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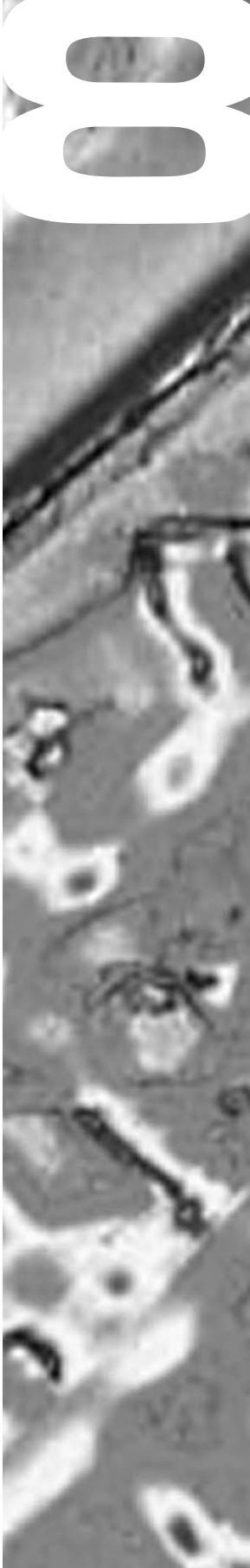
General disussion and summary



SUMMARY

This thesis focuses on the role of phospholipids (PL) in absorption and metabolism of essential fatty acids (EFA), under physiological and bile-deficient conditions. EFA cannot be synthesized *de novo* by the body, but are crucial for normal function and development, either as such or after metabolization into long-chain polyunsaturated fatty acids (LCPUFA). EFA deficiency is associated with dietary fat malabsorption, growth retardation, steatosis and impaired neurological development, although symptoms can be rather aspecific and only become apparent after an extended subclinical course. Children have limited adipose tissue stores of EFA and high EFA requirements during growth and development. These characteristics make children particularly dependent on adequate supply and absorption of EFA via the diet. Dietary EFA are predominantly esterified into triglycerides (TG), which are profoundly malabsorbed during impaired enteral lipolysis or solubilization, as in cystic fibrosis or cholestasis, respectively. EFA esterified into PL are more easily absorbed under these conditions, since the polar PL molecules do not require bile for solu-bilization in an aqueous environment, and can partially be absorbed intact, without (phopspho-)lipolysis[6; 22]. Additionally, PL have a high post-absorptive availability for the body[8; 10; 11].

We aimed to characterize the impact of EFA deficiency on intestinal and liver function and to develop a dietary treatment strategy for prevention or correction of EFA deficiency in susceptible conditions, like cholestasis and cystic fibrosis.

Fat malabsorption in EFAD mice is not due to impaired bile formation

In chapter 2, we investigated whether EFA deficiency-induced fat malabsorption in mice is mediated by impaired bile production. Bile salts and bile PL facilitate dietary fat absorption by enabling intraluminal solubilization and by providing surface coat material for CM formation, respectively. In rats, EFA deficiency does not affect lipolysis, but decreases bile secretion[17; 20]. In mice, however, we demonstrated that bile production was profoundly increased during EFA deficiency, and bile salt composition was unchanged, excluding impaired bile production as a cause for EFA deficiency-induced fat malabsorption. To specify the role of biliary PL secretion in fat malabsorption, we compared the effects of EFA deficiency in normal and in genetically modified mice that secrete PL-free bile (*Mdr2* $^{-/-}$ mice). If alterations in secreted bile PL were involved in EFA deficiency-associated fat malabsorption, fat absorption in *Mdr2* $^{-/-}$ mice should be unaffected by EFA depletion. However, fat absorption and

bile flow were equally affected by EFA deficiency in *Mdr2* $^{-/-}$ and wildtype mice.
Our data indicate that fat malabsorption during EFA deficiency in mice is not due to decreased bile production but, by inference, is more likely related to impaired intracellular processing of dietary fat in enterocytes, possibly due to EFA depletion of plasma- and/or microsomal membranes.

EFA deficiency in mice is associated with steatosis and secretion of large VLDL

In addition to fat malabsorption and increased bile secretion, EFA deficiency in mice is associated with hypotriglyceridemia and hepatic steatosis. In chapter 3 we evaluated whether impaired hepatic VLDL secretion contributes to these metabolic consequences of EFA deficiency in mice. Both *in vivo* and *in vitro*, EFA deficiency increased hepatic TG levels, but hepatic VLDL-TG secretion rate was quantitatively normal. Interestingly, EFA deficiency induced hepatic production of remarkably large VLDL particles compared to the non-deficient condition.
EFA are suppressors of lipogenesis via down-regulation of the transcription factor SREBP1c, therefore, hepatic synthesis of non-EFA can increase during EFA deficiency. The observed hepatic accumulation of TG in EFA-deficient mice may additionally be related to increased VLDL clearance. Indeed, hepatic expression of apoAV and apoCII, genes involved in VLDL catabolism, was increased. Although HL and LPL activities were normal in EFA-deficient mice, large VLDL particles may be subject to increased clearance rates. As EFA depletion affects the physicochemical and biological characteristics of PL bilayer membranes, it may similarly influence PL monolayers on the surface of nascent lipoproteins. Both lipoprotein size, i.e., curvature of the lipoprotein surface, and low EFA content can disturb the physical VLDL surface structure, increasing accessibility to lipases or affinity for apoC-II or apoA-V. Steatosis and hypotriglyceridemia in EFA-deficient mice could be a combined result of increased hepatic lipogenesis, unimpaired hepatic VLDL-TG secretion and production of large VLDL particles that may be subject to rapid clearance. We hypothesize that the effects of EFA deficiency on VLDL particle size are related to PL availability for lipoprotein assembly. Increased biliary PL secretion in EFA-deficient mice (chapter 2) may limit hepatic PL availability for VLDL assembly, inducing secretion of large lipoprotein particles.

Lymphatic CM size is inversely related to biliary PL secretion in mice

We tested the hypothesis that PL availability determines lipoprotein size in chapter 4, by investigating whether the size of intestinal lipoproteins is also inversely related to

PL availability. Since biliary PL secretion is absent in *Mdr2* $^{-/-}$ mice and strongly increased in EFA-deficient mice, we determined lymphatic chylomicron (CM) size after mesenteric lymph duct cannulation in these two mouse models. CM were considerably larger during intestinal lack of biliary PL (*Mdr2* $^{-/-}$ mice), whereas hyper-secretion of bile PL into the intestine (EFAD-deficient mice) induced secretion of smaller CM into lymph. These observations confirmed our hypothesis that PL availability is a major determinant of lipoprotein size. Since EFA-deficient mice secrete larger hepatic VLDL than EFA-sufficient controls, secretion of small lipo-proteins is apparently not an intrinsic feature but rather an organ-specific feature of EFA deficiency. Similar to the metabolism of VLDL particles, altered CM size and fatty acid composition affect intravascular processing[13]. Large CM may enter the lymph slower, but are likely to be subsequently metabolized with high efficacy, since large CM have a greater affinity for lipases and are cleared more rapidly than small parti-cles. In addition, EFA-rich CM are cleared faster than EFA-depleted CM. This could partially explain the observation that, although post-prandial plasma appearance of ingested lipid is delayed both in EFA-deficient and bile-PL-deficient (*Mdr2* $^{-/-}$) mice, only EFA-deficient mice have net dietary fat malabsorption. This supports the concept that intraluminal PL are not crucially important for quantitative intestinal absorption of dietary lipids, but all the more for their absorption kinetics and post-absorptive metabolism.

No indications for altered EFA metabolism in two murine models for CF

Bijvelds *et al*.[5] demonstrated in mouse models for cystic fibrosis (CF) that *cftr* $^{-/-CAM}$ mice have a profound dietary fat malabsorption, which could result in EFA deficiency. Indeed, deficiencies of EFA or LCPUFA are still reported in CF patients, despite hypercaloric nutrition and pancreas enzyme replacement therapy. It has been suggested that CFTR malfunction directly affects LCPUFA synthesis, increasing pro-inflammatory n-6 and decreasing anti-inflammatory n-3 LCPUFA levels in CF-affected organs, which would contribute to CF symptoms. Supplementation with n-3 LCPUFA, but not with the precursor ALA, was postulated to alleviate phenotypic manifestations of the disease.

To determine whether the CF condition merits specific supplementation of EFA and/or LCPUFA, we analyzed EFA status in two mouse models for CF, with and without fat malabsorption. In both CF models, we demonstrated that organ LCPUFA profiles were highly similar to those of healthy sex-matched littermates. *In vivo* conversion of ^{13}C-EFA into AA and DHA was unimpaired in CF mice compared to littermate controls, indicating that impaired LCPUFA synthesis is not an intrinsic

feature of CF phenotype, and that fat malabsorption does not strongly affect EFA status in CF mice.

Many CF mouse models are available, which display great phenotypic variability, similar to the situation in CF patients. Apart from the specific CFTR mutation and environmental influences, phenotypic variability in CF is related to independently inherited disease-modifying genes, encoding proteins that may partially compensate for effects of CFTR dysfunction. We demonstrated that diet and age, but primarily genetic background is an overriding determinant of EFA status in CF mice. In the studies suggesting that LCPUFA synthesis is controlled by CFTR, thus affecting CF pathology, the CF mice were compared to control mice of a different mouse strain. For any meaningful comparison of EFA status between CF mouse models, and particularly for inferring observations to CF patients, meticulous verification of mouse genetic backgrounds and use of littermate controls are a prerequisite.

We conclude that CFTR dysfunction does not impair LCPUFA synthesis, and altered PUFA levels in CF are more likely secondary to inflammation or malnutrition. Extrapolating these conclusions to CF patients would implicate that sufficient oral EFA intake should effectively prevent EFA or LCPUFA deficiency in CF. Since fat malabsorption did not strongly affect EFA status in CF mice, specific EFA supplementation does not seem required in this condition, and we further concentrated on developing EFA supplementation strategies for patients with cholestatic liver disease.

Oral treatment of EFA deficiency with TG or PL in cholestatic conditions

EFA deficiency is common during cholestasis, due to malabsorption of dietary EFA which are predominantly esterified into hydrophobic TG molecules. The amphiphilic PL are more soluble than TG in the aqueous intestinal lumen and more readily absorbed during bile deficiency. PL are absorbed intact or after digestion to lyso-PL. In chapter 5, we demonstrated in EFA-deficient mice with acute cholestasis that EFA supplementation with oral TG or PL was equally effective in preventing decrease of EFA concentrations in RBC, yet in brain and liver, PL were highly superior to TG and significantly improved EFA-derived LCPUFA levels. In addition, oral PL prevented weight loss during EFA deficiency and cholestasis, in contrast to oral TG, supporting the concept that enteral PL have a facilitating effect on lipid absorption during bile deficiency.

In chapter 6, we compared the efficacy of oral EFA supplementation as PL and TG for treatment of EFA deficiency in children with chronic cholestasis, who have a high incidence of compromised EFA status[16]. Three-month supplementation with EFA as TG or PL prevented the deterioration in RBC LA, mead acid and total n-6 fatty acids as observed in non-supplemented children. Although we could not directly demon-

strate differential effects of TG or PL supplementation on RBC EFA upon group-wise comparisons, oral PL clearly induced a positive monthly increase in RBC LA compared to non-supplemented children, whereas oral TG did not.
Interestingly, in cholestatic mice, the differential effects of TG and PL EFA supplementation during cholestasis were not evident in RBC, but the beneficial effects of oral PL were highly evident in EFA target organs liver and brain.

In line with Korotkova and Rioux, we question the paradigm that RBC fatty acid profiles are a tentative index for overall body EFA status. Due to their long half-life and short-term independence of post-prandial plasma fatty acid levels, RBC may be a stable and easily accessible compartment for evaluation of body EFA status. Yet it seems that specific channeling of EFA occurs to target organs (brain, liver, intestine), at the expense of less critical tissues as RBC. Apart from international consensus on validated cut-off values defining biochemical EFA-deficiency, there would be great merit in developing sensitive functional tests to assess deficiency, sufficiency and requirements of EFA and LCPUFA in patients and animal models[7; 21].

The mechanism underlying the remarkably efficient CNS uptake of LCPUFA is not fully understood. To a certain extent, body stores may provide EFA for the brain[9], and although astrocytes are capable of synthesizing AA and DHA from EFA, the plasma compartment is the main source of brain LCPUFA. In plasma, LCPUFA are present esterified in lipoproteins, unesterified bound to albumin, or as lyso-PL. The latter are preferentially incorporated by the brain[4; 18; 19]. Dietary PL are absorbed from the intestine intact and as lyso-PL. In addition, oral PL could be a source of plasma (lyso-)PL as components of HDL-particles, derived from excess CM surface material shed during lipolysis. Possibly, PL on the CM surface are preferentially targeted to HDL before exchanging with other lipoproteins and RBC[6; 15; 22]. Either as albumin-bound lyso-PL or as HDL-PL, oral PL provide a highly accessible source of LCPUFA for the brain, supporting the concept of high post-absorptive bioavailability of enteral PL.
Obviously, human brain and liver are ethically not accessible for analysis of EFA composition. Yet in light of the beneficial effects of PL supplementation on brain and liver in mice, and the slightly better effects of supplementory PL compared to TG on RBC EFA in cholestatic children, EFA supplementation in the form of PL may still be preferred compared with TG for prevention or correction of EFA deficiency in children with end-stage liver disease.

CONCLUSIONS

Before the present studies were performed, it was apparent from existing literature that EFA deficiency has important consequences for fat absorption and metabolism. Evidence has been provided that EFA deficiency:
- decreases bile formation in rats;
- does not affect intraluminal lipolysis or enterocyte uptake of dietary fat;
- decreases LA and AA levels in intestinal mucosal membranes;
- leads to accumulation of fat in enterocytes after oral administration
- influences post-absorptive metabolism, i.e., alters plasma lipid profiles, lipoprotein composition and lipolytic enzymes.

Still, the specific role of EFA deficiency in various steps of fat absorption was poorly understood. Present results indicate that lipid malabsorption during EFA deficiency is not due to decreased bile formation nor to altered EFA contents of biliary PL. Considering the effects of EFA deficiency on lipoprotein formation, we hypothesize that EFA deficiency-induced fat malabsorption may be due to EFA depletion of PL membranes, which affects lipoprotein processing in intestine and in liver. In both organs, the exit step of lipid transport appears to be changed during EFA deficiency; in both, lipoprotein size is altered and fat accumulation occurs[3]. PL synthesis and membrane incorporation proceeds during deficiency of EFA, as EFA acyl chains are substituted with available n-9, n-7 or saturated fatty acids. The mechanisms that determine which fatty acid species are incorporated into PL, or which type of PL is inserted into lipoprotein monolayers or cell membrane bilayers, are not fully known. There are strong indications that the chemical structure of dietary fat, TG or PL, is relatively maintained after intestinal digestion and absorption[1]. This observation, in line with our results on specific targeting of EFA from PL to liver and brain, suggests that separate intracellular fatty acid pools exist. It will be challenging to elucidate the mechanisms underlying the fatty acid or PL sorting towards different compartments in enterocytes or hepatocytes, and the subsequent targeting to specific organs. Endothelial lipase activity at the blood-brain-barrier has been suggested to play a role in the specific EFA uptake by the central nervous system, as well as SR-B1-mediated uptake of HDL-PL EFA by brain capillary endothelial cells[2;12;14]. In cholestatic mice, we clearly demonstrated the preferential capture by the brain of dietary EFA in the form of PL compared to TG, supporting this form of supplementation for children with chronic liver disease. In future studies, it would be of great physiological interest and probably of therapeutic value to elucidate the mechanisms underlying the specific EFA/LCPUFA accretion by the brain, and the targeting of dietary EFA-PL towards brain and liver.

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