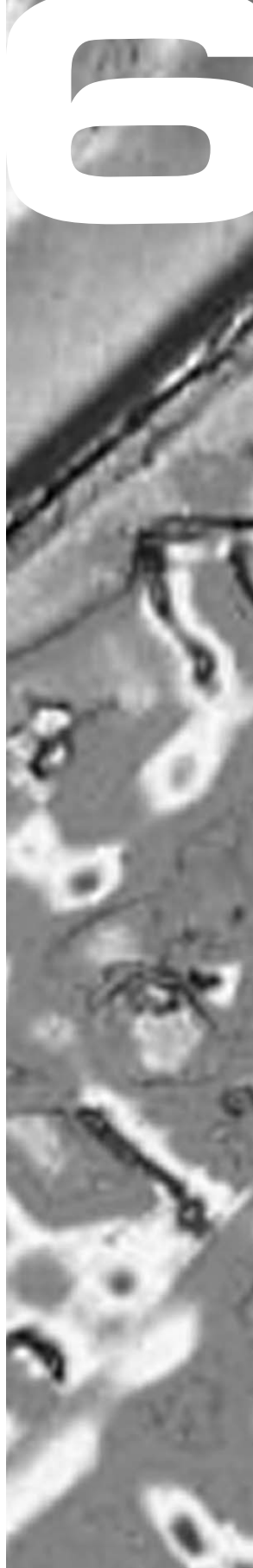


Treatment of essential fatty acid deficiency with dietary triglycerides or phospholipids in a murine model of extrahepatic cholestasis

Am J Physiol Gastrointest Liver Physiol. 2004; 286(5): G822-832

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ABSTRACT

Background: Essential fatty acid (EFA) deficiency during cholestasis is mainly due to malabsorption of dietary EFA⁽¹⁾. Theoretically, dietary phospholipids (PL) may have a higher bioavailability than dietary triglycerides (TG) during cholestasis. We developed murine models for EFA deficiency with and without extrahepatic cholestasis, and compared the efficacy of oral supplementation of EFA as PL or TG. **Methods:** EFA deficiency was induced in mice by feeding a high-fat EFA-deficient (EFAD) diet. After three weeks on this diet, bile duct ligation (BDL) was performed in a subgroup of mice to establish extrahepatic cholestasis. Cholestatic and non-cholestatic EFA-deficient mice continued on the EFAD diet (controls), or were supplemented for three weeks with EFA-rich TG or EFA-rich PL. Fatty acid composition was determined in plasma, erythrocytes, liver and brain.

Results: After four weeks of EFAD diet, induction of EFA deficiency was confirmed by a six-fold increased triene/tetraene ratio (T/T-ratio) in erythrocytes of non-cholestatic and cholestatic mice ($p < 0.001$). EFA-rich TG and EFA-rich PL were equally effective in preventing further increase of the erythrocyte T/T-ratio, which was observed in cholestatic and non-cholestatic non-supplemented mice (12- and 16-fold the initial value, respectively). In cholestatic mice, EFA-rich PL was superior to EFA-rich TG in decreasing T/T-ratios of liver triglycerides and phospholipids (each $p < 0.05$), and in increasing brain phospholipid concentrations of the long-chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid and arachidonic acid (each $p < 0.05$). **Conclusions:** Oral EFA supplementation in the form of PL is more effective than in the form of TG in increasing LCPUFA concentrations in liver and brain of cholestatic EFA-deficient mice.

INTRODUCTION

Essential fatty acids (EFA) and their long-chain polyunsaturated metabolites have been recognized to play a role in growth, development of the central nervous system, eicosanoid production and control of lipid homeostasis. Due to the inability of mammalian cells to synthesize EFA *de novo*, adequate EFA levels depend entirely on sufficient dietary intake and absorption. Conditions leading to fat malabsorption, such as cholestasis, have been associated with a high incidence of essential fatty acid deficiency (EFAD)⁽²⁻⁴⁾. In a rat model of cholestasis, we recently demonstrated that impaired intestinal absorption of EFA is the main contributor to EFA deficiency in cholestatic conditions, rather than altered post-absorptive EFA metabolism⁽¹⁾. So far, no adequate oral strategies for prevention or treatment of EFA deficiency during cholestasis have been developed. In regular diets, 90% of EFA is present as acyl esters in triglycerides, and only 10% is present as acyl esters in phospholipids and cholesterol esters⁽⁵⁾. Under physiological conditions, however, the intestine receives significant amounts of EFA as biliary phospholipids, which contain up to 40 mol% of EFA (mostly linoleic acid), esterified at the sn-2 position. Triglycerides and phospholipids have different intestinal absorption mechanisms. Due to their hydrophobic nature, triglycerides are insoluble in the aqueous intestinal lumen, and products of triglyceride lipolysis, i.e., free fatty acids and monoglycerides, highly depend on bile components for solubilization into mixed micelles. After absorption by the enterocyte, fatty acids and monoglycerides are re-esterified into triglycerides and secreted in lymph as the major core components of chylomicrons. Phospholipids, on the other hand, are relatively independent of bile components for intestinal absorption. Phospholipids have a higher tendency than triglycerides to interact with water and can associate into liquid crystals (bilayers)⁽⁶⁾, which have been suggested to play a role in luminal lipid solubilization under bile-deficient conditions⁽⁷⁾. Phospholipids can be absorbed intact or after partial digestion to lysophospholipids and free fatty acids⁽⁸⁻¹⁰⁾. Phospholipids of luminal origin are predominantly used by enterocytes for assembly of the surface coat of chylomicrons secreted into lymph. In conditions of cholestasis, i.e. during decreased or absent bile formation, triglyceride absorption is severely impaired. Phospholipids can facilitate dietary lipid absorption under bile deficient conditions by enhancing luminal lipid solubilization and by providing surface components for lipoprotein assembly^(6,11). Additionally, phospholipids have been postulated to have greater post-absorptive bioavailability⁽¹²⁻¹⁶⁾. Based on these characteristics, we hypothesized that phospholipids could be a better vehicle for oral EFA supplementation during cholestasis than triglycerides. In this study, we compared the efficacy of EFA-rich PL and of EFA-rich

TG for treatment of EFA deficiency under cholestatic conditions in mice. For this purpose, we developed a model for both EFA deficiency and cholestasis by feeding mice an EFA-deficient (EFAD) diet followed by bile duct ligation. Subsequent oral supplementation with EFA either as TG or PL demonstrated that the latter resulted in higher concentrations of EFA-derived LCPUFA in target organs as brain and liver.

MATERIALS AND METHODS

Animals

Wildtype mice with a free virus breed (FVB) background were obtained from Harlan (Horst, the Netherlands). Male mice (bodyweight 25-35 g) were housed in a light-controlled (lights on 6 AM-6 PM) and temperature-controlled (21°C) facility and were 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 diets

The EFA-deficient (EFAD) diet contained 20 energy% protein, 46 energy% carbohydrate and 34 energy% fat, respectively, and had the following fatty acid composition: 41.4 mol% palmitic acid (C16:0), 47.9 mol% stearic acid (C18:0), 7.7 mol% oleic acid (C18:1n-9) and 3 mol% linoleic acid (C18:2n-6). An isocaloric EFA-sufficient (EFAS) diet was used as control diet, containing 20 energy% protein, 43 energy% carbohydrate and 37 energy% fat with 32.1 mol% C16:0, 5.5% C18:0, 32.2 mol% C18:1n-9 and 30.2% C18:2n-6 (custom synthesis, diet numbers 4141.08 (EFAD) and 4141.07 (EFAS), Hope Farms BV, Woerden, the Netherlands). For EFA supplementation, EFAD chow pellets were finely pulverized and homogeneously mixed with either triglyceride oil or phospholipid oil (lecithin), dissolved in water en ethanol. Both TG and PL oil were purified from crude soybean oil and had the following fatty acid profile (weight%): 53.5% C18:2, 7.6% C18:3, 22.8% C18:1, 10.7% C16:0, 4.0% C18:0 and <0.5% of C14:0, C16:1, C20:0, C20:1, C22:0 and C24:0 each (TG); and 56.1% C18:2, 8.2% 18:3, 13.2% C18:1, 16.1% C16:0, 4.5% C18:0 and <0.5% of C14:0, C16:1, C20:0, C20:1, C22:0 and C24:0 each (PL), respectively.

We aimed to supplement the EFA-deficient mice with 2.5 mg LA per day. For the TG-oil, the percentage of fatty acid mass relative to the glycerol backbone mass is 95.5%, of which 53.5% is linoleic acid. For the PL-oil, the fatty acid mass vs. the mass of the glycerol-phosphate-choline backbone is 72.3%, of which 56.1% is linoleic acid. To obtain equimolar amounts of LA in the two EFA-supplemented diets, we added

5.6 g TG (=0.006 mol TG) per kg chow and 5.8 g PL (=0.007 mol PL), resulting in 0.003 mol LA per kg of chow for both oils. Based on average daily chow intake of 3 g (measured in pilot experiments with EFAD mice), this resulted in 2.5 mg of LA supplementation per day. The PL and TG soybean oils were a generous gift from Unimills BV, Zwijndrecht, the Netherlands.

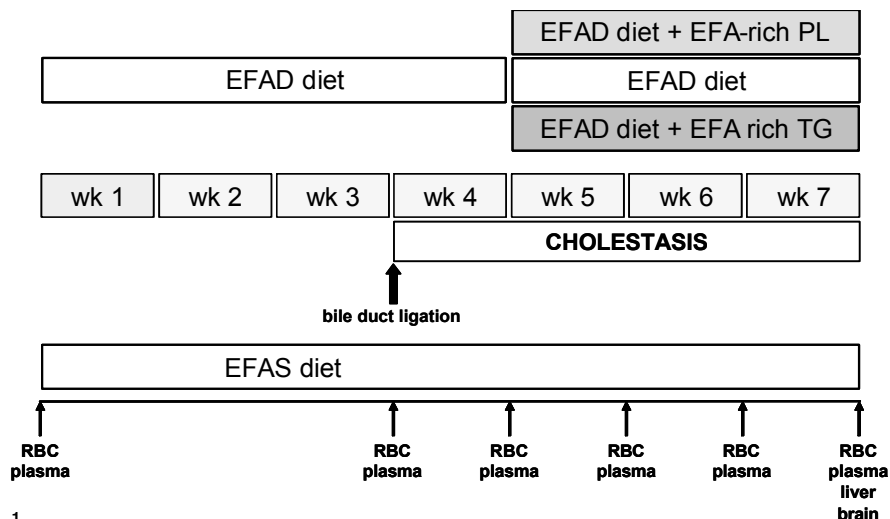
Experimental procedures

Induction of EFA deficiency in mice and oral administration of EFA-rich TG or PL

Mice were fed standard laboratory chow containing 6 weight% fat (RMH-B, Hope Farms BV, Woerden, the Netherlands) from weaning. Before starting the EFAD diet, a blood sample was obtained by tail bleeding under halothane anesthesia for determination of baseline EFA status. Blood was collected in micro-hematocrit tubes containing heparin, and plasma and erythrocytes were separated by centrifugation at 2400 rpm for 10 min (Eppendorf Centrifuge, Eppendorf, Germany). Plasma and erythrocyte samples were hydrolyzed and methylated the same day⁽¹⁷⁾ for gas-chromatographic analysis of fatty acid profiles. All mice were then fed a high-fat (16 weight% fat), EFAD diet for four weeks. Subsequently, mice were randomly assigned to EFAD diet supplemented with either EFA-rich TG or EFA-rich PL, or continued on the EFAD diet for three weeks (n=5 per group). At weekly intervals mice were weighed, chow containers were weighed to monitor food intake and blood samples were taken by tail bleeding for determination of EFA status.

Bile duct ligation in EFAD mice and oral administration of EFA-rich PL or TG

A separate group of mice was fed the EFAD diet for three weeks, after which their bile ducts were ligated by placing three sutures proximal to the gallbladder under halothane/NO₂ anesthesia. Animals were allowed to recover from surgery for one week during which the EFAD diet was continued. Subsequently, i.e. after four weeks on EFAD diet, mice were randomly assigned to EFAD diet enriched with either EFA-rich TG or EFA-rich PL, or continued on the EFAD diet for three weeks. A separate control group of non-bile duct ligated control mice received an EFA sufficient (EFAS) diet for seven weeks (n=6 for each dietary group). During the entire experiment, bodyweight, chow ingestion, plasma liver enzymes and EFA status were determined at weekly intervals. After three weeks of supplementation, mice were terminated under anesthesia by heart puncture, by which a large blood sample (0.6 - 1.0 ml) was obtained, and liver and brain were removed and stored at -80°C for fatty acid analysis. A schematic overview of the experimental design is depicted in Figure 1.



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Figure 1: Experimental design of EFA supplementation to cholestatic EFA-deficient mice. EFA deficiency was induced by feeding the EFAD diet for 3 weeks, followed by surgical bile duct ligation. After one week of recovery, mice were assigned to EFAD diet enriched with TG or PL, or continued on the EFAD diet for 3 weeks. A group of non-bile duct ligated control mice received an EFA sufficient (EFAS) diet for 7 weeks ($n=6$ for each dietary group). Blood samples were taken weekly and liver and brain were removed for fatty acid analysis at the end of the experiment.

Analytical techniques

Fatty acid status was analyzed by hydrolyzing, methylating and extracting total plasma lipids and erythrocyte membrane lipids as described by Muskiet *et al.*⁽¹⁷⁾. For fatty acid analysis of brain and liver, tissue samples were mechanically homogenized in 0.9% NaCl and lipids were extracted from aliquots of tissue homogenate as described by Bligh and Dyer⁽¹⁸⁾. Lipid extracts were fractionated into phospholipids, cholesterol esters, triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids using thin-layer chromatography (TLC) (20x20 cm, Silica gel 60 F254, Merck), with hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as solvent. TLC plates were dried and colored by iodine, PL and TG spots were scraped off and methylated⁽¹⁷⁾. To account for losses during lipid extraction, heptadecanoic acid (C17:0, Sigma Chemical Company, St. Louis, MO, USA) was added to all samples as internal standard prior to extraction. Butylated hydroxytoluene was added as antioxidant. Chow pellets were freeze-dried and mechanically homogenized and from aliquots of each diet, lipids were hydrolyzed, methylated and extracted as described above. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard gas chromatograph model 6890, equipped with a 50m x 0.2mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA) and a FID detector, using program conditions as described previously⁽¹⁹⁾. Individual fatty acid methyl esters were quantified by relating areas of their chromatogram peaks to that

of the internal standard C17:0. Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP) and cholesterol concentrations were determined using routine clinical procedures. Plasma total bile salt levels were determined according to Mashige *et al.*⁽²⁰⁾. Liver and brain histology was examined on frozen tissue sections after Oil-Red-O (ORO) staining, which colors neutral lipids (mainly triglycerides), and on paraformaldehyde-fixed, paraffin-embedded tissue sections after hematoxylin-eosin (HE) staining by standard procedures.

Calculations and statistics

Relative concentrations (mol%) of plasma, RBC, liver and brain fatty acids were calculated by summation of all fatty acid peak areas and expression of individual fatty acid areas as a percentage of this amount. EFA status was evaluated by comparing mol% of individual EFA and LCPUFA, and by calculating markers for EFA deficiency such as the triene/tetraene-ratio in different body compartments⁽²¹⁾. All results are presented as means \pm S.D. for the number of animals indicated. Data were statistically analyzed using Student's t-test or, for comparison of more than two groups, ANOVA-test with post-hoc Bonferroni correction. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

RESULTS

EFA deficiency in non-cholestatic mice

In a previous study⁽¹⁹⁾, we developed a murine model for diet-induced EFA deficiency by feeding mice an EFAD diet for 8 weeks, which resulted in a pronounced dietary fat malabsorption. In the present study, feeding the EFAD diet for 4 weeks did not decrease bodyweight, but mice stopped growing (baseline: 32.4 ± 2.9 g; at 4 weeks: 34.1 ± 3.3 g, NS). After 3 weeks of EFA supplementation, no differences in bodyweight were found between mice fed EFA-rich TG (33.6 ± 3.9 g) or EFA-rich PL (33.1 ± 1.9 g, NS). Chow intake was 3.5 ± 0.5 g/day in both dietary groups throughout the experimental period, indicating an average daily LA intake of 3 mg. Since EFA deficiency has been reported to have species-specific effects on bile formation^(19,22), we measured the effects of dietary EFA deficiency and -supplementation on plasma bile salts. EFA deficiency was associated with increasing plasma bile salt levels (21 ± 4 μ M at baseline to 40 ± 7 μ M after 4 weeks of EFAD diet). Three weeks of supplementation with TG or PL tended to reverse the increased plasma bile salt levels (32 ± 10 and 38 ± 6 μ M, respectively), but differences were not statistically significant.

Figure 2 shows the molar percentages of relevant PUFA in erythrocyte membrane lipids of non-cholestatic mice during EFAD diet feeding (control), EFA-rich PL supplementation or EFA-rich TG supplementation.

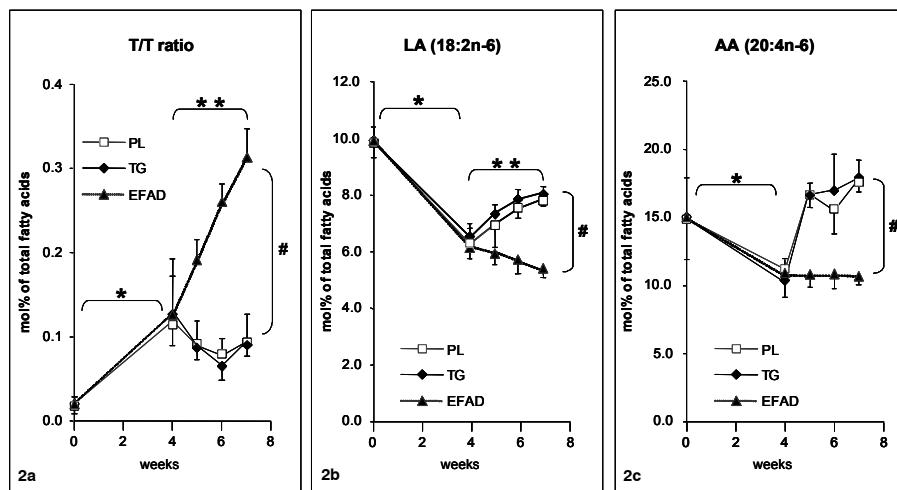


Figure 2: Triene/tetraene ratio (T/T-ratio), linoleic acid (LA) and arachidonic acid (AA) in red blood cells (RBC) of non-cholestatic mice fed EFAD diet for 4 weeks, and EFA-rich PL, EFA-rich TG or no EFA for 3 weeks. Fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means \pm SD of 5 mice per group. * $p < 0.001$ for week 0 vs. week 4 for all fatty acids, and ** $p < 0.005$ for the TT-ratio and LA in week 4 vs. week 7. # $p < 0.001$ for EFAD vs. PL or TG in week 7. No significant differences were found between PL- or TG-fed mice.

The classic biochemical parameter describing EFA status is the triene/tetraene-ratio (T/T-ratio), i.e., the molar ratio between the non-essential fatty acid eicosatrienoic acid (C20:3n-9) and arachidonic acid (C20:4n-6). Development of EFA deficiency is associated with an elevated T/T-ratio. Compared to baseline (0.02 ± 0.01), the T/T-ratio in erythrocytes of non-cholestatic mice increased more than 6-fold ($p < 0.001$) after four weeks, and 16-fold after another three weeks of EFAD diet ($p < 0.001$, Figure 2a). EFA supplementation, either with EFA-rich PL (0.09 ± 0.02) or EFA-rich TG (0.09 ± 0.03) completely prevented this further increase in T/T-ratio ($p < 0.001$ for either PL or TG vs. EFAD) and tended to decrease the ratio compared to four-week values. Molar percentages of linoleic acid (LA, Figure 2b), and of its corresponding long-chain polyunsaturated metabolite arachidonic acid (AA, Figure 2c) were decreased after four weeks on EFAD diet (both $p < 0.001$). After another three weeks on this diet, LA levels had further decreased ($p < 0.001$) while AA levels remained at a stable low level. Supplementation with either EFA-rich TG or PL reversed the decreased LA and AA concentrations with similar efficacy ($p < 0.005$) (Figure 2b, 2c).

In control mice fed the high-fat EFA-sufficient (EFAS) diet for seven weeks, LA and

AA levels remained stable (LA: 8.9 ± 0.6 , AA 15.7 ± 0.6 ; not depicted in the figure). Molar percentages of the n-3 EFA ALA were low in all dietary groups and did not change significantly during four or seven weeks of EFAD diet, nor during three weeks of EFA supplementation (data not shown).

EFA deficiency in cholestatic mice

Under physiological (non-cholestatic) conditions, mice fed a high-fat, EFA-sufficient (EFAS) diet for 7 weeks gradually increased in bodyweight from 25.8 ± 0.6 g to 40.2 ± 2.2 g (Figure 3). Development of EFA deficiency and cholestasis interrupted physiological growth in mice. Non-supplemented and TG-supplemented EFAD mice significantly lost weight during the seven-week experiment (from 25.8 ± 1.5 g at baseline to 22.7 ± 1.2 g and 23.2 ± 1.5 g, respectively; each $p < 0.01$). Supplementation of EFA with PL, however, prevented this weight loss completely, resulting in a stable body weight throughout the experiment (PL: 27.0 ± 2.5 g, $p < 0.05$ for PL vs. TG and EFAS).

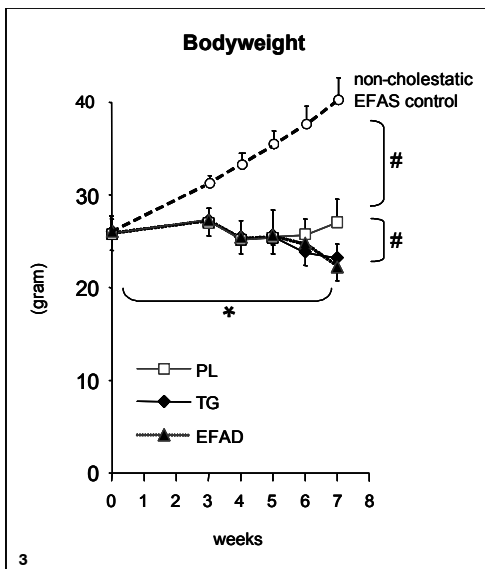


Figure 3: Body weight in bile duct-ligated mice fed EFAD diet or EFAD diet supplemented with PL or TG, and of non-cholestatic EFA-sufficient control mice. Data represent means \pm SD of 5 mice per group. * $p < 0.01$ for TG, EFAD and EFAS in week 0 vs.7. # $p < 0.05$ for PL vs. TG, EFAD and EFAS in week 7. For PL-fed mice, bodyweight was not significantly different in week 7 compared to baseline.

Plasma bile salt concentrations significantly increased from 21 ± 4 μ M at baseline to 46 ± 11 μ M after three weeks of EFAD diet. In the fourth week of bile duct ligation, plasma bile salt concentrations had strongly increased in all groups. However, EFA supplementation in the form of either PL or TG profoundly mitigated the increase in plasma bile salt concentration compared to non-supplemented mice (PL: 1242 ± 163 μ M, TG: 1104 ± 252 μ M, EFAD: 1983 ± 510 μ M; $p < 0.05$ for either TG or PL vs. EFAD in week 7; $p < 0.005$ for all groups in week 3 vs. week 7). As expected, alanine amino-

transferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) activity in plasma strongly increased after induction of extrahepatic cholestasis (Table 1). No profound differences between the three experimental groups were noted in these parameters. Plasma cholesterol concentrations had remained fairly constant after four weeks of EFAD diet / one week of BDL. After another three weeks of cholestasis, however, plasma cholesterol levels were significantly higher in PL- and TG-supplemented mice compared to non-supplemented mice.

	wk 0			wk 4 (=1wk after BDL)			wk 7 (=4 wk after BDL)		
	PL	TG	EFAD	PL	TG	EFAD	PL	TG	EFAD
AST (U/l)	48 ± 30			659 ± 323 *			549 ± 23	451 ± 116	739 ± 288
ALT (U/l)	34 ± 14			354 ± 199 *			261 ± 82	253 ± 60	406 ± 192
AP(U/l)	81 ± 31			895 ± 228 *			1885 ± 1097 #	1520 ± 278 #	1167 ± 385
Cholesterol (mM)	3.2 ± 1.2			3.3 ± 1.7			9.2 ± 2.8 ##	9.4 ± 0.5 ##	6.4 ± 2.7 **

Table 1: Plasma aspartate transaminase (AST, Units/L), alanine transaminase (ALT, Units/L), alkaline phosphatase (AP, Units/L) and cholesterol (mM) at baseline (week 0), after one week of bile duct ligation (week 4) and after 4 weeks of bile duct ligation (week 7) in TG-supplemented, PL-supplemented or non-supplemented mice. Data represent means±SD of 4-6 mice per group. *p<0.001 for AST, ALT and AP concentrations in week 4 compared to baseline. #p<0.001 for TG and PL supplemented mice in week 4 vs. week 7. ##p<0.01 for cholesterol levels in TG and PL supplemented mice in week 7 vs. 4. **p<0.005 for cholesterol levels in EFAD vs. TG and PL supplemented mice in wk 7.

The T/T-ratio (C20:3n-9/C20:4n-6) in total plasma lipid increased during development of EFA deficiency and cholestasis from 0.01 ± 0.00 at baseline to 0.51 ± 0.28 in week four ($p < 0.001$, Figure 4). Upon supplementation with PL or TG, the T/T-ratio decreased to 0.32 ± 0.17 and 0.36 ± 0.03 , respectively, whereas in non-supplemented mice the T/T-ratio further increased to 0.61 ± 0.20 . Plasma molar percentages of LA and AA were significantly lower in week four compared to baseline values ($p < 0.001$). A further decrease in LA levels was partially prevented only after supplementation with PL and not with TG ($p < 0.05$ for PL vs. EFAD at week 7; TG vs. EFAD NS).

Since total plasma lipid fatty acid composition in non-fasted animals is strongly determined by postprandial dietary TG, we also analyzed fatty acid profiles of the isolated plasma PL fraction of the terminal blood sample in week seven (Figure 5). The T/T-ratio tended to be lower and LA, AA and DHA-concentrations higher in TG- and PL-fed mice compared to non-supplemented mice, but differences were not statistically significant. Fatty acid concentrations were not significantly different between the two supplementation groups.

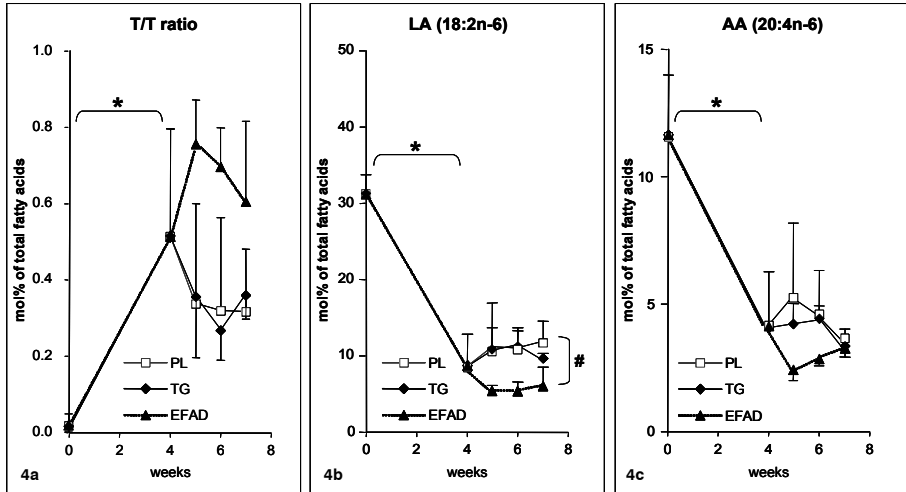


Figure 4: Triene/tetraene ratio (T/T-ratio), linoleic acid (LA) and arachidonic acid (AA) in plasma of bile duct ligated mice fed EFAD diet for 4 weeks, and EFA-rich PL, EFA-rich TG or no EFA for 3 weeks. Individual fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means \pm SD of 4-6 mice per group. * $p < 0.001$ for the TT-ratio, LA and AA in week 0 vs. week 4. # $p < 0.05$ for PL vs. EFAD in week 7; LA in TG-fed mice was not significantly different from EFAD mice in week 7.

Plasma PL cholestatic EFAD mice

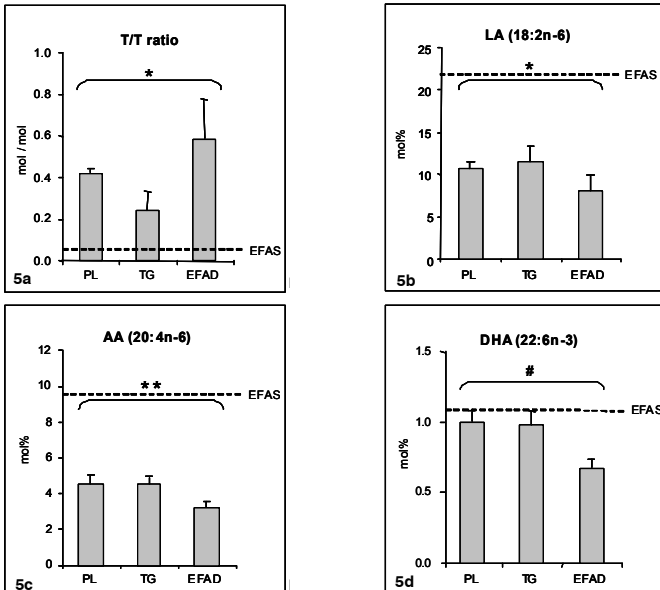


Figure 5: Triene/tetraene ratio (T/T-ratio), linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) in the plasma PL-fraction of cholestatic mice, after 7 weeks of experimental diet feeding. Fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means \pm SD of 4-6 mice per group. * $p < 0.001$ for the T/T-ratio and LA in PL-, TG- and non-supplemented mice compared to non-cholestatic EFAS mice. ** $p < 0.05$ for AA in PL, TG and non-supplemented mice compared to non-cholestatic EFAS controls. # $p < 0.05$ for DHA in EFAD vs. EFAS mice in week 7; DHA in TG-fed mice and PL-fed mice was not significantly different from non-cholestatic EFAS mice.

The fatty acid composition of the plasma compartment is largely determined by the plasma triglyceride fraction, which reflects dietary fatty acid composition to a certain extent. Fatty acid profiles in erythrocyte membranes are assumed to be a more stable reflection of overall essential fatty acid status. The T/T-ratio in erythrocytes was 0.02 ± 0.01 at baseline and increased to 0.12 ± 0.06 in week four (i.e., one week after bile duct ligation, $p < 0.001$; Figure 6). Continuation of the EFAD diet in cholestatic mice induced a further increase of the erythrocyte T/T-ratio, which was prevented by supplementation with either PL or TG (Figure 6). Linoleic acid (LA) decreased from 10.5 ± 0.5 mol% at baseline to 6.4 ± 1.7 mol% in week four (Figure 6b). Supplementation with PL for three weeks resulted in higher LA concentrations compared to the EFAD group (5.3 ± 1.5 vs. 3.5 ± 1.5 mol%, respectively; $p < 0.005$). TG-supplemented mice had intermediate LA levels at 7 weeks (4.3 ± 0.8 ; TG vs. EFAD: NS). AA molar percentages in cholestatic mice remained remarkably stable during the entire experiment in all dietary groups: 15.3 ± 2.9 at baseline; 15.7 ± 1.0 in week four (Figure 6c); and 15.3 ± 4.0 (PL), 13.3 ± 4.3 (TG) and 13.2 ± 1.8 (EFAD) in week seven of the study period, respectively (NS). Surprisingly, when comparing AA values of cholestatic with those of non-cholestatic EFA-deficient mice (Figure 2c), we observed that bile duct ligation completely prevented the decline in AA during EFAD diet feeding. AA levels in cholestatic EFAD mice remained similar to those in non-cholestatic EFAS mice (15.4 ± 0.5 , NS).

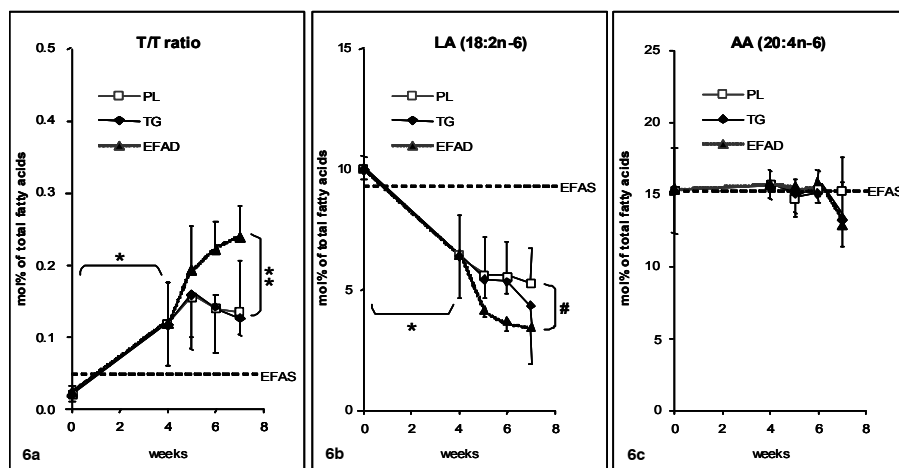


Figure 6: Triene/tetraene ratio (T/T-ratio), linoleic acid (LA) and arachidonic acid (AA) in red blood cells (RBC) of cholestatic mice during the 7-week period of experimental diet feeding. Individual fatty acid concentrations are expressed as molar percentages of total fatty acids. Data represent means \pm SD of 4-6 mice per group. * $p < 0.001$ for the T/T-ratio and for LA in week 0 vs. 4. ** $p < 0.05$ for T/T-ratios of EFAD mice compared to PL- and TG-supplemented mice in week 7. # $p < 0.005$ for LA in PL-supplemented vs. non-supplemented EFAD mice in week 7; LA concentrations in TG-fed mice were not significantly different from those of EFAD mice. AA levels were not significantly different between non-cholestatic EFAS mice and any of the cholestatic EFAD mice throughout the experimental period.

Since the liver is the principal site of desaturation and elongation of EFA into their long-chain polyunsaturated metabolites, we analyzed the fatty acid composition of liver triglyceride and liver phospholipid fractions. In liver phospholipids (Figure 7), the T/T-ratio was significantly lower in PL-fed mice than in TG-fed and EFAD mice after four weeks of cholestasis ($p < 0.05$, Figure 7a). Linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) concentrations were not significantly different between PL-, TG-, and non-supplemented cholestatic mice. Yet, LA- and AA-levels of liver phospholipids were significantly lower in cholestatic animals than in non-cholestatic EFAS mice ($p < 0.001$).

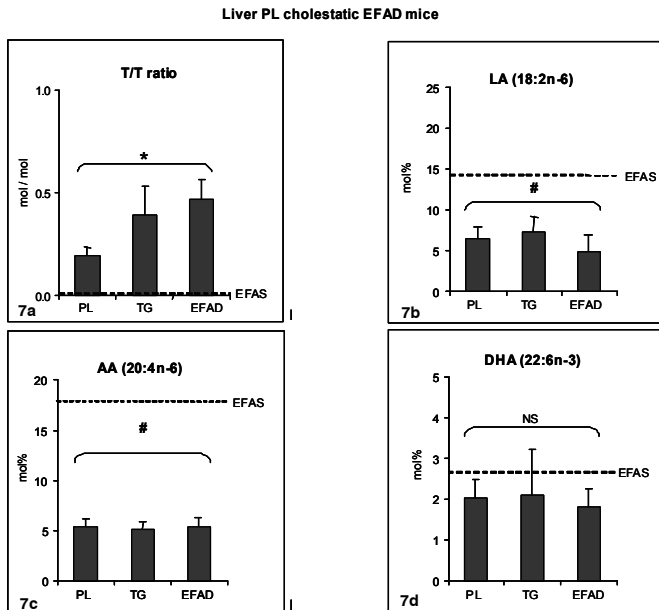


Figure 7: T/T-ratio, linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) in liver PL of cholestatic mice after 7 weeks of experimental diet feeding, and of non-cholestatic EFAS controls (dotted line). Fatty acid concentrations are mol% of total fatty acids. Data represent means \pm SD of 4-6 mice per group. * $p < 0.05$ for T/T-ratios of PL-fed and EFAS mice vs. TG-fed and EFAD mice. # $p < 0.001$ for LA and AA of PL-, TG- and non-supplemented cholestatic mice vs. non-cholestatic EFAS mice.

Differential effects of TG and PL supplementation were also observed in the liver triglyceride fraction (Figure 8). Although LA levels were similar in all 3 cholestatic groups, the T/T-ratio of PL-supplemented mice was comparable to that of non-cholestatic EFAS mice (Figure 8a), whereas T/T-ratios of TG-supplemented and EFAD mice were significantly higher ($p < 0.05$). Arachidonic acid was significantly higher in PL-supplemented mice compared to TG- or non-supplemented mice (PL: 1.4 ± 0.4 mol%; TG: 0.7 ± 0.2 mol%; EFAD: 0.7 ± 0.2 mol%, $p < 0.05$). Similarly, DHA levels were higher in the PL-group compared to the TG- and EFAD-groups, and even higher than in EFAS control mice (Figure 8d).

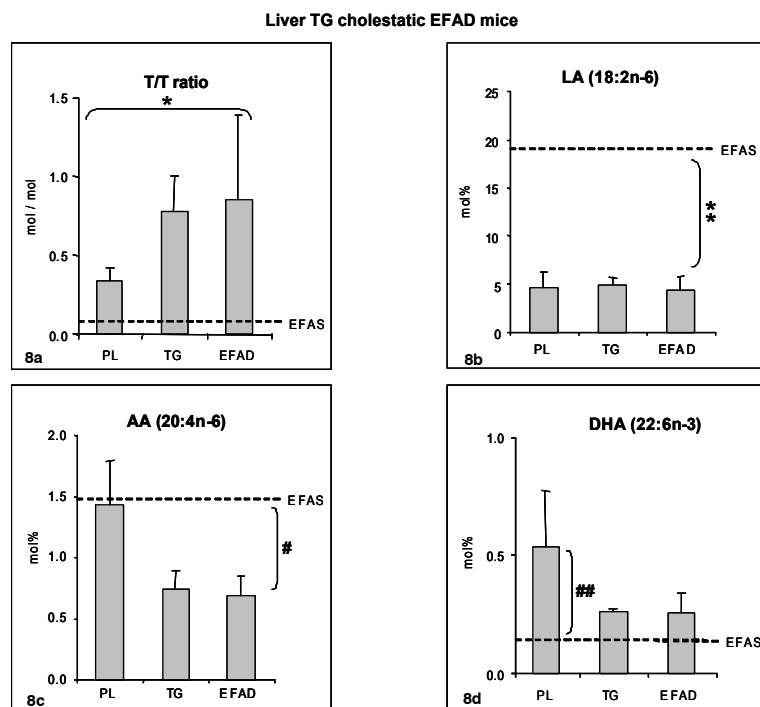


Figure 8: Triene/tetraene ratio (T/T-ratio), linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) in liver TG of cholestatic mice after 7 weeks of experimental diet feeding. Fatty acid concentrations are expressed as mol% of total fatty acids. Data represent means \pm SD of 4-6 mice per group. * $p < 0.05$ for the T/T-ratio of PL-fed and EFAS mice (dotted line) vs. TG-fed and EFAD mice. ** $p < 0.001$ for LA concentrations in cholestatic mice vs. non-cholestatic EFAS mice. * $p < 0.05$ for AA in PL-fed and EFAS mice vs. TG-fed and EFAD mice. ** $p < 0.05$ for DHA levels in PL-fed mice vs. TG-fed, EFAD and EFAS mice.

Oil red O staining for neutral lipids on frozen liver sections showed lipid accumulation in periportal (zone 1) but not in perivenous (zone 3) hepatocytes of EFA-deficient non-cholestatic mice. EFA-sufficient non-cholestatic mice did not show hepatic lipid accumulation. In paraffin-embedded HE-stained liver sections of cholestatic mice, extensive bile duct proliferation was observed in all three dietary groups, with extensive hepatocyte damage due to toxic bile accumulation. No accumulation or zonal distribution of lipid was observed in livers of cholestatic mice. There were no overt histological differences between the three cholestatic groups (data not shown).

Since the central nervous system is a well-known target organ for LCPUFA and contains particularly high levels of docosahexaenoic acid, we analyzed fatty acid profiles of brain tissue samples. PL-supplemented mice had significantly higher AA and DHA concentrations in phospholipids isolated from brain tissue than TG-fed and EFAD mice (each $p < 0.05$ for differences between PL vs. TG and EFAD, Figure 9), and even slightly higher DHA concentrations than non-cholestatic EFAS mice,

although the latter difference was not significant. The third major brain fatty acid, C22:4n-6, was similarly lower in TG-fed and EFAD mice than in non-cholestatic controls (1.69 ± 0.13 mol% (TG), 1.63 ± 0.12 mol% (EFAD), 2.09 ± 0.20 mol% (EFAS), $p < 0.01$), whereas PL-supplemented mice had C22:4n-6 concentrations comparable to non-cholestatic EFAS mice (1.83 ± 0.13 mol%, NS; data not shown).

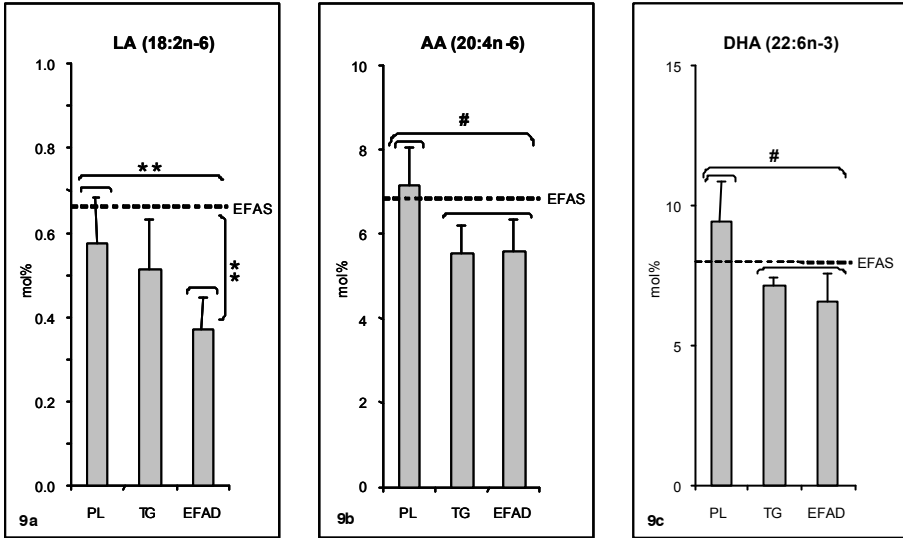


Figure 9: Linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) in brain PL of cholestatic mice after 7 weeks of experimental diet feeding, and of non-cholestatic EFAS controls (dotted line). Individual fatty acid concentrations are expressed as molar percentages of total fatty acids. Data represent means \pm SD of 4-6 mice per group. * $p < 0.05$ for T/T-ratios of PL-fed and TG-fed mice compared to EFAD mice, and for all cholestatic groups compared to non-cholestatic EFAS mice (dotted line). ** $p < 0.05$ for LA concentrations of EFAD mice compared to PL-fed and EFAS mice. AA and DHA were significantly higher in brain PL of PL-fed mice compared to TG-fed and EFAD mice (* $p < 0.01$).

DISCUSSION

We compared the efficacy of EFA-rich triglycerides (TG) and EFA-rich phospholipids (PL) for correcting EFA deficiency under cholestatic conditions in mice. We hypothesized that PL would be more effective than TG for oral treatment of EFA deficiency in cholestatic liver disease, since dietary TG are profoundly malabsorbed during cholestasis and PL are less dependent on bile for intestinal absorption. Present results indicate that, indeed, oral supplementation with PL is superior to oral TG in increasing EFA-derived LCPUFA concentrations in brain and liver.

We used and adapted a murine model for diet-induced EFA deficiency that we developed and characterized previously⁽¹⁹⁾. Ligation of the common bile duct in mice fed an EFAD diet for three weeks resulted in acute extrahepatic cholestasis, combined with a diet-induced EFA deficiency. Both our cholestatic and non-cholestatic mouse models for EFA deficiency developed the characteristic biochemical hallmarks of EFA deficiency in plasma and erythrocyte fatty acid profiles^(21;23;24). Plasma and erythrocyte EFA and LCPUFA concentrations strongly decreased, and levels of non-essential fatty acids such as mead acid (C20:3n-9) and oleic acid concomitantly increased. The cessation of growth that we observed in EFA-deficient mice is likely related to impaired dietary fat absorption during EFA deficiency, which had been described previously^(22;25;26).

Superimposing extrahepatic cholestasis on EFA deficiency in mice subsequently resulted in weight loss. Bile-diverted rats compensate for bile deficiency-induced fat malabsorption by increasing their chow ingestion⁽²⁷⁾, in contrast to rats with bile duct ligation⁽¹⁾. In our bile duct-ligated EFA-deficient mice, chow intake similarly remained constant, suggesting that two causes for fat malabsorption are present in our mouse model; bile deficiency and EFA deficiency, the combination of which presumably induced weight loss. Bile duct ligation profoundly elevated plasma bile salt concentrations, which were already slightly elevated by prior EFAD diet feeding. Liver histology revealed extensive bile duct proliferation and parenchymal damage in cholestatic mice, accompanied by jaundice within days after bile duct closure. The rapid onset of these biochemical and morphological parameters of EFA deficiency and cholestasis in our mice provided us with an effective model for studying EFA deficiency under acute cholestatic conditions.

In cholestatic EFAD mice, oral administration of EFA-rich PL completely restored DHA and AA levels in the target organs brain and liver, in contrast to EFA-rich TG which did not improve these parameters relative to continuation of the EFAD diet. It is well-known that the high levels of DHA and AA in the excitable retinal and synaptosomal membranes of the central nervous system are crucial for adequate membrane reactivity and function of receptor proteins⁽²⁸⁻³⁰⁾. The molecular mechanism underlying the high concentrations of DHA and AA in the brain is unknown. Scott and Bazan⁽³¹⁾ demonstrated that the majority of brain DHA originates from hepatic elongation and desaturation of n-3 fatty acids and subsequent redistribution to the brain. Additionally, astrocytes and brain endothelial cells are capable of synthesizing LCPUFA from EFA, which may constitute a minor but still relevant source of DHA and AA for the central nervous system⁽³²⁻³⁵⁾. The plasma compartment appears to be the main source of brain DHA and AA. LCPUFA are partly present in plasma as lip-

protein components, but Purdon and Rapoport demonstrated in rats that LCPUFA esterified within circulating lipoproteins do not enter the brain to a measurable extent, and that only the unesterified form is incorporated^(36;37). Unesterified LCPUFA bound to albumin are a well recognized plasma source of PUFA for the brain⁽³⁸⁾, but concentrations of particularly n-3 fatty acids are low, and it is questionable whether the free fatty acid compartment accounts for the majority of LCPUFA supply to the brain⁽³⁹⁾. LCPUFA are also present in plasma as lyso-PL, bound to albumin⁽⁴⁰⁾. Thies and Bernoud^(41;42) reported data suggesting that the brain preferentially absorbs DHA as sn-2 lyso-PL compared to unesterified DHA. Since dietary PL are partly absorbed as lyso-PL and partly as intact PL molecules, the highly efficient increase in brain LCPUFA concentration after PL supplementation in our study could be in line with these observations, supporting the presumed high bioavailability of dietary PL^(12;13;15;16). Surprisingly, PL-supplementation seemed to increase brain DHA to an even slightly higher level than in non-cholestatic mice fed the EFAS diet. However, since both EFAD and EFAS diets contain very low amounts of ALA, prolonged EFAS diet feeding may result in marginal DHA levels. The TG- and PL-enriched diets equally provided supplementary ALA, which has the capacity to increase DHA levels, even compared to EFAS mice. However, comparison between the TG- and the PL-fed groups indicated that only PL supplementation increased brain DHA.

We did not find indications that EFA deficiency and cholestasis affected activities of hepatic desaturation and elongation enzymes required for AA and DHA synthesis from their respective precursors. Ratios between C20:3n-6 and C20:4n-6, estimating delta-5 desaturase activity, and between C22:5n-3 and C22:6n-3 marking delta-6 desaturase activity, were similar between supplemented and non-supplemented mice, nor between cholestatic and non-cholestatic EFAD mice. Present observations are in agreement with previous results in EFAS bile duct- ligated rats, which showed no indications for altered post-absorptive EFA metabolism during cholestasis⁽¹⁾. Oral PL more efficiently improved LCPUFA concentrations in brain and in liver than oral TG. Interestingly, PL and TG reversed parameters of EFA deficiency in plasma and erythrocytes with equal efficacy. Demarne *et al.* and Minich *et al.* demonstrated in cholestatic rats that absorption of unsaturated fatty acids, and of EFA in particular, is relatively preserved compared to that of saturated species^(1;11). Quantitative absorption studies would be required to fully exclude differences in net enteral uptake of TG-EFA and PL-EFA. In addition, the products of TG and PL lipolysis (2-MG and 1-lyso PL) follow qualitatively different routes within the enterocyte, possibly resulting in different post-absorptive metabolic pathways of the attached EFA.

Although we could not demonstrate differential effects of the TG and PL supplements in the plasma PL fraction, fatty acid profiles of plasma PL closely corresponded with fatty acid profiles as measured in liver PL. If PL on the surface of chylomicron particles were specifically targeted for EFA delivery to the brain, then plasma PL fatty acid analysis is likely to miss differential effects of oral PL over TG if blood samples are not taken during or immediately after fat absorption, due to the rapidity of chylomicron clearance⁽⁴³⁾.

Our present data are in contradiction with the paradigm that the erythrocyte membrane fatty acid composition is a tentative index for overall body EFA status. Rather, our data are in line with the recent questioning by several authors of the validity of extrapolating erythrocyte membrane fatty acid profiles to their status in target organs for EFA. Korotkova and Strandvik⁽⁴⁴⁾ demonstrated that EFA deficiency in rats differentially affects fatty acid profiles of erythrocyte-, serum-, jejunum-, ileum- and colon-PL. Rioux and Innis⁽⁴⁵⁾ reported that in piglets, plasma PL are good indicators for liver and bile PL-fatty acids, but not for brain fatty acid composition. Our results in mice support these observations, since, indeed, fatty acid profiles of the isolated plasma PL fraction closely corresponded with those of liver PL but not of brain PL. Data suggest that specific channeling of PUFA to target organs as the central nervous system occurs, at the expense of less critical tissues such as erythrocytes.

Supplementation of EFA as PL not only improved biochemical parameters of EFA deficiency in cholestatic mice, oral PL also prevented weight loss during EFA deficiency and cholestasis, whereas oral TG did not. Nishioka *et al.*⁽⁷⁾ recently demonstrated that enteral infusion of PL-cholesterol liposomes partially corrects lipid malabsorption in bile-diverted rats, compatible with a facilitating effect of enteral PL supplementation on fat uptake during bile deficiency. Although no information on fat balance is available from the present studies, it is tempting to speculate that EFA-rich PL supplementation partially corrects fat malabsorption in EFAD cholestatic mice.

A surprising observation was the remarkably stable AA concentration in erythrocytes of cholestatic EFAD mice, when compared to non-cholestatic EFAD mice. While in the latter AA levels decreased after starting the EFAD diet, bile duct ligation *per se* appeared to maintain AA concentrations at a normal level. Tso *et al.*⁽⁵⁾ reported that human bile PL provide up to 1.7 g AA to the intestine per day. An average Western adult diet supplies 1.8 g AA daily⁽⁴⁶⁾, indicating that biliary PL secretion into the intestine provides a significant portion of enteral AA. Our results support the concept that biliary secretion of AA in the form of PL quantitatively affects overall body AA homeostasis.

EFA deficiency in pediatric cholestatic patients has been proven difficult to correct by merely increasing EFA ingestion. It should be realized that in patients, in contrast to the presented mouse model, cholestasis usually develops gradually, and EFA deficiency is not due to lack of dietary EFA content. Yet, our present experiments suggest that oral administration of EFA-rich PL might be an effective treatment strategy for reversing EFA deficiency in patients with cholestatic liver disease. The efficacy of oral PL supplementation for treatment of EFA deficiency in pediatric patients with cholestasis is currently under investigation.

Acknowledgements

The authors would like to thank H. Helmink and W. de Groot from Unimills Zwijndrecht (the Netherlands) for their generous gift of the soybean TG and PL oils, and Ingrid Martini, Juul Baller and Renze Boverhof for their technical expertise and assistance in the experiments described in this article.

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