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Essential fatty acid deficiency and the small intestine

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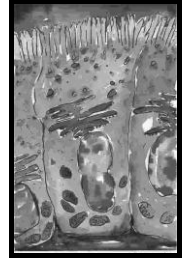
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CHAPTER 7



SUMMARY AND FUTURE PERSPECTIVES

S. Lukovac

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SUMMARY

The experiments described in this thesis aimed to characterize and unravel the effects of essential fatty acid (EFA) deficiency on the function of the small intestine. EFA deficiency is common in pediatric patients with cholestasis (characterized by decreased or absent hepatic secretion of bile into the intestine), where it is associated with fat malabsorption and severely impaired nutritional status. Often, EFA deficiency aggravates the cholestasis induced failure to thrive (CIFTT) in pediatric patients. In our laboratory, a mouse model for EFA deficiency has been developed. Previous studies in this model focused on the absorption and metabolism of EFA in hepatic disorders.^{1,2} In this mouse model, as well as in other species, it has become apparent that EFA deficiency likely affects the small intestine.^{1,3,4,5} However, detailed information about the effects of EFA deficiency on the small intestinal morphology and function had remained scarce. In order to improve the nutritional status of pediatric patients encountering EFA deficiency, improvement of the intestinal function is essential. Therefore, we studied in detail several effects of EFA deficiency on the small intestinal function in the mouse model for EFA deficiency. We analyzed the effects of EFA deficiency on the absorption and digestion of several nutrients and on the enterohepatic circulation of bile salts by means of a stable isotope dilution technique. We determined the effects of EFA deficiency on the intestinal morphology *in vivo* and *in vitro*. Finally, we performed experiments *in vitro* to determine the intracellular effects of EFA deficiency and to analyze whether some of the symptoms of EFA deficiency can be reversed. Increased knowledge on the role of the small intestine in EFA deficiency could lead to a development of improved nutritional therapies in pediatric patients with CIFTT awaiting liver transplantation.

Essential fatty acid deficiency in mice impairs lactose digestion and alters jejunal cholesterol metabolism

Previous studies in rat and mouse models of EFA deficiency revealed that EFA deficiency by itself leads to fat malabsorption, even in absence of cholestasis. However, the effects of EFA deficiency on digestion and absorption of other (dietary) compounds remained unclear. Theoretically, if EFA deficiency is associated with overall impaired small intestinal function, not only the absorption of fat but also that of other nutrients, like carbohydrates and cholesterol, could be expected to be decreased during EFA deficiency. A general absorptive defect would have consequences for the nutritional treatment of these conditions.

First, we assessed the capacity of EFA-deficient mice to digest and absorb carbohydrates, using stable isotope methodology and administration of the disaccharide [$1\text{-}^{13}\text{C}$]lactose and the monosaccharide [$\text{U-}^{13}\text{C}$]glucose (**chapter 2**). While the absorption of the monosaccharide glucose was unaffected in EFA-deficient mice, digestion of the disaccharide lactose was significantly delayed. The functional observation corresponded with severely reduced mRNA expression and enzyme activity of the hydrolyzing enzyme of lactose, lactase, in the small intestine of EFA-deficient mice. These data underscored the observation that EFA deficiency functionally impairs the small intestine. Biochemical analysis suggested that the digestive function correlated with the linoleic acid (LA) concentration in the phospholipids of the enterocytes; upon EFA deficiency, the LA

concentration decreased, simultaneously with a decrease in the lactase expression and activity. Whether digestion of lactose is impaired in pediatric cholestatic patients, with or without EFA deficiency, is not known. Studies in (non-EFA deficient) bile duct ligated rats (cholestatic rat model) revealed no significant differences in absorption of glucose or sucrose between control and bile duct ligated rats.⁶ However, lactose digestion was not studied in these rats, which limits the extrapolation of these results to our mouse model for EFA deficiency. Future studies should focus on determining whether EFA deficiency is associated with specifically impaired lactose digestion or with a more general defect in disaccharide digestion and absorption. In addition, studies should be performed to determine whether it would be beneficial to increase the dietary intake of monosaccharides compared to that of disaccharides, in order to improve the nutritional status of patients with CIFTT.

Cholesterol is quantitatively and metabolically an important lipid that enters the intestine via the diet and via biliary secretion. We studied cholesterol absorption and metabolism in jejunal intestinal segments in a mouse model of EFA deficiency (**chapter 3**). The fecal cholesterol excretion was 57% higher in EFA-deficient mice compared with control mice, indicating reduced cholesterol absorption. In accordance with reduced cholesterol absorption, the marker for cholesterol absorption (plasma plant sterols/cholesterol ratio) was significantly decreased in EFA-deficient mice. Niemen-Pick C1-like 1 protein (NPC1L1) is the critical player in the absorption of intestinal sterols expressed at the apical surface of enterocytes. *Npc1l1* mRNA expression was decreased in jejunum of EFA-deficient mice.⁷ EFA deficiency had no effect on total cholesterol concentrations in jejunal mucosa, what could be due to a compensatory increase in jejunal cholesterol synthesis. Additional analysis of triglyceride and fatty acid metabolism revealed elevated jejunal triglyceride content, accompanied by increased concentrations of oleic acid. Interestingly, microarray analysis revealed that the mRNA expression of all the genes involved in cholesterol synthesis was increased in jejunum EFA-deficient mice. Cholesterol and fatty acid metabolism are mainly regulated by the sterol regulatory element binding proteins, SREBP2 and SREBP1C, respectively.⁸ When cholesterol concentration in the cell decreases, SREBP2 is cleaved by proteases and transported to the nucleus where it binds to the DNA to increase the mRNA expression of genes involved in cholesterol synthesis. SREBP2 can also be activated by increased oleic acid concentration. In jejunum of EFA-deficient mice *Srebp2* mRNA expression was significantly increased, resulting in increased mRNA expression of its target genes involved in cholesterol metabolism. *Srebp1c* mRNA expression was also increased in jejunum of EFA-deficient mice, leading to the increased induction of mRNA expression of *Srebp1c* target genes involved in fatty acid metabolism.

Transcriptional analysis further revealed that the pathway involved in the inhibition of the proteasome was the most significantly affected cellular process by the EFA deficiency in jejunum. Reduced proteasome activation leads to prolonged expression and activity of several cellular proteins. Recently, the activity of several transcription factors, proteins and enzymes involved in cholesterol synthesis (SREBP, ABCA1, HMGCR) has been shown to be regulated by the ubiquitin-proteasome pathway in a sterol-dependent manner.^{9,10} In addition, preliminary data of Hamel imply fatty acids, mainly oleic acid, as possible regulators of the proteasome pathway.¹¹ Thus, on one hand increased oleic

acid can induce jejunal cholesterol synthesis in EFA-deficient mice and on the other hand, increased oleic acid inhibits the proteasome pathway leading to enhanced expression of genes involved in cholesterol synthesis. In summary, our data show that EFA deficiency in mice is associated with cholesterol malabsorption, and with increased cholesterol biosynthesis. This increase in cholesterol synthesis during EFA deficiency is not specific for jejunal tissue, as it occurs in epidermal tissue as well.¹² Furthermore, we demonstrated that the mRNA expression of HMGCR, rate limiting enzyme of cholesterol synthesis, is increased in livers of EFA-deficient mice (**chapter 3**). We speculate that modifications in the jejunal proteasome regulation pathway, which prolong the expression of relevant proteins of lipid metabolism, may be a compensatory mechanism for the malabsorption of lipids. It is of interest to study whether cholesterol malabsorption during EFA deficiency in mice leads to reduced membrane cholesterol concentrations and structural changes within the enterocyte membrane. If this would be the case, it would be worthwhile to determine to what extent the (postulated compensatory) increase in cholesterol synthesis in jejunum is capable to correct for reduced membrane cholesterol content. In order to improve the intestinal function during EFA deficiency, further studies on cholesterol content in the membranes of the enterocytes in the intestinal epithelium are relevant.

Essential fatty acid deficiency in mice leads to enhanced ileal bile salt reabsorption and to persistent hepatic bile salt synthesis in mice

Our previous studies revealed impaired small intestinal function during EFA deficiency, mainly located at the level of the mid small intestine, i.e. corresponding to the jejunum (**chapter 2 and 3**). In order to determine whether EFA deficiency affects other parts of the small intestine, we studied bile salt reabsorption, what mainly occurs in the terminal ileum, i.e. the last part of the small intestine (**chapter 4**). It has been shown by Werner et al. that EFA-deficient mice have increased bile production and enhanced biliary secretion of bile salts.¹ Using a stable isotope methodology, we characterized relevant kinetic and quantitative parameters of the enterohepatic circulation of bile salts in EFA-deficient mice, without interrupting the normal enterohepatic circulation. EFA deficiency-enhanced bile flow and biliary bile salt secretion were associated with increased ileal bile salt reabsorption and unexpectedly elevated bile salt synthesis rate. The persistent hepatic bile salt synthesis was most likely to be explained by the reduction in ileal mRNA expression of *Fgf15* (inhibitor of bile salt synthesis¹³). To confirm our *in vivo* findings, we additionally measured expression of relevant intestinal genes in the enterohepatic circulation of bile salt synthesis in EFA-deficient (post-confluent) Caco-2 cells. The cells were stimulated with chenodeoxycholic acid and GW4064 compound, both potent agonists of farnesoid X receptor (FXR), relevant regulator of the bile salt synthesis. Stimulation of EFA-deficient Caco-2 cells resulted in lower induction of the mRNA expression of relevant genes (FGF19, IBABP) compared with control Caco-2 cells. Together, these data clearly show that besides the effects on the jejunum, EFA deficiency affects the terminal ileum function, with respect to the enterohepatic circulation of bile salts. Interestingly, EFA deficient mice have fat malabsorption, despite the observation that mice with EFA deficiency have an increased biliary bile salt secretion, and thus more specifically increased intestinal availability to bile salts. EFA

deficiency has some species specific phenotype: in rats, bile salt secretion is decreased during EFA deficiency, coinciding with a more prominent fat malabsorption, compared with mice. It seems thus that mice, at least in part, compensate for severe fat malabsorption by increasing the bile salt concentrations in the intestine. It remains to be elucidated whether these high bile salt concentrations in the intestine of EFA-deficient mice are toxic to intestinal tissue in long terms, leading to more functional problems.

In vitro model of essential fatty acid deficiency reveals increased permeability and impaired mRNA expression of brush border enterocyte markers, which are not rapidly reversible by linoleic acid (LA) supplementation

In order to study the intracellular effects of EFA deficiency, we established an *in vitro* model of EFA deficiency in post-confluent Caco-2 cells (**chapter 5**). Upon confluence, these cells differentiate towards the small intestinal phenotype, as indicated by dome formation, microvilli, and expression of brush-border enzymes.¹⁴

Caco-2 cells were cultured in medium containing normal or delipidated FCS (control and EFA-deficient cells, respectively) for one week post-confluent. From previous studies this condition has been shown to be sufficient for reproducible induction of the small intestinal phenotype and of EFA deficiency.^{14,15} To study EFA deficiency in an *in vitro* model, with a phenotype similar to *in vivo* situation, we have reproduced and further characterized an *in vitro* model originally described by Spalinger et al.^{15,16,17} EFA deficiency severely reduced the expression of relevant brush border markers of the small intestine, and impaired the cellular permeability, as demonstrated by increased transepithelial electrical resistance (TER). These effects were not rapidly reversible by LA supplementation to EFA-deficient Caco-2 cells. Theoretically, persistent effects on permeability and mRNA expression of lactase and sucrase isomaltase after LA supplementation might be caused by absent capability of EFA-deficient Caco-2 cells to incorporate the supplemented LA into the cellular phospholipids. However, we demonstrated that EFA-deficient Caco-2 cells were capable of LA incorporation into phospholipids to similar extent as control Caco-2 cells during 6 days of LA supplementation. Despite similar LA concentrations in the phospholipids of EFA-deficient and control cells, EFA-deficient cells retained increased TER values and severely reduced mRNA expression of the brush border enzymes. Another possibility is that EFA deficiency leads to reduced differentiation of the small intestinal enterocytes, as demonstrated by the decreased expression of the enterocytic markers. Reduced differentiation might, at least in part, be caused by impaired transcriptional regulation of the mRNA expression of certain enterocytic markers and could be difficult to reverse by LA supplementation. Prolonged treatment with (higher concentration) of LA might restore the enterocyte function during EFA deficiency. Furthermore, additional supplementation with α -linolenic acid (C18:3 ω -3, ALA) along with LA might be useful in order to reverse the effects of EFA deficiency. Theoretically, increased permeability would be expected to influence the transmucosal transport of dietary compounds. Yet, EFA deficiency in mice is associated with reduced nutrient absorption. Studies in EFA-deficient pigs revealed decreased lipid fluidity within the brush border membrane of the enterocytes.⁵ Further *in vivo* studies in mouse model of EFA deficiency are warranted to determine whether increased permeability indeed is a common feature of EFA deficiency *in vivo*.

Nevertheless, we expect that this *in vitro* model will allow performing more detailed studies on the underlying (molecular) mechanism(s) of the EFA-deficient phenotype in the small intestinal enterocyte.

Gelucire®44/14 improves fat absorption in rats with impaired lipolysis

EFA deficiency, by itself or in combination with CIFTT or cystic fibrosis, is associated with severe fat malabsorption. Apart from improving the small intestinal function (in)directly, the supplementation with exogenous compounds might enhance the absorption of dietary fat. Gelucire®44/14 is a semi-solid self-micro-emulsifying excipient, frequently used as an absorption enhancer of water insoluble drugs.¹⁸ In **chapter 6** we show that Gelucire®44/14 can enhance fat absorption under conditions of impaired lipolysis, but not during impaired solubilization in rats. Possibly, Gelucire®44/14 stabilizes and improves residual lipolytic enzyme activity *in vivo*. This could be of therapeutic value in clinical conditions of fat malabsorption due to impaired lipolysis, but probably not in case of EFA deficiency, since EFA-deficient mice seem to have normal lipolysis. However, Levy et al. showed in rats impaired intraluminal steps of fat absorption.³ If lipolysis indeed would be reduced in (pediatric) patients with EFA deficiency, Gelucire®44/14 might help reduce the severe fat malabsorption. We reasoned, however, that experiments with Gelucire®44/14 supplementation to mouse model of cystic fibrosis could be helpful in this respect, before patient studies would be designed and planned.

OVERALL CONCLUSION AND IMPLICATIONS

Our studies on the effects of EFA deficiency on the small intestine in mice and in immortalized *in vitro* model of EFA deficiency clearly show that EFA deficiency leads to a variety of functional changes in the small intestine. More specifically, lipid malabsorption and disaccharide digestion are impaired during EFA deficiency in mice. Increased intestinal reabsorption of bile salts is insufficient to normalize the decreased lipid absorption, underscoring previous implications in mice that intracellular rather than intraluminal steps of fat absorption are impaired during EFA deficiency in mice. The (isolated) supplementation of LA does not seem to reverse the effects of EFA deficiency on the small intestinal enterocytes as revealed by our *in vitro* study. Maintenance and/or improvement of the nutritional status of cholestatic patients with EFA deficiency is relevant since the number of patients on the waiting list for liver transplantation increases. In order to improve the nutritional strategies of CIFTT patients with EFA deficiency, further studies on the effects of EFA deficiency on the intestinal function are warranted as the follow up to our presently obtained results in mice. Stable isotope dilution techniques represent an elegant methodology that is applicable in pediatric patients to assess the absorption of several nutrients.¹⁹ Patients studies using stable isotope-labeled macronutrients, i.e. lipids, carbohydrates and proteins, will further assess nutritional status of children with CIFTT. We expect that these mechanistic nutritional studies will help to develop and rationalize nutritional therapies for pediatric patients with impaired digestion or absorption, including patients with EFA deficiency.

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