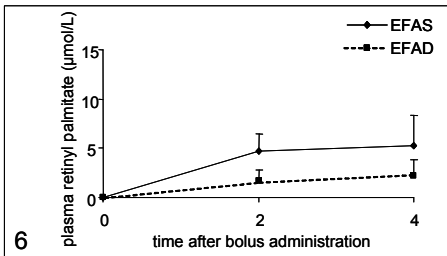
	EFAS	EFAD
Triglyceride	101.0 ± 22.2	225.8 ± 95.9 *
Total cholesterol	45.8 ± 2.1	81.4 ± 11.8 *
Cholesterol esters	11.6 ± 1.5	41.7 ± 7.6 *

**Table 6:** Hepatic lipid concentrations in *Mdr2*<sup>-/-</sup> mice fed EFA-containing or EFA-deficient chow for 8 weeks. Concentrations are given in nmol/mg protein; \* p<0.05; n=7 mice per group

The fecal fat balance revealed a decreased dietary fat absorption in EFA-deficient *Mdr2*<sup>-/-</sup> mice compared to EFA-sufficient controls (p<0.01, Figure 5). Absorption coefficients for saturated fatty acids (palmitic and stearic acids) were lower than absorption coefficients of unsaturated fatty acids (oleic and linoleic acids) in EFA-deficient *Mdr2*<sup>-/-</sup> mice. Plasma concentrations of retinyl palmitate after intragastric administration of retinol were significantly lower in EFA-deficient deficient *Mdr2*<sup>-/-</sup> mice compared to EFA-sufficient controls (p<0.01, Figure 6)



**Figure 5.** Fat absorption coefficients of total dietary fat, and separately of major dietary fatty acids (C16:0, C18:0, C18:1n-9, C18:2n-6) in *Mdr2*<sup>-/-</sup> mice fed EFAS or EFAD chow for 8 weeks. Feces were collected after a 72h period in which chow intake was monitored by weighing chow containers. Absorption was calculated by subtracting fecal excretion of these fatty acids after 72h from their dietary intake in 72h and multiplying the result by 100. Data represent means ± SD of 7 mice per group. For all lipid classes p<0.01.



**Figure 6.** Time course of retinyl palmitate concentration in plasma of *Mdr2*<sup>-/-</sup> mice fed EFAS (circles) or EFAD chow (squares) for 8 weeks after intragastric administration of 5000 IU retinol at time zero. Data represent means ± SD of 7 mice per group. p<0.05 at 2 and 4 hours after bolus administration.

### Bile secretion and composition

As in EFAD *Mdr2*<sup>+/+</sup> mice (Table 3), bile flow was increased in EFAD *Mdr2*<sup>-/-</sup> mice compared with their EFAS counterparts (p<0.05). Bile flow and bile salt secretion were higher in *Mdr2*<sup>-/-</sup> mice than in *Mdr2*<sup>+/+</sup> mice (p<0.01) (Table 7). Bile salt composition was virtually identical between EFAS and EFAD *Mdr2*<sup>-/-</sup> mice (Table 8).

	EFAS	EFAD
Bile flow <sup>1</sup>	6.57 ± 1.57	9.27 ± 2.17 *
Bile salts <sup>2</sup>	260 ± 53	540 ± 182 *
Cholesterol <sup>2</sup>	0.97 ± 0.28	1.31 ± 0.35
Phospholipids <sup>2</sup>	0.10 ± 0.26	0.07 ± 0.16

**Table 7:** Bile flow and biliary secretion rates in *Mdr2*<sup>-/-</sup> mice fed EFA-containing or EFA-deficient chow for 8 weeks. Biliary output rates are given in <sup>1</sup>  $\mu\text{l}/\text{min}/100\text{g}$  body weight or <sup>2</sup>  $\text{nmol}/\text{min}/100\text{g}$  body weight. \*  $p < 0.05$ ;  $n = 5-7$  mice per group.

	EFAS	EFAD
Cholate	63.7 ± 7.3	56.2 ± 3.4
Beta-muricholate	28.0 ± 8.0	35.9 ± 4.1
Omega-muricholate	4.8 ± 1.7	5.2 ± 1.1
Alpha-muricholate	1.0 ± 0.6	1.1 ± 0.3
Deoxycholate	1.6 ± 0.9	0.8 ± 0.4
Chenodeoxycholate	0.9 ± 0.4	0.7 ± 0.3

**Table 8:** Biliary bile salt composition in *Mdr2*<sup>-/-</sup> mice fed EFA-containing or EFA-deficient chow for 8 weeks. >90% of all bile salts represented. Minor metabolites (<1% of total area) have been excluded. Values expressed as percentage of total amount. Data represent means ± SD;  $n = 5-7$  mice per group.

## DISCUSSION

We investigated the mechanism of fat malabsorption in EFA deficiency in mice, with particular emphasis on the possible role of altered bile formation as proposed by Levy *et al.* based on studies in rats<sup>(3,6)</sup>. In order to specify the contribution of biliary phospholipid secretion, studies were performed in EFA-deficient and EFA-sufficient wildtype and *Mdr2*<sup>-/-</sup> mice; the latter are unable to secrete phospholipids into their bile. Yet, we first had to develop and characterize a murine model for EFA deficiency. Our data indicate that dietary fat absorption is reduced in EFA-deficient mice compared to EFA-sufficient controls. The mechanism underlying this EFA deficiency-associated fat malabsorption does not likely involve alterations in bile formation (including bile flow, bile salt secretion rate, bile salt composition or phospholipid secretion rate) nor changes in fat digestion (lipolysis). Rather, our data strongly indicate that EFA deficiency in mice affects intracellular events of fat absorption that occur in the enterocyte.

Biochemical EFA deficiency, conventionally defined by a molar ratio of eicosatrienoic acid (C20:3n-9) and arachidonic acid (C20:4n-6) above 0.20 in plasma<sup>(19)</sup>, was already reached after only 2 weeks of EFA-deficient diet feeding to mice. This rapidity of onset makes the mouse an attractive and versatile model for studying EFA deficiency.

Similar to EFA-deficient rats<sup>(21;22)</sup>, EFA-deficient mice experienced changes in plasma and liver fat contents. Specifically, EFA-deficient mice have decreased plasma triglycerides and increased hepatic triglyceride and cholesterol levels. In EFA-deficient rats, Wanon *et al.*<sup>(23)</sup> observed alterations in HDL-composition, with defective translocation of HDL-cholesterol into bile and concomitantly increased hepatic VLDL-

cholesterol secretion. Lipoprotein abnormalities were also found by Levy *et al.*<sup>(24)</sup> in EFA-deficient cystic fibrosis (CF) patients. Compared to their EFA-sufficient counterparts, EFA-deficient CF-patients had increased plasma triglyceride levels (specifically in the VLDL, LDL, HDL<sub>2</sub> and HDL<sub>3</sub> fractions), and decreased plasma HDL- and LDL-cholesterol. Also, lipoprotein size was altered in these EFA-deficient patients, with larger VLDL, LDL, and HDL<sub>2</sub> particles and smaller HDL<sub>3</sub> particles. It could be speculated that EFA, as constituents of triglycerides, phospholipids and cholesterol esters, may be essential for regulation of lipoprotein metabolism. Although the effects of EFA-deficiency are species specific, it appears that an adequate EFA-status is required for efficient intestinal and hepatic processing of lipoproteins.

In addition to the changes in plasma and liver lipids, EFA deficiency in mice was associated with fat malabsorption. The coefficients of fat absorption in EFA-deficient mice (60-70%) were somewhat lower than corresponding values in EFA-deficient rats (80-90%)<sup>(24,5)</sup>. In all of these studies, EFA deficiency was induced by feeding the animals high-fat EFA-deficient diets almost entirely composed of saturated fatty acids. Apart from species specificity, the difference in coefficients of fat absorption could be related to the amount and type of fat in the diet. In the present study, mice were fed high fat chow (16 weight%) whereas Hjelte *et al.*<sup>(2)</sup> used chow diets containing only 7 weight% fat. Levy *et al.*<sup>(9)</sup> reported that lipolytic activity in EFA-deficient rats was unchanged compared with control rats. Our present results in EFA-deficient mice are compatible with this observation. The appearance of [<sup>3</sup>H]-triolein in plasma after its intragastric administration was similar in EFA-deficient mice and control mice. If anything, the [<sup>3</sup>H]-label was recovered from plasma of EFAD mice even at higher concentrations compared to EFAS controls. The explanation for this phenomenon may involve the choice of the lipid, oleic acid. The absorption of dietary oleic acid was only mildly impaired during EFA deficiency (Figure 1). In addition, a tracer effect could not be excluded. When we investigated the absorption of a less polar lipid, retinol, after its intragastric administration, both EFA-deficient wildtype and EFA-deficient *Mdr2*<sup>-/-</sup> mice showed decreased plasma concentrations compared to their EFA-sufficient controls, which underlines the occurrence of lipid malabsorption during EFA deficiency.

Rather than differences in fat digestion (lipolysis), it could have been expected that alterations in bile formation contributed to fat malabsorption in EFA-deficient mice, in analogy to the situation in EFA-deficient rats<sup>(6)</sup>. The pathophysiology of EFA deficiency-associated fat malabsorption in mice could be due to decreased biliary bile salt

secretion rates, analogous to previous data in rats. However, bile flow and biliary bile salt and phospholipid secretion rates were increased during EFA deficiency. Robins and Fasulo<sup>(25)</sup> reported that EFA-deficient hamsters have increased hepatic bile flow and biliary bile salt and cholesterol secretion compared to controls. It is not known, however, whether EFA-deficient hamsters have a decreased coefficient of fat absorption. Our observation in EFA-deficient mice, together with the available data on EFA-deficient rats and hamsters, indicate that the effects of EFA deficiency on bile formation are species specific. Present data exclude that EFA-deficient fat malabsorption is due to decreased rates of biliary bile salt secretion in mice, in contrast to the situation in rats<sup>(3,6)</sup>. Theoretically, an increase in the contribution of hydrophilic bile salts (to total bile salts) could contribute to impaired solubilization of dietary fats. Yet, biliary bile salt composition was virtually unchanged in EFA-deficient mice compared to controls. The increased biliary secretion rate of bile salts, immediately after interruption of the enterohepatic circulation, strongly suggests an expansion of the bile salt pool size in EFA deficiency.

Bile salts negatively affect their own biosynthesis by repressing the expression of Cyp7A via the FXR-SHP1-LRH1-Cyp7A pathway, with Cyp7A encoding the enzyme cholesterol 7 $\alpha$ -hydroxylase that catalyzes the first step of the neutral pathway in bile salt synthesis<sup>(26-30)</sup>. In our experiments however, Cyp7A and Cyp27 mRNA levels were similar in livers from EFAS and EFAD mice. We speculate that EFA deficiency in mice impairs the capacity of bile salts to exert negative feedback inhibition on their own hepatic biosynthesis, but the mechanism hereof remains unclear.

Not only biliary bile salts, but also biliary phospholipids play a role in dietary fat absorption, for example in supplying surface components for the assembly of chylomicron particles in enterocytes<sup>(31,32)</sup>. Under physiological conditions, overall fat absorption in *Mdr2*<sup>-/-</sup> mice is only slightly decreased compared to control mice (95 vs. 98%), based on 72h fecal fat balance measurements. On the other hand, kinetics of chylomicron formation are clearly delayed in *Mdr2*<sup>-/-</sup> mice<sup>(33)</sup>. Therefore, a quantitative alteration in biliary phospholipid secretion was not likely to contribute to EFA deficiency-associated fat malabsorption. Yet, fat malabsorption during EFA deficiency could still be due to qualitative changes in biliary phospholipid composition. Replacement of polyunsaturated acyl chains (linoleoyl-, arachidonoyl-) by monounsaturated or saturated species could theoretically be responsible for impaired chylomicron assembly and secretion. In accordance with findings by Bennett Clark *et al.* in EFA-deficient rats<sup>(4)</sup>, the acyl chain composition of biliary PL in EFA-deficient mice showed less essential (i.e., C18:2n-6 and C20:4n-6) and more non-essential fatty acids (i.e., C18:1n-9, C18:1n-7, C16:1n-7). If acyl chain



composition of bile phospholipids were important for fat malabsorption during EFA deficiency, one would expect that EFA deficiency would not, or to a much lesser extent, affect fat absorption in *Mdr2*<sup>-/-</sup> mice. Present data, however, clearly indicate that fat absorption in EFA-deficient *Mdr2*<sup>-/-</sup> mice was affected similarly as in EFA-deficient wildtype mice. In EFA-deficient *Mdr2*<sup>-/-</sup> mice, as in EFA-deficient wildtype controls, bile flow and biliary bile salt secretion were increased. Based on the similar fat absorption coefficients we found in EFA-deficient mice with and without biliary PL secretion, we conclude that the effect of biliary PL acyl chain composition is not of pathophysiological relevance for EFA deficiency-associated fat malabsorption.

Rather than to alterations in bile secretion, fat malabsorption during EFA deficiency may be due to other steps involved in fat absorption. Intestinal mucosal phospholipids normally contain large amounts of C18:2n-6 and C20:4n-6, and during EFA deficiency the levels of these fatty acids are markedly decreased<sup>(34-36)</sup>. The resultant structural changes in membranes, and the increased cellular turnover rate in the intestinal mucosa reported in EFA-deficient rats<sup>(5)</sup> could be responsible for decreased dietary fat absorption. Based on our study and previous studies in EFA-deficient rats<sup>(3,4)</sup>, the intraluminal events involved in fat absorption (i.e., lipolysis of dietary triglyceride by pancreatic lipase, solubilization of lipolytic products and uptake by the enterocyte) seem to be relatively undisturbed in EFA deficiency. By inference, it is therefore more likely that defects in one of the several intracellular events (i.e., re-esterification, chylomicron assembly and/or secretion) are involved in EFA deficiency-associated fat malabsorption.

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