INTRODUCTION TO THE THESIS

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CHAPTER 1

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INTRODUCTION

Essential fatty acids (EFA) cannot be synthesized de novo by humans or animals and thus can only be obtained by means of dietary intake. EFA are involved in many biological processes; they are essential for normal neurodevelopment and regulation of membrane function in several tissues like the brain, retina, liver, kidney, adrenal glands and gonads.\(^1\) In addition, metabolites of EFA are precursors of eicosanoids, which strongly modulate processes like platelet aggregation and chemotaxis of the immune system.\(^2\) Accordingly, a shortage of EFA, also known as EFA deficiency, leads to various clinical consequences, such as impaired cognitive and motor development, reduced growth rate, dry skin, hair loss and functional changes in organs like heart and liver.\(^2\) EFA deficiency is a condition that can develop due to insufficient dietary intake or absorption, or due to enhanced metabolism of EFA. This condition is described in detail in one of the paragraphs of this chapter. Pediatric patients with cholestatic liver disease often encounter EFA deficiency, which is one of the determining factors for failure to thrive in these patients. In order to improve the nutritional status of patients with Cholestasis-induced failure to thrive (CIFTT), maintenance of intestinal absorptive capacity is essential.

Previous studies on EFA deficiency mainly focused on its effects on the liver, brain and heart.\(^1\) However, little is known about the effects of EFA deficiency on the function and physiology of the small intestine. In order to improve the nutritional status of pediatric patients, knowledge of (the effects of EFA deficiency on) the small intestinal function is essential. Recent studies suggested that EFA deficiency by itself might deteriorate the intestinal function, as demonstrated by EFA deficiency induced fat malabsorption.\(^3\) Rather than intraluminal effects, intracellular defects in the small intestinal enterocytes were suggested to contribute to fat malabsorption during EFA deficiency in mice.\(^4\)

The aim of this thesis was to characterize the effects of EFA deficiency on the function, morphology and (patho)physiology of the small intestine in a murine model. To study the intracellular effects of EFA deficiency in more detail, an in vitro model was established. Insight into the pathophysiology of EFA deficiency, regarding the small intestinal function, might help improve the nutritional status of patients with CIFTT and other conditions associated with EFA deficiency.

ESSENTIALS FATTY ACIDS (EFA)

The two EFA, also known as “parental” EFA, are linoleic acid (C18:2\(\omega\)-6, LA) and \(\alpha\)-linolenic acid (C18:3\(\omega\)-3, ALA). By means of a cascade of desaturation and elongation of the carbon chain, LA and ALA can be converted into their long chain fatty acid metabolites (LCPUFA: long chain polyunsaturated fatty acids) of the \(\omega\)-6 and the \(\omega\)-3 families, respectively (Figure 1).\(^5,6,7\)

Enzymes responsible for desaturation steps are being competed for by different LCPUFA. The enzymes have preferred affinity for the \(\omega\)-3 family of LCPUFA over the \(\omega\)-6 family members. The affinity for these two EFA families, on the other hand, is preferred over the affinity for non-EFA of \(\omega\)-9 and \(\omega\)-7 fatty acids. Desaturation and elongation of fatty acids depend on the needs and availability of the LCPUFA in the organism.\(^8\) LA and
ALA are not only converted to LCPUFA; part is used as substrates for β-oxidation, representing a source for energy for the organism. Another relevant function of EFA and their LCPUFA metabolites is their role as constituents of the membrane lipids (mainly of phospholipids). Within the membrane, they regulate its fluidity, but also the function and localization of the proteins within these membranes. EFA and their LCPUFA metabolites can also serve as precursors of eicosanoids and leukotrienes which are important signaling molecules in inflammation and second messengers of the central nervous system. Recently, EFA and LCPUFA (along with other non-essential fatty acids) were reported as potent ligands for nuclear receptors, which regulate the gene expression of genes involved in several metabolic processes.

**Figure 1** Essential fatty acids of the ω-3 and ω-6 family, with their source and long chain polyunsaturated metabolites (LCPUFA) and enzymes involved in desaturation and elongation of EFA and LCPUFA.

Under certain circumstances, for example during excessive intake of dietary LA or during low metabolism of LA, LA can be stored in adipose tissue for future use. Since (preterm) infants have limited amounts of adipose tissue and are rapidly growing and developing, they are highly dependent on sufficient and continuous intake of dietary EFA.

Within the enterocytes of the small intestine, absorbed EFA and LCPUFA are mainly re-acylated into triglycerides and subsequently assembled into chylomicrons in order to be excreted into the lymph. However, resident EFA are incorporated in membrane phospholipids, which are mainly rich in LA and its metabolite arachidonic acid (C20:4ω-6, AA). The relatively short lifespan of the enterocytes requires a continuous and rapid supply of EFA and their metabolites, either from dietary, from biliary or from systemic origin. Around 40% of bile consists of EFA- or LCPUFA-acyl chains, making it a very important supplier of intestinal EFA. High EFA requirements are needed for
morphological and dynamic structural changes in the intestinal mucosa. Therefore, the intestinal mucosa is highly sensitive and adaptive to dietary changes in EFA.

As stated above, EFA deficiency in the small intestine can develop in times of low dietary intake, enhanced metabolism, and/or malabsorption of (essential) fatty acids. In general, severe EFA deficiency can lead to growth retardation, skin lesions, reduced vision, impaired cognitive development and steatosis. The symptoms caused by ω-6 fatty acid deficiency are more obvious than those caused by ω-3 fatty acid deficiency.

**Essential fatty acid (EFA) deficiency**

The supply of EFA in the Western diet is usually sufficient to fulfill the metabolic needs. Some chronic intestinal disorders can lead to severe fat malabsorption and thus to EFA deficiency. However, most common is the EFA deficiency due to reduced fat absorption as a consequence of reduced bile secretion in patients with cholestatic liver diseases or reduced activity of pancreatic enzymes, like for example in patients with cystic fibrosis (CF).\(^{13,14,15}\) EFA deficiency itself aggravates the fat malabsorption in these patients leading to even more severe symptoms.\(^{4,3,16}\)

Symptoms of EFA deficiency are usually not immediately obvious, especially not for isolated ALA deficiency. It is therefore important to have another, preferably biochemical, marker to assess EFA deficiency in (pediatric) patients. Plasma measurements of total lipid LCPUFA are relatively easy and can function as an indication of EFA status. Yet, plasma EFA composition may not correspond to the EFA status of various organs, but may rather correlate more closely with recent dietary EFA intake. The EFA composition in membrane phospholipids of erythrocytes may be a better indicator of body EFA status, based on their relatively long half lives.\(^{17}\) This, on the other hand, might only be relevant during EFA assessment in severe, long lasting EFA deficiency. Neither plasma nor red blood cell phospholipid measurements are likely completely representative for complete EFA status, since different tissues are known to have their own specific requirements and metabolism of EFA. Unfortunately, it is clinically impossible to determine the EFA status in the most relevant tissues, such as for example the central nervous system, and therefore plasma or erythrocyte composition of LCPUFA is the most commonly used parameter to assess EFA status. For estimation of the severity of combined deficiency of ω-3 and ω-6 EFA, the so called triene:tetraene ratio has been introduced by Holman in 1960.\(^{18}\) In case of reduction of both ω-3 and ω-6 EFA, the synthesis of non-essential fatty acids of the ω-9 family increases, leading to an enhanced production of the long chain metabolite eicosanoic acid (C20:3ω-9, also known as mead acid) from oleic acid (C18:1ω-9). The increase of the mead acid is an indicator of LA and ALA deficiency. Since sufficient supply of one of the two EFA will prevent an increase in mead acid, this ratio can only be used when the concentrations of both EFA are decreased. Although the triene:tetraene ratio has been regarded for long as “the biochemical marker of EFA deficiency”, it does not provide an overall picture of the EFA and their LCPUFA metabolites.\(^{19}\) More common is the use of triene:tetraene ratio in combination with other determinations of EFA (e.g. plasma profile) in order to obtain a more complete and accurate picture of the EFA status in patients. The nature of the disease may influence what the best clinically relevant marker is for a certain disease. Magbool et al. have
recently demonstrated that in pediatric patients with CF, assessment of serum LA status as the clinical indicator of EFA status is more relevant than the triene:tetraene ratio.\textsuperscript{19} Fatty acid compositions are most often represented as molar percentages, which indicate the percentage of an individual fatty acid (or a group of fatty acids) as the percentage of total fatty acids in plasma or other compartments. The relative, molar percentages are often more relevant than absolute concentrations, since the latter do not indicate the changes in membranes, which are mainly influenced by the composition. As stated above, a high incidence of EFA deficiency has been reported during cholestasis or CF. In both conditions, the small intestinal function seems to be affected.\textsuperscript{13,14,15,20} The association of cholestasis and CF in relation to EFA deficiency will be discussed in more detail in the next two paragraphs.

**Essential fatty acid (EFA) deficiency in cholestasis**

Cholestatic liver diseases (CLD) are characterized by decreased or absent hepatic secretion of bile into the intestine, either caused by congenital or acquired diseases.\textsuperscript{21,22} CLD are associated with several nutritional complications, including EFA deficiency.\textsuperscript{23} In general, neonatal and pediatric patients are more affected by CLD than adults. EFA deficiency is one of the contributors to “failure to thrive” in pediatric patients with cholestasis, known as *cholestasis induced failure to thrive* (CIFFT). Several treatment options aim to reduce the cholestatic symptoms in pediatric patients, as well as their negative impact on the nutritional condition. However, CIFFT can be very resistant to treatment options, particularly in young children with end stage liver disease who require liver transplantation.\textsuperscript{24,25} The most common cause of CLD in children requiring liver transplantation is biliary atresia. Biliary atresia is a progressive disorder characterized by an inflammatory reaction towards the extrahepatic and intrahepatic bile ducts, leading to destruction and subsequent replacement of the normal tissue by fibrotic scar tissue. The etiology of biliary atresia remains unknown, although an inflammatory reaction to a detrimental stimulus seems to play an initiating role.\textsuperscript{26} Another group of causes for pediatric CLD involve Progressive Familial Intrahepatic Cholestasis (PFIC). PFIC constitute a group of genetically transmitted disorders, inherited in an autosomal recessive fashion. Three phenotypic forms of PFIC have been characterized and attributed to gene defects in three different genes (PFIC1-3; official symbols: ATP8B1, ABCB11, ABCB4).\textsuperscript{27} Another cause of CLD is non-syndromic paucity of the intrahepatic bile ducts, whose etiology is still enigmatic, infections, chromosomal disorders and metabolic disorders have been suggested to play a role.\textsuperscript{28} Inborn errors in bile acid synthesis account for another part of the children with CLD. Defects have been identified in enzymes catalyzing cholesterol catabolism and bile acid synthesis.\textsuperscript{28} CLD in adolescents and young adults is often due to autoimmune hepatitis, primary biliary cirrhosis or primary sclerosing cholangitis.\textsuperscript{28,29} Poor dietary intake is an important factor in the pathophysiological basis of malnutrition in children with CLD. The nutritional status may be further compromised by decreased absorption of the macronutrients, fat, carbohydrates and proteins. At infant age, fat accounts for the most important dietary energy source (up to 50\% of total ingested energy). It is therefore, not surprising that up to 70\% of children with CLD requiring liver transplantation have biochemical indications of EFA and LCPUFA deficiency.\textsuperscript{24,25}
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Several studies demonstrated the decreased uptake and/or intracellular processing in the enterocyte as the main reason for decreased EFA and LCPUFA concentrations during EFA deficiency,\textsuperscript{3,4} rather than the decreased activity of desaturases and/or elongases as proposed earlier by Socha et al.\textsuperscript{14} In addition, cholestasis has been proposed to impair the β-oxidation pathway and can therefore interfere with the last step of DHA and DPA metabolism from their precursors.\textsuperscript{2,30} However, in an animal model for cholestasis (rats with bile duct ligation), Minich et al. showed no major difference in LA oxidation, thus showing no support for this concept.\textsuperscript{31}

**Essential fatty acid (EFA) deficiency in cystic fibrosis**

Cystic fibrosis (CF) is still one of the most common genetic disorders among the Caucasian population.\textsuperscript{20} It is an autosomal recessive disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The encoded CFTR protein mainly functions as a chloride channel.\textsuperscript{20} Over 1500 mutations have been identified in the CFTR gene. However, for only a small number of these mutations the functional importance has been elucidated. Symptoms of CF are age- and patient-dependent, but most of them involve gastrointestinal, pulmonary, endocrine and reproductive disorders.\textsuperscript{32}

Gastrointestinal problems include meconium ileus (obstructive condition of the small intestine) and pancreatic insufficiency, which both lead to malnutrition and failure to thrive.\textsuperscript{33,34} In a sub selection of patients, cirrhosis and cholestatic symptoms may develop in CF patients which contribute to an even further deterioration of the nutritional status.\textsuperscript{35,36} EFA deficiency has been common in CF, particularly in the era that many patients were treated with low-fat diets to counteract the steatorrhoea, and was mainly characterized by low plasma levels of linoleic acid (C18:2ω-6, LA).\textsuperscript{37,38} Number of events has been suggested to contribute to EFA deficiency in CF patients, like pancreatic insufficiency, solubilization defects, altered intestinal microclimate and altered enzyme activity of desaturases and elongases involved in EFA metabolism. Additionally, increased energy expenditure is thought to contribute to the poor nutritional status in CF patients.\textsuperscript{39} Several attempts to correct for EFA deficiency, with pancreatic enzyme replacement therapy and linoleic acid supplementations, in CF patients have shown variable effects.\textsuperscript{40,41,42,43}

**SMALL INTESTINE**

The small intestine is one of the largest and most metabolically active tissues. It is continuously renewed by processes of proliferation and differentiation, leading to a highly ordered tissue architecture.\textsuperscript{44} Enterocytes are responsible for the absorption of dietary and endogenous compounds. Enterocyte differentiation can be studied by assessment of the expression of brush border enzymes, such as lactase and sucrase-isomaltase. The three transcription factors Gata-4, Hnf1α and Cdx2 regulate the expression of the corresponding genes.\textsuperscript{45} Cdx2 also plays an essential role in the organogenesis of the midgut into the small intestine.\textsuperscript{46} Enterocytes located within different regions of the small intestine (duodenum, jejunum and ileum) vary in their functional capacities; while for example the carbohydrate absorption mainly takes place in the more proximal part, bile
salt uptake occurs mainly in the terminal ileum. Small intestinal morphology is characterized by two distinct axes, the horizontal axis, i.e. the proximal to distal (or anterior to posterior) small intestine, and the vertical axis, representing the crypt-to-villus distinction in the enterocytes.\textsuperscript{47}

**Crypts and villi**

Already during the formation of the primitive gut at gestational age of 9 weeks in humans, the morphogenesis of nascent villi and crypts occurs within the epithelium (Figure 2). At this point, cellular proliferation is concentrated mainly within the intervillus region. During development, the intervillus regions are transformed into crypts by means of cellular penetration into the mesenchyme.

![Histological staining of mouse small intestine indicating the crypt and villus regions. Crypts are located at the bottom and contain stem cells which migrate towards the upper located villus region. Fully differentiated cells represent mainly the absorptive cells, the enterocytes.](image_url)

It has been accepted that the small intestinal epithelium is maintained by a population of tissue-specific stem cells.\textsuperscript{48} Developed crypts contain a small, proliferating group of stem cells which give rise to different intestinal cell types, subsequently migrating towards the adjacent villi.\textsuperscript{48,49} In most mammals, re-differentiation of the intestine starts after birth, simultaneous with increased proliferation. Increased proliferation eventually leads to development of larger crypt depth and increased villus height. Parallel with the development of the crypt and villi, different cell lineages develop from the immature cells, including absorptive cells (enterocytes), mucus secreting cells (goblet cells), various enteroendocrine cells and enzyme- and antibacterial peptides secreting cells (Paneth cells).\textsuperscript{48,50,51,52} All the epithelial cells, originate from the same multipotent stem cells that proliferate from the bottom of the crypt. Enterocytes account for almost 90\% of all epithelial cells within the small intestine. Research described in this thesis focuses on the enterocytes, the most relevant intestinal cell type with regard to absorption and metabolism of dietary compounds.

**Apical and basolateral compartment in the enterocyte**

Enterocytes are absorptive intestinal cells characterized by two domains within the cell,
namely the apical and the basolateral domain, separated by the tight junctions. It is the existence of these domains that plays an important role in the maintenance of the intestinal barrier function. One of the most remarkable features of the absorptive enterocytes is the presence of the so called brush border membrane (BBM) at the apical site of the cell which consists of many closely packed microvilli. Apical and basolateral domains differ in their expression of different enzymes and transporters. The histocompatibility antigens are specifically located at the basolateral membrane of the enterocyte. Enzymes (hydrolases) appear only within the BBM at the end of the physiological differentiation process of the enterocyte, i.e. when the proliferating cells reach the villi during their migration from the crypts. For this reason, many hydrolytic enzymes, like lactase and sucrase isomaltase, are used as the markers for the differentiation status of the absorptive enterocytes. The exact pathways by which enterocytes deliver different newly synthesized proteins from the Golgi apparatus to the apical or basolateral site are the subject of intense cell biological research.

**Enterocyte function**
Small intestine is one of the first barriers to be encountered by nutrients after their dietary ingestion. Absorption of most important dietary and hepatobiliary compounds is described below. Figure 3 shows a short schematic summary of enzymes and proteins involved in processes of absorption and metabolism of the small intestine.

**Fat absorption**
Dietary fat is mainly absorbed as triglycerides in the human diet and in smaller amounts as phospholipids (~10%). Intestinal absorption of fat can be separated in intraluminal and intracellular events, and these have been reviewed extensively. Intraluminal steps of fat absorption can be divided into emulsification, lipolysis, and solubilization, followed by translocation across the epithelial apical membrane. Emulsification involves mechanical disruption and partial hydrolysis of triglycerides within the stomach and results in increasing the oil-water surface area by decreasing the median size of the fat droplets from the diets. This process is stimulated mechanically by shear force in the stomach and the pylorus and biochemically by generating the lipolytic products of triglycerides, namely diacylglycerol and free fatty acids. The emulsified dietary fat subsequently enters the first part of the small intestine, the duodenum, where it is subjected to lipolysis by pancreatic lipases into monoacylglycerol and free fatty acids. Triglyceride lipolysis by pancreatic lipases requires a co-factor, pancreatic co-lipase, which is able to facilitate proper binding of the lipase to the oil-water surface of the fat emulsion. The secretion of the pancreatic lipase into the duodenum is often associated with gallbladder contraction and cholecystokinin release. Digestion of phospholipids derived from diet and bile, occurs within the duodenum by the enzyme phospholipase A2. However, as demonstrated by studies in PLA2-deficient mice, additional enzyme(s) can compensate for pancreatic PLA2 in catalyzing phospholipid digestion. Hydrolysis of phospholipids results in production of lyso-phospholipids and free fatty acids, which can subsequently be translocated across the enterocyte apical membrane. Lipolytic products must be solubilized in order to be soluble and thus transportable in the aqueous phase of the intestinal lumen and across the so called...
unstirred water layer, which is the border between the luminal site of the intestine and the BBM of the enterocytes. Solubilization of the lipolytic products is performed by biliary bile salts and phospholipids by means of their mixed micellar formation with the products of lipolysis. Mixed micellar solubilization increases the solubility of the lipolytic products up to 1000-fold. Compared to diglycerides and fatty acids, phospholipids are more independent of bile salts for the mucosal uptake, since they can interact more easily with water molecules.

**Figure 3** Major small intestinal enzymes, proteins and nuclear receptors involved in fatty acid, carbohydrate, cholesterol and bile salt absorption and metabolism. On the left, the apical site with the brush border membrane and on the right the basolateral site of the enterocyte is indicated. ABCA1, Abc-transporter a1; ABCG5/8, Abc-transporter g5/g8; ACAT2, Acyl-coenzyme A:cholesterol Acyltransferase 2; ASBT, Apical sodium dependent bile acid transporter; DGAT1/2, Acyl coenzyme A:diacylglycerol acyltransferase 1/2; FABP, Fatty acid bindind protein; FAT, Fatty acid transporter; FATP4, fatty acid transport protein; FGF15, Fibroblast growth factor 15; FGFR4, Fibroblast growth factor receptor 4; FXR, Farnesoid X receptor, GLUT2/5, Glucose transporters 2/5; IBABP, Ileal bile acid binding protein; LDLR, Low density lipoprotein receptor; LXR, Liver X receptor; MGAT, monoacylglycerol O-acyltransferase 1; MTTP, microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick C1 like 1; OSTα/β, Heteromeric organic solute transporter alpha-beta; PPAR, Peroxisome proliferator-activated receptors; RXR, Retinoid X receptor; SI, Sucrase isomaltase; SGLT, Sodium glucose cotransporter; SHP, short heterodimer partner; SRBI, scavenger receptor BI.

After lipolysis and solubilization, fatty acids dissociate from different lipid classes (micelles, liposomes, liquid crystalline vesicles or free phospholipids) within the unstirred
Translocation of the free fatty acids and across the BBM subsequently occurs. Whether this translocation occurs only via passive diffusion, or in addition via fat transporters in the enterocytes, remains unclear. Several candidate transporters have been identified to facilitate fat transport across the BBM of the enterocyte, including the fatty acid transport protein 4 (FATP4; official symbol SLC27A4) and the fatty acid translocase (FAT; official symbol CD36), both located at the BBM of the enterocytes. However, FATP4 has been shown to localize within the enterocyte as well and thus not exclusively at the BBM. More importantly, several studies in mice with deletions in these transporters clearly have indicated that these transporters are not essential. Rather, they might co-facilitate in dietary fatty acid absorption or influence their intracellular processing, as these mice do not show severe signs of fat malabsorption. Within the enterocyte re-esterification and chylomicron formation occur, starting with the binding of fatty acids to the intestinal fatty acid binding protein (IFABP; official symbol FABP2) or liver fatty acid binding protein (LFABP; official symbol FABP1) which escort them to the endoplasmatic reticulum. Interestingly, I-FABP deficiency in mice does not lead to fat malabsorption, indicating that I-FABP is not essential for sufficient absorption of dietary fat.

Within the smooth endoplasmatic reticulum absorbed fatty acids are acylated into triglycerides via two different pathways. Under physiological conditions, the so called monoacylglycerol pathway is the predominant one in which 1 acetylated fatty acid molecule and 2 molecules of monoacylglycerol are re-esterificated into triglycerides. The enzymes involved in the two steps of monoacylglycerol pathway are acyl-CoA:monoacylglycerol acyltransferases (MGATs). These enzymes convert monoacylglycerol and fatty acyl-CoA into diacylglycerol. Acyl-CoA: diacylglycerol acyltransferases (DGATs), on the other hand, convert diacylglycerol into triglycerides. The second, physiologically less prominent route is the α-glycerophosphate pathway, which becomes of major importance under conditions of fat malabsorption. In the first two steps, glycerol-3-phosphate is converted into phosphatidic acid by means of glycerol-3-phosphate acyltransferases and 1-acylglycerol-3-phosphate O-acyltransferases. Subsequently, phosphatidic acid is converted to diacylglycerol by PA phosphatases. Diacylglycerol produced by this pathway is preferentially used to synthesize new phospholipids. The rest of the diacylglycerol is used to produce triglycerides, which are thought to be slightly different from triglycerides produced by the monoacylglycerol pathway, since the triglycerides from the latter pathway are transported faster across the basolateral membrane of the enterocytes.

The last step of lipid absorption involves the assembly of newly produced triglycerides, phospholipids, cholesterol, cholesteryl esters and apolipoproteins (mainly apoB48) into pre-chylomicrons. This process requires the microsomal triglyceride transfer protein (MTTP) within the smooth endoplasmatic reticulum. Afterwards, these pre-chylomicrons are transported towards the Golgi apparatus, where they transform into mature chylomicrons. These are eventually released in the cytoplasm and exocytosed into the interstitium, ending up in lymph.
proliferator-activated transcription factors (PPARs) α, β/δ and γ which, like the other nuclear receptors, heterodimerizes with retinoid X receptor (RXR).

Although the functions of PPARs have been studied extensively in the liver, their role in the intestine is still emerging. PPARα activation in the intestine has recently been demonstrated to activate the transcription of several genes involved in fatty acid, triacylglycerol, sterol and bile acid metabolism. PPARδ activation, on the other hand, was recently shown to reduce intestinal cholesterol absorption efficiency. PPARγ within the intestine has recently been implied in modulating epithelial and mucosal inflammation.

**Carbohydrate absorption**

Carbohydrates in diet are derived from starch (polysaccharides, 75%) or sugars (di- and monosaccharides). Starch is composed of amylase and amylpectin, and is digested by salivary and pancreatic amylases. Afterwards, final hydrolysis to glucose at the brush border of the enterocytes in the proximal part of the small intestine occurs by sucrase-isomaltase and maltase-glucoamylase. Glucose can subsequently be taken up by the sodium-dependent glucose transporter (SGLT1; official symbol SLC5A1). Lactose and sucrose are the quantitatively most important dietary disaccharides. They are hydrolyzed into glucose and galactose or fructose, respectively. Hydrolysis of lactose and sucrose is catalyzed by the enzymes lactase and sucrase isomaltase, respectively, anchored within the brush border of the enterocytes. Monosaccharides are transported directly across the BBM by means of SGLT1 (glucose and galactose) or GLUT5 (fructose; official symbol SLC2A5), without requiring hydrolysis. Subsequently, basolateral transport of all carbohydrates occurs via the universal GLUT2 (official symbol SLC2A2) transporter. Studies in rats with bile duct ligation demonstrated that cholestasis is not associated with severely affected absorption and digestion of carbohydrates. However, whether EFA deficiency affects digestion and absorption of dietary carbohydrates is not known.

**Cholesterol absorption**

Between 25% and 85% of dietary cholesterol is absorbed from the small intestine in humans. Once in the lumen of proximal small intestine, cholesterol and plant sterols are most likely transported into the enterocyte by means of the recently identified, apical transporter Niemann-Pick C1-like 1 protein (NPC1l1). The function of this protein can be illustrated by the phenotype of mice lacking NPC1l1 protein, showing severely reduced cholesterol absorption compared to their wild type littermates. Within the enterocytes, cholesterol is esterified into cholesteryl esters by means of the acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2), which has a high affinity for cholesterol, but not for plant sterols. This results in packaging of the cholesteryl esters into chylomicrons, which are subsequently secreted into the circulation. Recent studies demonstrated that a fraction of the enterocytic cholesterol can be secreted into the circulation independent from the chylomicron pathway. Direct secretion across the basolateral membrane occurs in monomeric form, to be subsequently incorporated into the HDL particles. This basolateral transport occurs via the ATP binding cassette transporter 1 (ABCA1). Scavenger receptor class B, member 1 (SR-BI; official symbol SCARB1) and LDL receptor (LDLR), localized at the basolateral site of the enterocyte, can reabsorb selectively the cholesteryl esters, without absorption of the remnants of the
HDL particle. Unesterified plant sterols are not assembled for basolateral secretion, but are transported back to the intestinal lumen along with unesterified cholesterol. This apical transport of plant sterols and unesterified cholesterol from the enterocyte into the lumen is facilitated by an ABC heterodimeric transporter ABCG5/ABCG8. Within the enterocyte, the nuclear liver X receptor (LXRα and LXRβ; official symbols NR1H3 and NR1H2) is expressed, which tightly regulates cholesterol and fatty acid metabolism by inducing the transcription of genes involved in these metabolic pathways (ABC transporters, SREBP1c and SREBP2; official symbols SREBF1 and SREBF2). Until recently, hepatobiliary secretion of cholesterol has been thought as the most prominent way of cholesterol excretion from the body. This is rather peculiar, since already in 1927 an alternative pathway has been proposed, involving direct secretion from the intestine. However, this latter pathway has never been validated or paid sufficient scientific attention. Recently, the alternative pathway has become re-appreciated, since in various conditions and models the fecal excretion of neutral sterols was higher than the sum of dietary and biliary cholesterol entering the intestinal lumen. Direct transintestinal pathway for cholesterol excretion (TICE) has been demonstrated in mice by van der Velde et al. The capacity of the intestinal cholesterol excretion pathway was exactly sufficient to account for the missing cholesterol and twice as high as the quantitative hepatobiliary secretion. This observation indicated the relevance of TICE in excretion of cholesterol in mice. Importance of TICE in other species has not been studied in detail so far. TICE was demonstrated to depend on the dietary fat content. The EFA deficiency might, therefore, be associated with alterations in TICE. However, the effects of EFA deficiency on cholesterol metabolism in the intestine have not been studied so far.

Small intestine and the enterohepatic circulation of bile salts
Bile salts are synthesized in the liver from cholesterol via the neutral or the acidic pathway. Under physiological conditions, bile salts are subsequently secreted via bile into the intestine. Within the intestine the bile salts are almost completely reabsorbed; only around 5% of the endogenous bile salts escape the reabsorption and is excreted via the feces every day. Unconjugated bile salts in the small intestine and in colon can be transported passively. However, conjugated bile salts require facilitated transport across the BBM. This is achieved by means of the apical sodium-dependent bile salt transporter (ASBT/ISBT; official symbol SLC10A2), mainly expressed in the terminal ileum. The intracellular transport of bile acids from the apical to the basolateral compartment was thought to be facilitated by the ileal bile acid binding protein (IBABP; official symbol FABP6), however, the exact role of IBABP in the intracellular trafficking of bile salts is still under debate. Within the cell, bile salts can bind to and activate the nuclear hormone farnesoid receptor (FXR; official symbol NR1H4), which is an important regulator of bile salt homeostasis. Activated FXR initiates the transcription of a whole cascade of genes important for bile salt metabolism. One of these genes is the small heterodimer partner (SHP; official symbol NR0B2), which leads to subsequent ASBT repression. Another intestinal protein which is tightly regulated by the activated FXR is the fibroblast growth factor 19 (FGF19, mouse homologue is Fgf15). Upon FXR activation, FGF19 is released into the circulation, in order to be
transported to the liver. In the liver, FGF19 binds to the fibroblast growth factor receptor 4 (FGFR4) on the hepatocyte cell membrane. This binding leads to the activation of the JNK pathway and repression of cholesterol 7-α-hydroxylase (CYP7A1) and sterol 12-α-hydroxylase (CYP8B1), resulting in decreased bile salt synthesis. Recent studies demonstrated that in addition to intestinal/hepatic FGF19/FGFR4 signaling pathway, liver FGFR4/FGF19 pathway might exist to protect the liver under conditions of bile salt accumulation. Another study reported expression of FGFR4 at the basolateral site of the enterocytes and in cholangiocytes, suggesting the existence of a feedback loop mechanism of FGF19/FGFR4 within the intestine and bile ducts. Excretion of bile salts in the enterocytes occurs via basolaterally localized heterodimeric organic solute transporter OSTα-OSTβ.

**Manifestation of the impaired small intestinal function in common intestinal disorders**

Two common small intestinal disorders with a profound impact at pediatric age are celiac disease and Crohn’s disease. Both conditions can severely affect small intestinal morphology and function, and lead to malabsorption to nutrients and to growth failure. Celiac disease is a form of autoimmune disease of the small intestine leading to nutrient malabsorption and immune reaction to transglutaminidase in genetically predisposed subjects. It is a life-long condition characterized by villous atrophy (blunted villi), enhanced cell proliferation, increased number of crypts and increased infiltration of lymphocytes upon ingestion of gluten. Symptoms vary largely among the patients and disappear upon a gluten-free diet. Crohn’s disease is anti-inflammatory disease which can affect the whole gastrointestinal tract. Within the small intestine neutrophil infiltration into the epithelium can occur along with atypical crypt branching and finally with villous blunting. Intestinal permeability might also be profoundly increased, associated with an impaired barrier function. Together, these pathophysiological factors can lead to malabsorption of nutrients and growth failure. The exact factors involved in pathophysiology of EFA deficiency in the small intestine which lead to nutrient absorption remain unclear. Therefore, it is useful to study how EFA deficiency affects the small intestinal function and morphology.

**AIM AND THE OUTLINE OF THE THESIS**

Clinical conditions associated with EFA deficiency are accompanied by impaired nutritional status. In children with cholestasis, EFA deficiency aggravates the cholestasis induced failure to thrive (CIFTT). In animal models, EFA deficiency by itself is associated with malabsorption of fat, even in absence of cholestasis or CF. Previous studies suggested that defects in the small intestine during EFA deficiency were located at the intracellular level. *We aimed to characterize and unravel the effects of EFA deficiency on the pathophysiology and the function of the small intestine.*

First, we studied the epithelial histology and function by analyzing the morphology and nutrient absorption of the small intestine during EFA deficiency (chapter 2). We describe the effects of EFA deficiency in mice on the absorption of carbohydrates and on the expression of lactose, relevant small intestinal differentiation marker. By means of the
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administration of stably labeled glucose and lactose, we determined the absorption and digestion of these compounds in vivo. In chapter 3 we further characterized the effects of EFA deficiency on intestinal physiology by determining the jejunal cholesterol absorption and metabolism during EFA deficiency. The results obtained are based on the physiological parameters and the microarray analysis of mouse jejunal tissue.

In chapter 4 we determined the effects of EFA deficiency on the enterohepatic circulation (EHC) of bile salts. Bile salt (re)absorption is a small intestinal function which does not depend on the jejunal intestinal epithelium, but rather on that of the terminal ileum. In order to study whether EFA deficiency differentially affects different small intestinal segments, we studied the EHC in EFA-deficient mice. Small intestine plays an important role in the EHC of bile salts by regulating the feedback mechanism of the hepatic bile salt synthesis. Previous studies in EFA-deficient mice revealed elevated bile salt secretion and bile flow. The underlying mechanism of this finding remained unclear. We determined several parameters of the enterohepatic circulation of bile salts using the stable isotope dilution technique, combined with bile duct cannulation. Small intestinal regulatory mechanisms of the enterohepatic circulation were assessed by analyzing the expression of the intestinal genes implicated in bile salt metabolism.

In order to study in more detail the intracellular effects of EFA deficiency on the small intestine, an in vitro model of EFA deficiency has been established. Differentiating Caco-2 cells cultured in EFA-deficient or control medium were characterized and validated as a model for EFA deficiency (chapter 5). We described the effects of EFA deficiency on cell differentiation, gene expression and morphology, based on several in vitro experiments in EFA-deficient Caco-2 cells.

To optimize nutritional condition during cholestatic liver disease, one could aim to decrease the fat malabsorption by administration of exogenous absorption enhancers. Chapter 6 describes experiments in two different rat models of fat malabsorption; one with impaired lipolysis (pancreatic insufficiency model) and one with reduced solubilization (cholestatic model). In these rat models we studied the effects of the compound Gelucire®44/14 on fat malabsorption in vivo. Gelucire®44/14 is currently used to improve the absorption of poorly soluble drugs. Fat absorption was assessed in both models, at the level of lipolysis and solubilization, respectively, after the administration of Gelucire®44/14.

Chapter 7 provides a summary of the most relevant findings in this thesis and future perspectives for EFA deficiency-related research.

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CHAPTER 1
