Essential Fatty Acid Absorption and Metabolism

RIJKSUNIVERSITEIT GRONINGEN

Essential Fatty Acid Absorption and Metabolism

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“L’essentiel est invisible pour les yeux.”

Le Petit Prince, Antoine de Saint-Exupery

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Groningen, July 1999

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List of frequently used abbreviations*

AA	Arachidonic acid, 20:4n-6
ADA	Adrenic acid, 22:4n-6
ALA	Alpha-linolenic acid, 18:3n-3
BSSL	Bile salt stimulated lipase
CF	Cystic fibrosis
DAG	Diacylglycerol
DGLA	Dihomo-gamma-linolenic acid, 18:3n-6
DHA	Docosahexaenoic acid, 22:6n-3
DPA	Docosapentaenoic acid, 20:5n-3
EFA	Essential fatty acid
EFAD	Essential fatty acid deficiency
EPA	Eicosapentaenoic acid, 20:5n-3
FABP	Fatty acid binding protein
FFA	Free fatty acid
GLA	Gamma-linolenic acid, 18:3n-6
IFABP	Intestinal fatty acid binding protein
LA	Linoleic acid, 18:2n-6
LCPUFA	Long chain polyunsaturated fatty acid
LDL	Low-density lipoprotein
LFABP	Liver fatty acid binding protein
MA	Mead acid, 20:3n-9
MAG	2-monoacylglycerol
*mdr*2	Multidrug resistance 2 P-glycoprotein
MTG	Microsomal transfer protein
OA	Oleic acid, 18:1n-9
PA	Palmitic acid, 16:0
PC	Phosphatidylcholine
POA	Palmitoleic acid, 16:1n-7
SA	Stearic acid, 18:0
TAG	Triacylglycerol
TPN	Total parenteral nutrition
VLDL	Very low-density lipoprotein

***Plural forms of the terms listed here are designated in this thesis by the addition of an 's' after the abbreviation.*

CHAPTER 1

Introduction

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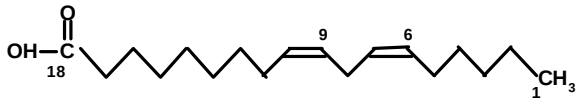
1.1 Importance of essential fatty acids (EFAs)

The concept that specific fat components may be necessary for the proper growth and development of animals and possibly humans was introduced over 65 years ago [1,2]. Burr and Burr (1929-1930) [1,2] demonstrated that rats reared on a fat-free diet failed to grow and reproduce and also developed renal disease, fatty liver, dermatitis, and necrosis of the tail. Subsequent addition of saponified lard to the fat-free diet alleviated the skin lesions and the retarded growth. The fatty acids that prevented the symptoms of fat deficiency were specific unsaturated fatty acids from the n-6 and n-3 series with two or more double bonds, namely linoleic acid (9,12-octadecadienoic acid, LA, 18:2n-6[1]) and alpha-linolenic acid (9,12,15-octadecatrienoic acid, ALA, 18:3n-3) (Fig. 1) [2,3]. These fatty acids became known as *essential fatty acids*[2] (EFAs) because they are not synthesized *de novo* by mammals but are nevertheless necessary for proper physiological functioning [3]. EFAs were not considered to be important for human nutrition until the early 1970s when signs of clinical deficiency such as dermatitis became apparent in infants fed a skim milk-based formula with a low LA content [4] and in adults given lipid-free parenteral nutrition [5]. During the past decade, increasing attention has been focused on the role of n-6 and n-3 EFAs and their *long-chain polyunsaturated fatty acids*[3] (LCPUFAs) (Fig. 2) in normal and neonatal development [6-9].

(a) **n-6**



(b) **n-3**

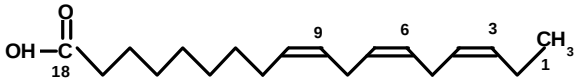


Figure 1. *The structural formulas of the n-6 and n-3 essential fatty acids; (a) linoleic acid (18:2n-6; LA) and (b) alpha-linolenic acid (18:3n-3, ALA).*

Dietary EFAs have been considered part of the lipid supply necessary for energy, growth and cellular metabolism [10]. More specifically, fatty acids of the n-6 and n-3 families are structural components of membrane phospholipids. Their presence in the membrane can alter the physiochemical characteristics (microviscosity or fluidity) of the membrane lipid matrix, which, in turn, can influence the conformation, mobility and function of a wide variety of intrinsic and extrinsic membrane-bound proteins; these constitute the hormone receptors, ion gates and channels and membrane-bound enzymes [11]. LCPUFAs synthesized from LA, such

[1]The nomenclature used for fatty acids in this thesis is as follows: the number of carbon atoms, followed by a colon and the number of double bonds. The fatty acid family is indicated by designation of the position of the first double bond from the methyl end of the carbon chain.

[2] EFA or EFAs as used in this thesis refers exclusively to the 'parent' essential fatty acids, linoleic and linolenic acids and not to their metabolites.

[3] LCPUFA or LCPUFAs as used in this thesis refers exclusively to the n-3 or n-6 metabolites of linolenic and linoleic acids, respectively.

as dihomo-gamma-linolenic acid (DGLA, 20:3n-6) and arachidonic acid (AA, 20:4n-6), are precursors of eicosanoids [12,13]. Eicosanoids are potent messenger substances that regulate a wide variety of processes including gastric acid secretion, uterus contraction, reproduction, blood pressure control, and inflammation [14].

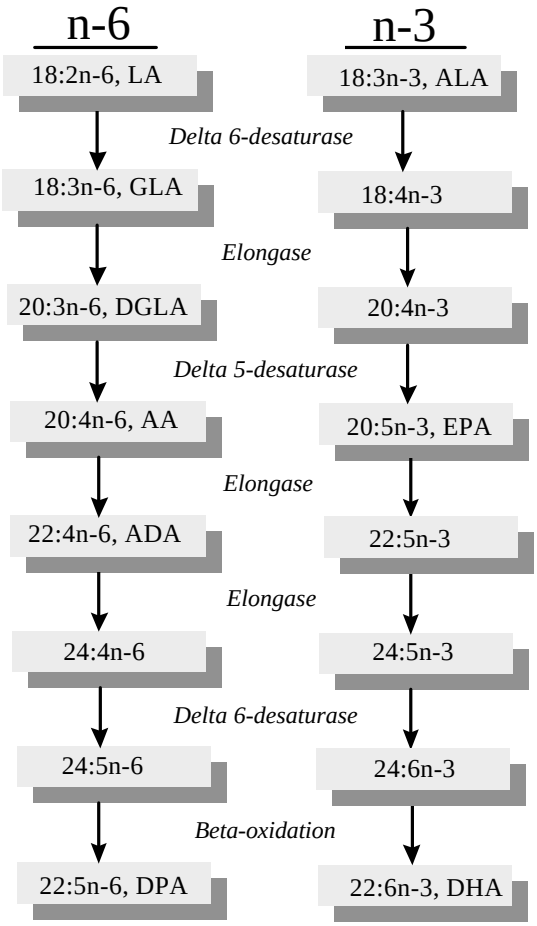


Figure 2. *Classical n-6 and n-3 fatty acid LCPUFA synthesis pathways [15-17].* LA: linoleic acid; GLA: gamma-linolenic acid; DGLA: dihomo-gamma-linolenic acid; AA: arachidonic acid; ADA: adrenic acid; DPA: docosapentaenoic acid; ALA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexanenoic acid.

A majority of EFA research has been directed towards LA, which is the EFA most commonly found in the diet of individuals from Western countries. Evidence for a specific function for ALA other than being a precursor of n-3 LCPUFAs has not yet been reported [18,19]. The most important fatty acids derived from ALA are timnodonic acid (9,12,15,18,21-eicosapentaenoic acid, EPA, 20:5n-3) and cervonic acid (9,12,15,18,21,24-docosahexaenoic acid, DHA, 22:6n-3), which are commonly referred to as fish oil fatty acids. Epidemiologic studies on American, Dutch, and Japanese populations have provided data associating fish oil consumption with reduced risk of coronary heart disease and inflammatory disease [15,20-22]. Fish oils reduce very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) concentrations in plasma of normal and hyperlipidemic patients [23], reduce platelet aggregability and prolong

bleeding time [21]. It has been suggested that both EPA and DHA displace AA from membrane phospholipids and, thus, reduce its availability for eicosanoid formation.

Finally, as studied in preterm and term infants, n-6 and n-3 LCPUFA levels in the body have been related to development of visual acuity, cognitive functions and growth [24-29]. AA and DHA are found in high concentrations in the cerebral cortex and retina, suggesting their participation in neural and visual function [10,30-32]. DHA reaches levels of up to 50% of total fatty acids in the phospholipids of these tissues [10,33]. Reduced visual function has been found in animals fed diets deficient in ALA and with reduced brain and retinal concentrations of DHA [18,34,35]. However, a specific association of either AA or DHA with neural tissue function has not been fully defined [10].

1.2 Essential fatty acid (EFA) status

Theoretically, an adequate EFA status in the body will depend on a number of factors including quantity of endogenous stores, dietary EFA levels, and efficiency of lipid absorption. Cunnane [3] has proposed a diagram for the various fates of LA which are responsible for its levels in the body under physiologic conditions (Fig. 3). Although it occurs in only small amounts in green leafy vegetables, 16:2n-6 and 16:3n-3 can be converted to their respective 22-carbon LCPUFA [3]. Significant amounts of vegetables containing these fatty acids would have to be consumed in order to meet the requirement for EFAs and LCPUFAs in the body. If the dietary intake of EFAs is too low to meet the requirement of the body, the body stores of EFAs will be used as an additional supply [7]. Considerable amounts of LA are stored in adipose tissue and can readily been mobilized. However, adipose tissue contains hardly any ALA and LCPUFAs [7]. In particular, neonates and premature infants have a rather limited adipose reserve. Desaturation and elongation of EFAs to LCPUFA will depend on the need for LCPUFA in the body and on the available supply of EFAs for conversion. For example, the activity of these enzymes is increased during pregnancy but decreased in short-term or chronic undernutrition [3]. Several reports over the past three decades demonstrate that LA and ALA are relatively easily beta-oxidized to CO_2 in different model systems [36-39]. Results from a 90-day balance study in free-feeding rats revealed that 65-75% of dietary LA disappears from the whole-body n-6 LCPUFA pool when LA is consumed by nonpregnant-lactating rats at or above 2% of energy intake [40]. This value may be lower in pregnancy but can rise to >100% of intake (including losses from body stores) with chronic undernutrition during pregnancy [41]. It is not known if EFAs and LCPUFAs are spared from oxidation under these conditions.

Essential fatty acid deficiency (EFAD) and insufficiency

Animal experiments have shown that the young are more susceptible to EFAD than are adults [15]. Body stores of EFAs are limited in low-birth-weight infants, resulting in a deficiency state more rapidly evident than in adults [15]. Due to the ubiquity of EFAs and LCPUFAs in the Western diet, EFAD was thought to be uncommon in human adults. Therefore, the need for these fatty acids, at least in human adults, was questioned. With the advent of total parenteral

nutrition (TPN) based on a system of continuous fat-free infusion, EFAD became evident in human adults [5]. TPN is commonly administered as a continuous infusion of a glucose-containing solution, which results in a constant elevation of serum insulin. High serum insulin levels depresses the release of fat from adipose stores, which in normal patients contains more than 1000 g of EFAs [15]. This amount could otherwise sustain dietary needs for more than six months. However, it is difficult to estimate the precise onset of EFAD development since the supply of EFAs in the body and the extent of demand for EFAs varies depending on the individual. In the neonate, biochemical indications of EFAD may become apparent in five to ten days on fat-free TPN, whereas in adults, the biochemical evidence of deficiency may be seen by the end of two weeks [5,42]. Patients maintained on fat-free TPN for three weeks developed alopecia, brittle nails, desquamating dermatitis, and increased susceptibility to infection [5,15].

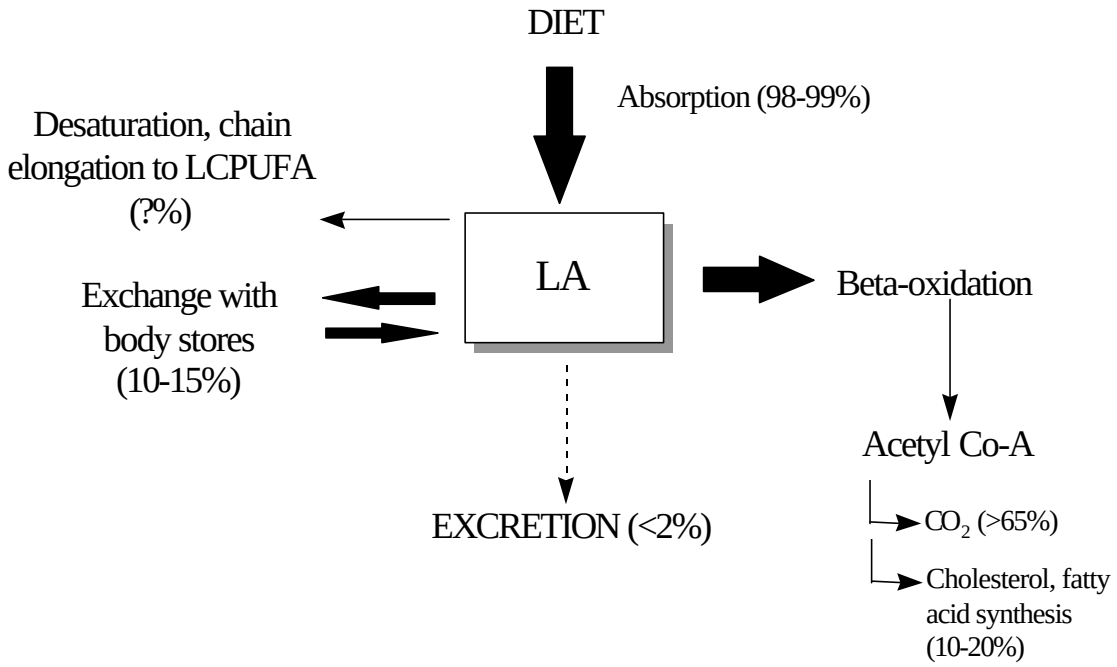


Figure 3. *Metabolic possibilities for LA, showing approximate partitioning among the various routes in parentheses. This scheme would apply to young male rats consuming LA at 2% of energy as the only n-6 fatty acid in the diet. Adapted from [3].*

Different clinical symptoms mark n-6 and n-3 deficiencies (Table 1). In human infants, signs of n-6 deficiency include dermatitis involving dryness, desquamation, and thickening of the skin, often accompanied by unsatisfactory growth, fatty liver and impaired water balance [43]. The skin symptoms and water loss are both examples of a general derangement of membrane structure and consequent function. There is an increase in capillary permeability and fragility. Erythrocytes become more fragile and susceptible to osmotic hemolysis [15]. In LA deficiency, clinical symptoms disappear shortly after the introduction of feeding diets providing 2% or more of energy as LA [42-44]. The minimal dietary amount of LA to prevent a deficiency state is about 5% of total calories for adults and 2% for pediatric patients [15].

Table 1. *Characteristics of n-6 and n-3 essential fatty acid deficiencies [34]*

	n-6	n-3
Clinical features	Growth retardation	Reduced learning capacity
	Skin lesions	Abnormal electroretinogram
	Reproductive failure	Impaired vision
	Fatty liver	Polydipsia
	Polydipsia	Numbness
	Brittle nails	Leg pain

In contrast to the more obvious clinical symptoms of n-6 fatty acid deficiency, evidence of n-3 deficiency are more subtle. The fur and skin of rats on a n-3 deficient diet do not show the conspicuous dermatitis found in n-6 fatty acid deficiency, nor does fatty liver occur. The first case involving ALA deficiency in humans was described in 1982 by Holman et al. [45]. A six-year old girl maintained for five months on TPN, which included a safflower oil emulsion rich in LA, but poor in ALA, experienced episodes of weakness, numbness, parethesia, inability to walk, leg pain and blurred vision. Serum analysis revealed very low levels of ALA and n-3 LCPUFAs. TPN was then changed to include soybean oil emulsion, which contains both LA and ALA. Within three months, all of the symptoms of deficiency disappeared. From this case study, the requirement for ALA was estimated to be 0.5-0.6% of calories. Bjerve et al. [46] described ALA deficiency in four adults fed by gastric tube and estimated the minimal daily requirement of ALA to be 0.2-0.3% of calories. There is evidence of the importance of n-3 LCPUFAs in retinal and brain function in rats and in rhesus monkeys [34,47]. A diet deficient in n-3 fatty acids leads to visual impairment, abnormalities of the electroretinogram, and polydipsia [48-50].

In general, EFAD in humans has been suggested to be implicated in behavioral and learning disorders [51-53], immunological impairment [54,55], and cardiovascular and neoplastic diseases [56-58]. The signs or symptoms of EFAD will depend on the extent of the deficiency and overall nutritional status, for example, whether there are concurrent deficiencies of vitamins or minerals [59]. Siguel and Lerman [60] have devised the term ‘EFA insufficiency’ to refer to patients who have fatty acid profiles with abnormalities in the same direction as those found in EFAD subjects, namely an enhanced conversion of n-3, n-6 and n-9 fatty acids to their derivatives and an accumulation of monounsaturated fatty acids. However, the degree of the biochemical abnormality is smaller than in EFAD and there is less evidence of the traditional clinical signs associated with EFAD such as dermatitis, impaired wound healing, impaired growth, neurological abnormalities, decreased learning ability, and histological abnormalities in most tissues [60,61]. According to Siguel and Lerman, EFA insufficiency is suspected of contributing to hyperlipidemia, hypertension, coronary heart disease, impaired wound healing and cell reproduction, and abnormal eicosanoid metabolism [60].

Biochemical assessment of essential fatty acid status

Certain biochemical markers have been used either alone or in conjunction with others to

indicate EFA status in the body (Table 2). Generally, animal studies using various amounts of LA in the diet, ranging from 0-4%, have demonstrated that as the caloric intake of LA increases, the fatty acids derived from it, such as AA, relatively increase and other fatty acids decrease [62,63]. Similarly, as dietary intake of ALA increases, the n-3 fatty acids increase and other fatty acids decrease. Thus, the total n-6 and n-3 are positive indicators of intake of LA and ALA, respectively. When concentrations of n-3 and n-6 fatty acids are low in the body, high amounts of n-9 long-chain fatty acids are produced via a microsomal enzyme system that allows further desaturation and chain elongation. Data from animal studies indicate that the binding affinity of fatty acids to the first enzyme in the cascade, delta-6-desaturase, is highest for ALA, high for LA and lowest for oleic acid (OA, 18:1n-9) [64]. Therefore, little OA is desaturated when EFAs are readily available. Only at low concentrations of LA and ALA is there appreciable conversion of OA to its long-chain metabolite, eicosatrienoic acid (Mead acid, MA, 20:3n-9); hence, an increase of this metabolite is considered to indicate deficiency of LA and ALA [65]. The increase of MA relative to that of AA, known as the triene:tetraene ratio (20:3n-9/20:4n-6), is the classical marker of EFAD introduced in 1960 by Holman [65]. This ratio is not useful in isolated deficiency of only n-6 or n-3 fatty acids because an adequate supply of either LA or ALA will prevent synthesis of MA. Although it is presently the classical method of determining EFAD, conclusions drawn from the triene:tetraene ratio may not provide a completely accurate analysis of EFA status [62]. Many investigators have used both the triene:tetraene ratio together with a plasma profile of LCPUFAs in determining EFA status, which reveals disproportions in the EFAs (n-6, n-3) and nonessential fatty acids (n-9, n-7).

Table 2. *Commonly used markers to measure essential fatty acid status in plasma*

Marker	Description	Deficiency	Reference
Triene:tetraene ratio	20:3n-9/20:4n-6	> 0.2	[65]
Molar percentage n-6 fatty acids	(Total n-6 fatty acids/ total fatty acids) * 100	↓	[35,66]
Molar percentage n-3 fatty acids	(Total n-3 fatty acids/ total fatty acids) * 100	↓	[35,66]

Both essential and nonessential fatty acids are compartmentalized into the various lipid classes, each of which has its unique composition and selectivity for incorporation of fatty acids. To describe the fatty acid composition of all the lipid classes, including phospholipid, triacylglycerol, cholesterol ester, free fatty acids, would perhaps be the best assessment of LCPUFA status, but with current methods this is not practical [62]. Modern capillary gas chromatography permits separation and measurement of nearly all the LCPUFAs which occur in these tissue lipids. According to Holman [62], total phospholipids in plasma are the richest lipid class with respect to LCPUFAs and respond in easily measurable fashion to changes in dietary EFAs. He suggests that the LCPUFA pattern of serum or plasma phospholipids as a group is probably the best practical single measure of LCPUFA status, although analysis of cholesteryl esters, triacylglycerols, and free fatty acids contribute additional information. Some

researchers prefer to measure long-term status of EFA and LCPUFA in erythrocyte membrane phospholipids due to their long half-lives. On the contrary, Siguel and Lerman [60] contend that EFAs within phospholipids remain relatively constant over a wide range of EFA status, whereas whole plasma is the net result of short-term factors such as diet, exercise and antioxidant status. Thus, in their opinion, phospholipids are poor indicators of EFA status except in severe EFAD [60].

It is difficult to draw an overall conclusion about EFA status from only measuring plasma fatty acids since it may not reflect the effect of EFAD in every tissue. Each tissue has its own LCPUFA metabolism and responds specifically to the dietary fatty acid composition [19] (Table 3). This idea is in agreement with Moussa et al. [67] who found that the effects of EFA-deficient diets on n-6 fatty acid levels in rats vary according to the organs studied. A better approach would be to measure fatty acid status in cells that specifically require EFA for functioning such as those found in brain, retina, and testes, when warranted. LCPUFA status may be measured by the percentage of LCPUFAs in total fatty acids of lipids, also known as the molar percentage, rather than by the absolute concentrations of LCPUFAs in the tissue or fluid since properties of membranes seem to be influenced by the proportion of fatty acids in the membrane rather than by the absolute concentration [60,62].

Table 3. *Comparison of the relative content of total n-6 and n-3 fatty acyl chains in various mammalian tissues and fluids [19]*

Tissue	% of total		Approximate ratio
	n-6	n-3	(n-6/n-3)
Retina	10-14	27-36	1:3
Spermatozoa	8-12	32-36	1:4
Testes	20-45	6-10	4:1
Liver	20-24	6-10	4:1
Heart	35-40	8-14	4:1
Kidney	35-40	6-8	5:1
Adipose	4-22	1-3	5:1

1.3 Intestinal absorption of (essential) fatty acids

On average, adult Western diets contain at least 100 g (40% of energy) of lipid, of which 92 to 96% is long-chain triacylglycerols (TAGs) [68,69]. The remainder consists of cholesterol esters, plant sterols and phospholipids [70]. The overall process of lipid absorption can be classified as a chain of events occurring after lipid ingestion, including lipolysis, solubilization, uptake into the enterocyte, re-esterification and transport into the lymph or portal blood (Fig. 4). The relative importance of each step depends strongly on the dietary fatty acid species. Under physiological conditions, the efficacy of lipid absorption ranges from 96-98% [68,71]. Various excellent reviews have appeared on the mechanisms involved in physiological absorption of dietary lipids in general [68,72-75]. However, information specific to the absorption of EFA

under physiological conditions is quite limited. Although EFA absorption does not differ in many ways from overall lipid absorption, some characteristic differences apply and are discussed shortly.

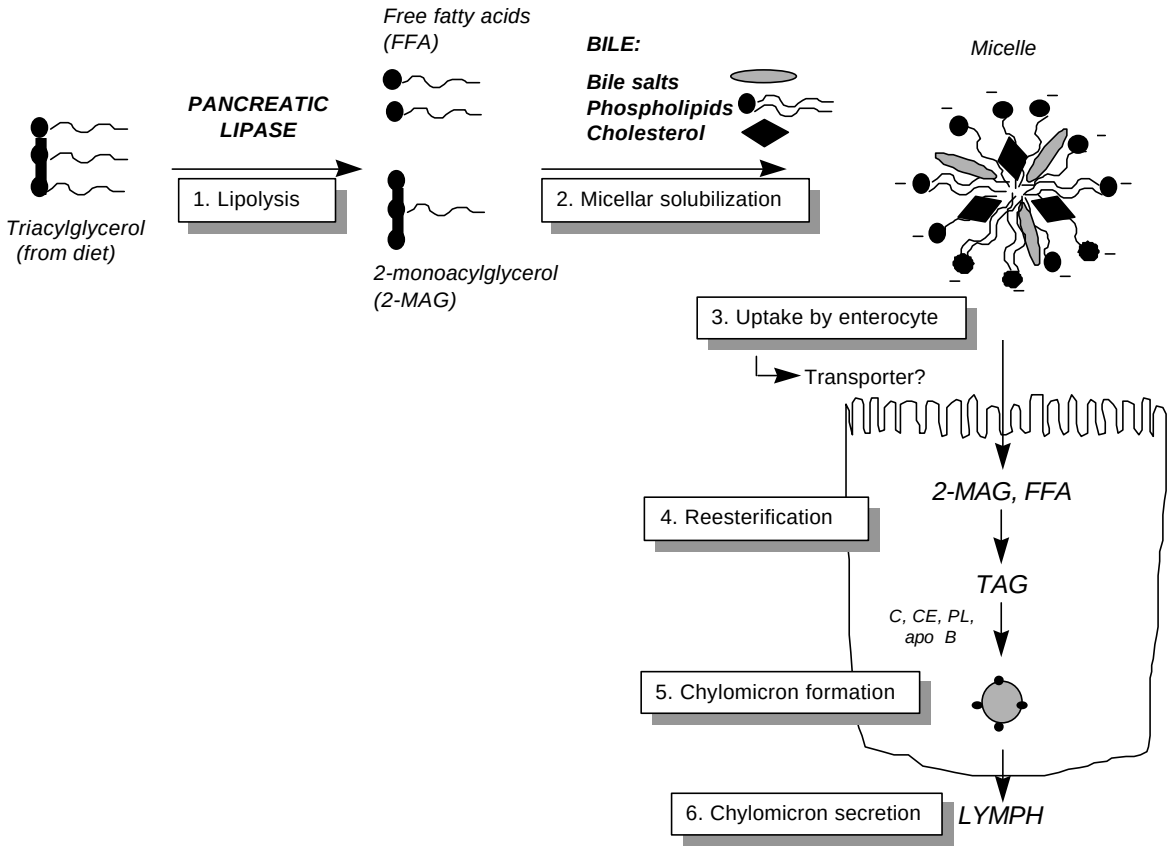


Figure 4. *Diagrammatic representation of the major steps in lipid absorption, as modified from [79]. These include: (1) the lipolysis of dietary triacylglycerol (TAG) by pancreatic enzymes; (2) micellar solubilization of the resulting lipolytic products (FFAs and 2-MAG) by bile salts, phospholipids and cholesterol; (3) uptake of lipolytic products by the enterocyte; (4) reesterification to TAG; (5) packaging of lipids (TAG, cholesterol;C, cholesterol ester, CE; phospholipid, PL;) into a chylomicron particle and addition of apoprotein B (apo B) and (6) secretion of the chylomicron particle into the lymph.*

Lipolysis

About 95% of the lipids in the human diet are composed of TAGs. TAGs require lipolysis at the sn-1 and sn-3 positions to produce two free fatty acids (FFAs) and 2-monoacylglycerol (MAG) for efficient solubilization and uptake into the enterocyte [75]. Hydrolysis of dietary lipids in humans is catalyzed by lipases from gastric and pancreatic origin, with pancreatic colipase-dependent lipase being functionally the most predominant enzyme under physiological conditions [76]. *In vitro* studies using purified pancreatic lipase have demonstrated a potent inhibitory effect of bile salts on lipolysis of TAGs at concentrations above the critical micellar concentration [77,78]. Colipase, a protein found within pancreatic juice, antagonizes the inhibitory action of bile salts. Colipase acts by attaching to the ester bond region of the TAG molecule. In turn, the lipase binds strongly to the colipase by electrostatic interactions, thereby

allowing the hydrolysis of the TAG by the lipase molecule [80]. Colipase is secreted as a procolipase and is converted to the active form through trypsin digestion [81]. The majority of luminal phospholipid is phosphatidylcholine (PC). Both biliary and dietary PC are hydrolyzed in the presence of pancreatic phospholipase A_2 to form lyso-PC and FFAs [74]. Carboxyl ester hydrolase is quantitatively a minor pancreatic enzyme, and is also known as cholesterol esterase, pancreatic nonspecific lipase, human milk lipase, carboxyl ester lipase, monoglyceride lipase, and bile salt stimulated lipase (BSSL) [71,74,82-84]. This enzyme is capable of hydrolyzing cholesterol esters, lipidic vitamin esters, glycerides (sn-1, sn-2 and sn-3 esters), and phospholipids (sn-1 and sn-2). Thus, carboxyl ester hydrolase can hydrolyze sn-2 monoglycerides, which are present in the intestinal lumen as products of pancreatic lipase, thereby completing the hydrolysis of dietary TAGs [74].

The impact of lipase activity on EFA and LCPUFA absorption, especially of EPA and DHA, has been extensively investigated using various lipid formulations (TAG, ethyl ester, FFA) and measuring subsequent appearance in the lymph [85-89]. This approach may not correctly quantify differences in lipolysis since lipolysis could be only one of rate-limiting steps for appearance into the lymph. Apart from this reservation, various studies are in agreement with the finding that longer chain fatty acids (>C20) are lipolyzed at a slower rate than shorter chain fatty acids (≤ C18) [83,86,90]. To optimize TAG sn-configurations with respect to EFA absorption, the influence of the sn-position on lipolysis, as measured by subsequent secretion into lymph, was investigated in several animal studies [76,91,92]. After intraduodenal administration, EFAs at the sn-2 position, compared with sn-1 and sn-3, have the highest absolute recovery in the lymph. Compared to all other configurations, intraduodenally administered TAG with LA in the sn-2 position made the most rapid appearance in lymph [93]. Similarly, Christensen et al. [76] found a higher percent recovery of LA in the lymph of pancreatic and biliary diverted rats when administered as sn-2 TAG compared to LA supply at random positions or as soybean oil. These observations may indicate a resistance towards the enzymatic hydrolysis of the sn-1 and sn-3 acyl chains by pancreatic colipase-dependent lipase, since whale oil TAGs, in which EPA and DHA are predominately esterified at the sn-1 and sn-3 positions, have been characterized as being lipase resistant [92]. The reason for this resistance to lipolysis is not known, but it can be speculated that the stereospecific conformation of EFAs and LCPUFAs may prevent efficient binding of lipase to the TAG molecule. Unfortunately, the kinetics of pancreatic lipolysis of EFAs and LCPUFAs from TAGs have not been thoroughly investigated, thereby, it is not possible to adequately compare these fatty acids with regard to lipase activity.

Bile

The presence of bile in the lumen of the intestine appears to be involved in more than one aspect of the absorption of dietary lipids. One of the important properties of bile is its ability to increase the solubility of the lipolytic products, FFAs and MAGs, in the aqueous intestinal lumen by the formation of mixed micelles. The micelles are aggregates of amphiphilic lipids that orient themselves with the hydrophobic regions on the inside of the micelles and the polar groups exposed to the aqueous environment. This macromolecular structure has a high water

solubility. Micellar solubilization increases the aqueous concentration of FFAs and MAGs 100-1000 times [74]. In the absence of or at low concentrations of bile salts, the absorption of fatty acids occurs to a relatively lower and slower extent [94]. It is known that the unsaturated, less hydrophobic structure of unsaturated fatty acids such as EFAs relies less upon bile for solubilization in the intestine compared with saturated long chain fatty acids [73-75,95-97]. This feature is illustrated by the dissociation rate constants for LA from transbilayers in small unilamellar PC vesicles, which are ten and five times greater compared to stearic acid (SA, 18:0) and OA, respectively [98]. Bile salts remain in the lumen of the small intestine to facilitate further digestion. They are finally absorbed in the distal ileum and are transported back to the liver by the portal blood in a cycle that constitutes the enterohepatic circulation [69].

In the absence of bile, unsaturated fatty acids are taken up more efficiently (~80%) than those that are saturated (~50%), though still significantly less efficient than in the presence of bile [99]. Under conditions of intestinal bile deficiency, long-chain saturated fatty acids such as palmitic acid (PA, 16:0) and SA tend to precipitate as insoluble calcium soaps in the feces [99]. Carey and associates have demonstrated the presence of two dispersed phases within the intestinal lumen, a phase of micelles saturated with lipids and cholesterol, and a phase of unilamellar vesicles (liposomes). Unilamellar vesicles may play an important role in the uptake of lipid in bile-salt deficient patients [71]. In an *in vitro* model system, Narayanan and Storch [94] demonstrated that the transfer of FFAs and MAGs is slower from vesicles compared with micelles, and could, in part, explain the slower lipid absorption during intestinal bile-salt deficiency.

In addition to the role of bile in solubilization, strong indications are available that biliary components are utilized for proper intestinal chylomicron assembly and secretion of lipid into the lymph, especially during high intestinal lipid loads [100-103]. Studies utilizing interruption of the enterohepatic circulation via cholestyramine feeding [104] consistently revealed a substantial accumulation of lipid in enterocytes. Under physiological conditions, phospholipids of lymph chylomicrons are derived predominantly from biliary rather than from dietary origin [105,106]. Enrichment of diets with PC was associated with increased biliary phospholipid secretion and increased transport of chylomicrons in rats [107]. Available *in vitro* data also indicate that the amount and species of phospholipid affects intestinal lipoprotein secretion. Using CaCo-2 cells, Mathur et al. [108] found that PC in the apical compartment above a concentration of 250 µM increased secretion of apoB-containing, TAG-rich lipoproteins. Although other phospholipids such as phosphatidylethanolamine and phosphatidylserine were also capable of stimulating lipid secretion, PC produced the greatest effect.

Apart from the obvious roles of biliary components as active participants in micelle and chylomicron formation, it may also be relevant that the acyl chain composition of the PC molecule is highly specific (Table 4). Biliary PC is highly enriched in EFAs, which could be important for a number of reasons relating to EFA absorption. Biliary PC, which compose

approximately 95% of the total biliary phospholipids [105,109], contain up to 40 mol% EFAs and LCPUFAs as measured in humans [110,111] and rats [112]. Daily enterohepatic circulation which provides roughly 12 g of biliary PC (subsequent 95% absorption) can therefore supply 2.3 - 3.4 g of LA and 0.5 - 1.7 g AA [74]. An average Western diet of 2367 kcal (9894 kJ) supplies ± 8.8 g LA and 1.8 g AA/day [113], indicating that biliary PC secretion in the intestine contributes a significant supply of EFAs. It can be speculated that the relatively hydrophilic conformation of EFAs play a role in micellar development. The effect of PC fatty acid composition, particularly of EFA acyl chains, on intestinal lipoprotein assembly or secretion has not been thoroughly studied. Although the mechanism has not been elucidated, apical exposure to luminal PC [114], as well as unsaturated fatty acids [115], has been noted to increase the basolateral secretion of lipid-rich, apo-B containing lipoproteins in polarized CaCo-2 cells, a frequently used *in vitro* model system for intestinal absorption.

Table 4. *Fatty acid composition of biliary phospholipids from suckling rats [109]*

		Mol %	
Fatty acid	PC[a]	PC (sn-1)[b]	PC (sn-2)[b]
10:0	0.14		
12:0	0.11		
14:0	1.67	2.65	0.54
16:0	42.30	79.40	5.56
16:1	1.08	2.47	0.64
18:0	3.32	8.65	0.57
18:1	4.86	2.86	7.19
18:2	27.73	2.89	46.33
18:3	0.18	0.13	0.13
20:0	0.19	0.26	0.69
20:4	13.53	0.42	28.04
22:5	0.47	0.08	0.33
22:6	4.42	0.19	9.77

[a]Phosphatidylcholine
[b]Fatty acids at the sn-1 or sn-2 position of phosphatidylcholine

Uptake into the enterocyte

For any molecule, the rate of uptake by the small intestine will depend on the number of molecules that are available for uptake by the brush border membrane. The mechanism by which fatty acids are taken up by the enterocyte across its apical membrane remains unresolved. Both facilitated [116-119] and passive diffusion processes [118, 120] have been hypothesized for LA translocation across the membrane. Uptake of EFAs and LCPUFAs by facilitated membrane translocation has been suggested to involve a membrane-bound fatty acid binding protein and/or a fatty acid translocase [116, 121]. A saturable uptake of long-chain (PA and OA), but not of short-chain (8:0) fatty acids has been demonstrated on the apical membrane of CaCo-2 cells [122]. In studies on isolated hamster intestinal cells, Gore et al. [123] found

indications that uptake of ALA is carrier-mediated and can be competitively inhibited by LCPUFAs.

Intracellular transport of fatty acids in the enterocyte

After absorption into the enterocyte, dietary lipids migrate to the endoplasmic reticulum. Approximately twenty-five years ago, it was suggested that migration of the lipids is mediated via fatty acid binding proteins (FABP) [124]. Since this observation, various members of the FABP family have been identified and characterized, of which two are located in the intestine, namely, the intestinal FABP (IFABP) and the liver FABP (LFABP). Based on its expression pattern, particularly in absorptive areas (jejunal villi) of the intestine, and on its responsiveness in expression to a high-fat diet [124], IFABP is hypothesized to be involved in the intracellular transport of fatty acids in the enterocyte. *In vitro* studies have indicated that saturated and unsaturated fatty acids [125, 126] can be bound by IFABP with similar, high affinity whereas the binding specificity of LFABP is considerably broader and includes long-chain fatty acids (OA, AA), lyso-PC, retinoids, bilirubin and carcinogens [127, 128]. Indirect support for the role of IFABP and LFABP in the intracellular transport of fatty acids can be derived from recent studies by Baier et al. [129]. A single amino acid substitution (Ala54Thr) in human IFABP is associated with altered binding of fatty acids to IFABP *in vitro* compared with the wild-type IFABP. A two-fold increased transport of PA and OA across CaCo-2 cells compared to that of the wild-type protein was observed. These findings suggest fatty acid binding to IFABP may influence intracellular transport and/or metabolism.

Chylomicron assembly and secretion

The transport of lipids from the small intestine into the systemic circulation requires that they be packaged into a physically stable transport moiety for an aqueous environment. Fatty acids are predominantly reacylated into TAGs inside the enterocyte and assembled into chylomicrons to be excreted and transported into the lymph. Before being transported into the lymph, TAGs are assembled together with phospholipids and apolipoproteins to form a lipoprotein particle. The apolipoproteins synthesized by the human small intestine are apo A-I, apo A-II, apo A-IV and apo B. Apart from stabilizing the surface of the lipoproteins, the apolipoproteins also provide a system of receptor identification that governs which cells in the body receive and metabolize these lipoproteins [69]. As yet, the only protein whose biosynthesis seems to be obligatory for the normal transport of chylomicrons is apo B (apo B48). Although there is but one copy of the apo B gene in the human genome [130], it can exist in two major forms: apoB100, which is of hepatic origin, and apoB48, which is produced by the intestine [131,132]. In the enterocyte, apo B is translocated across the endoplasmic reticulum during translation and lipidated via interactions with microsomal transfer protein [74,133]. Patients lacking the ability to synthesize apo B failed to transport chylomicrons, and the enterocytes in their small intestine are saturated with large lipid droplets [134,135].

The main lipoproteins responsible for transporting dietary fat from the intestine are the chylomicrons. However, the intestine is also able to secrete VLDL, and is estimated to be about

10% of VLDL that appears in the blood [69]. The chylomicrons are bigger than the VLDL (80-500 nm vs. 30-80 nm), and they have a higher content of TAGs (~95% vs. ~60%) [74]. However, the size and composition of the lipoproteins can vary according to the composition of the diet, and the rates of lipid absorption. Thus, with a high intake of dietary fat and at the peak of absorption, the chylomicrons tend to be larger and contain more TAGs than when the rate of lipid transport is lower [69].

The assembly of chylomicrons and VLDL begins with the acylation of absorbed fatty acids into TAGs, which takes place in the smooth endoplasmic reticulum in the apical region of the enterocyte. The main biochemical route for TAG synthesis under physiological circumstances is the MAG pathway, in which 2-MAGs (taken up by the enterocyte) are sequentially reacylated to diacylglycerols (DAGs) and to TAGs by MAG-acyltransferase and DAG-acyltransferase, respectively [74]. The other route of TAG synthesis, the alpha-glycerolphosphate pathway, involves conversion of glycerol-3-phosphate via phosphatidic acid to DAG and, subsequently, to TAG by various enzymes [74]. Under conditions of physiological lipid absorption, in which there is an ample supply of 2-MAG and FFA, the 2-MAG pathway predominates relative to the alpha-glycerophosphate pathway [74,136]. Lyso-PC, the phospholipase A2-metabolite of PC, can be routed to various metabolic pathways within the enterocyte: it can be reacylated to form PC [137-141], hydrolyzed into FFAs and glycero-3-phosphorylcholine [141], or two lyso-PC molecules can react to give PC and glycero-3-phosphorylcholine [142]. The addition of certain lipids to the diet may target the use of specific pathways. LA is preferentially routed into TAG synthesis, however, administration of lyso-PC produces an increase in the percent LA incorporated into the phospholipid fraction [143]. Administration of lyso-PC and [^{3}H]-AA to rats resulted in increased incorporation of [^{3}H]-AA into mucosal PC and increased transport of [^{3}H]-AA in the phospholipid fraction of lymph lipoproteins compared with [^{3}H]-AA administration alone [143].

The newly formed TAGs from either pathway are thought to be metabolically distinct, in that TAG made from 2-MAG are secreted more rapidly across the basolateral membrane compared to those originating from the alpha-glycerophosphate pathway [144]. The DAGs from each pathway have been suggested to enter into separate intracellular pools [74]. This hypothesis is supported by observations that DAGs from the alpha-glycerophosphate pathway are preferentially used for *de novo* synthesis of PC [145]. Although it has not been studied extensively, some indirect indications support selectivity of EFAs in the routing to TAG synthesis relative to PC synthesis [96,143,146]. Ockner et al. [96] compared the absorption of [^{14}C]-PA and [^{14}C]-LA in normal rats and found that the amount of LA esterified into TAG was twice that of PA when expressed as percentages of [^{14}C] in the enterocyte or as percent of [^{14}C] administered.

The TAGs appear in the form of lipid droplets in the cisternae of the smooth endoplasmic reticulum within minutes of exposure of the cells to the micellar lipid in the lumen. The droplets are stabilized by phospholipids and proteins produced in the rough endoplasmic reticulum. As time progresses, the droplets increase in number as the endoplasmic reticulum extends and

pinches off to form vesicles. It is hypothesized that these lipid-filled vesicles fuse with the Golgi apparatus. The nascent chylomicrons and VLDL are then carried to the lateral surfaces of the enterocyte by vesicles in the process of exocytosis. Fusion of the Golgi-derived vesicles and surface membranes occurs, and the chylomicrons and VLDL are secreted into the intercellular spaces which drain into the lymph vessels. The lipoproteins in these vessels pass via the thoracic duct and enter the circulation at the level of the jugular vein [69].

Within the group of unsaturated fatty acids, individual differences in the kinetics of lymphatic transport are evident. After an enteral infusion of a lipid emulsion containing [^{3}H]-LA and [^{14}C]-AA in rats, Nilsson et al. [147] reported that lymph recovery of labeled AA was slower and more extended compared with LA. It was demonstrated that a larger part of administered AA was retained in intestinal phospholipids compared to LA. To a quantitatively minor extent, LCPUFAs have also been shown to be transported portally. By intraduodenal infusion of labeled FFA into rats and subsequent analysis of their portal blood concentrations, Bernard and Carlier [73] reported evidence that the portal route could account for about 6% and 11% of the lymph route of LA and AA, respectively, when infused separately as fatty acids. With mixed infusates, the amount of LA transported portally was increased to 11%. However, Mansbach et al. [101,148] demonstrated that increasing the lipid load and including PC in the infused emulsion promoted lymph transport of enterally infused emulsions containing [^{3}H]-tri-OA. The enteral supply of PC, which may be rate-limiting for chylomicron assembly, led to less portal transport (0.5% for the low lipid load and 1.4% for the high lipid load), indicating that portal transport is relatively minor pathway for long-chain unsaturated fatty acids [101].

1.4 Malabsorption of (essential) fatty acids

Lipid malabsorption, also known as steatorrhea, is characterized by an increased excretion of mostly dietary lipid in the feces. The criterion for a diagnosis of steatorrhea on a 100 g fat challenge test in Europe and in the United States is fecal fat excretion >7 g/day, resulting in a coefficient of fat absorption of ≤ 93% [149]. Severe steatorrhea is defined as fecal fat excretion >20 g/day [149]. Under most conditions, clinical symptoms of steatorrhea can be reduced by limiting the dietary lipid intake. However, as discussed in detail in the previous sections, limiting dietary lipid intake would be disadvantageous since lipids serve energetic and structural purposes. In addition, impaired absorption of lipid soluble vitamins (A,D,E, and K) may lead to specific deficiency syndromes. As Table 5 demonstrates, the adequate supply of lipids to the body can become compromised due to defects at almost every step of lipid absorption, including the following: impaired lipolysis of dietary TAGs, abnormally low concentration of bile salts in the intestinal lumen due to biliary obstruction or liver disease, disturbed uptake of lipolyzed lipids by the enterocyte, and/or accumulation of dietary lipid in the enterocyte.

Table 5. *Abnormalities in lipid absorption [153]*

Steps	Abnormalities	Examples
Lipolysis	↓ Mixing of gastric lipase with substrate	Gastric surgery
	Intraluminal pH too low for lipase activity	Gastrinoma
	Pancreatic duct obstruction	Carcinoma of pancreas
	↓ Lipase synthesis	Chronic pancreatitis, cystic fibrosis
Solubilization by bile salt micelles	↓ bile flow - cholestasis	Hepatitis, alcoholic liver disease, common duct obstruction, extrahepatic bile duct atresia, metabolic liver disease
	Interrupted enterohepatic circulation	Ileal resection
	Altered intraluminal bile salt composition	Bacterial overgrowth
Uptake by enterocytes	Damaged absorptive cells and/or diminished mucosal surface area	Celiac-sprue, tropical sprue, invasive giardiasis, intestinal resection or bypass
Chylomicron formation and secretion	Genetically or pharmacologically decreased activity of microsomal transfer protein (MTP)	Abetalipoproteinemia, MTP inhibitors

Defects in lipolysis of dietary triacylglycerols in the intestinal lumen

Pancreatic lipase is the main enzyme responsible for hydrolysis of dietary TAGs in the intestinal lumen (Section 1.3). After a dietary lipid load, pancreatic juice containing lipase and colipase is secreted in excess into the intestinal lumen by the pancreas. Deficiency of pancreatic lipase is of clinical significance only when its secretion is below 10 to 15% of normal levels [150]. By inference, clinical symptoms due to disorders in lipolysis only become apparent when the decrease in pancreatic function is considerable. Frequently, insufficient pancreatic function interferes with bicarbonate secretion; hence, gastric chyme will not be adequately neutralized in the proximal small bowel [150]. An abnormally low pH inhibits lipase activity. Part of the unabsorbed TAG is split by bacterial lipases in the colon [151], but LCPUFAs are poorly absorbed by the colon [150]. These unsaturated fatty acids, particularly if they are metabolized to hydroxy-fatty acids by bacteria, can change the permeability of the colonic mucosa, resulting in an influx of water into the bowel lumen, causing diarrhea [150]. Conditions in which lipolysis is impaired include cystic fibrosis (CF), chronic pancreatitis,

pancreatic resection, or pancreatic carcinoma [152]. Steatorrhea caused by pancreatic insufficiency can be treated by fat restriction, oral lipase replacement with or without alkali, or of inhibitors of gastric acid secretion, or by substitution of LCPUFAs by medium-chain TAGs [152].

Defects in transport between intestinal lumen and epithelium

Biliary components are needed at more than one step in lipid absorption. Bile salts have been shown to affect the lipolytic activity of pancreatic lipase and to enable efficient transport of dietary lipids to the brush border membrane of the enterocyte after their hydrolysis by lipolytic enzymes (Section 1.3). Conditions in which bile secretion into the intestine is absent or decreased, such as in patients with a biliary fistula, biliary obstruction, chronic liver disease, or an interruption of the bile salt enterohepatic circulation by ileal resection, will result in reduced micellar formation and an expansion of the intraluminal oil phase. However, total absence of bile in the intestine of adults does not completely inhibit lipid absorption. Porter et al. [102] found that bile-diverted adults could absorb up to 80% of their dietary lipid. Furthermore, even at low concentrations of bile salt, the LCPUFAs have been shown to be solubilized more efficiently than saturated and shorter chain fatty acids [96]. As discussed in Section 1.3, an explanation for the high absorption percentage despite the absence of bile in the intestine could be a direct absorption of fatty acids from the expanded liquid crystalline phase [154]. Other important compensatory effects in bile diversion may be increased food ingestion [155] and increased villus and crypt height [156], as demonstrated in rats. However, absorption efficiency for sterols and fat-soluble vitamins was considerably lower, suggesting a higher dependence for these types of lipids on the availability of bile salt micelles in the intestine. Bile salts may arrive in the duodenum in sufficient quantity, but once there, may be altered so that the concentration of effective bile salts is too low. This occurs in chronic bacterial overgrowth of the small intestine where bacterial metabolism (deconjugation) results in more hydrophobic bile salts [153]. As a result, there is an increased loss of lipid in the stools. The concept that bile salts are important for efficient lipid absorption is supported by patients with cholestatic diseases. Cholestasis is functionally defined as an impairment or cessation of bile flow. Patients with a cholestatic condition often present symptoms of lipid malabsorption, such as malnutrition, growth retardation, EFA- and lipid-soluble vitamin deficiencies and steatorrhea [157].

In addition to disturbances in bile formation and/or secretion, lipolytic products may not be taken up by the enterocyte due to defects at the mucosal level. If inadequate cell surface area exists because of insufficient numbers of functional absorptive cells, FFAs and MAGs cannot be absorbed, and steatorrhea results. This is the case with massive intestinal resection or bypass and with a number of diseases in which there is a loss of mucosal surface area including celiac-sprue, tropical sprue and invasive giardiasis.

Therapy is directed toward either restoring the enterohepatic circulation of bile salts by surgical procedures such as hepatoenterostomy, or by dietary treatment with oral bile salts such as ursodeoxycholate in combination with high amounts of EFA-rich TAGs [152].

Defects in chylomicron formation

After their uptake by the enterocyte, FFAs and MAGs are subsequently reacylated into TAGs and assembled into chylomicrons to be excreted and transported into the lymph (Section 1.3). Chylomicrons consist of several components, including a hydrophobic core filled with TAGs, cholesterol and cholesterol ester and a hydrophilic surface coat consisting of phospholipids and apoproteins. Apart from the role of bile in solubilization, strong indications are available that biliary phospholipids are utilized for proper chylomicron assembly and secretion of lipid into the lymph, especially during high intestinal lipid loads [100-103]. Cassidy et al. [104] demonstrated that interruption of the enterohepatic circulation via cholestyramine feeding resulted in a substantial accumulation of lipids in the enterocyte. The individual component responsible for this effect may be the absence of biliary phospholipid for proper surface coat assembly. Under physiological conditions, phospholipids of lymph chylomicrons are derived predominantly from biliary rather than from dietary origin [105,106].

Another cause of defective chylomicron formation is the absence of microsomal triglyceride transfer protein (MTP), a resident lipid transfer protein within the endoplasmic reticulum of enterocytes [158]. It has been demonstrated that patients with abetalipoproteinemia, a recessive genetic disease in humans that is characterized by a defect in the assembly or secretion of plasma VLDL and chylomicrons, lack the MTP protein in the intestinal mucosa [159]. This finding indicates that a defect in MTP is the basis for this disease and the MTP is indeed required for lipoprotein assembly. Consequently, resynthesized TAGs accumulate in the absorptive cell and are unable to pass out of the lateral cell wall into the lacteals of the lymphatic system.

Treatment consists of substituting medium-chain TAGs in the diet, which are rapidly hydrolyzed to fatty acids in the lumen. The absorbed fatty acids are able to pass directly into the portal system, and chylomicron formation is not required. Medium-chain TAGs can supply fatty acids for the energetic demands, however, their ingestion cannot compensate for EFA-related functions.

1.5 Conditions of impaired essential fatty acid status associated with hepatobiliary disturbances

In addition to the many studies focusing on sufficient dietary amounts of EFAs for infants, a recent interest has been generated concerning the occurrence of impaired EFA status in patient groups with hepatobiliary disturbances in common. A high incidence of EFAD has been reported in pediatric patients with cholestasis [157,160-164] and CF [165-169]. Additionally, EFAD in itself can result in changes in bile flow and composition.

Cholestasis

Cholestasis can be defined clinically as a condition of pruritus and/or jaundice with conjugated hyperbilirubinemia, increased activity of serum alkaline phosphatase and liver transaminases,

and dietary lipid malabsorption due to reduced bile flow into the intestine [170]. As a result of the impaired bile entry into the intestine, the degree of fecal lipid excretion is increased. One of the possible parameters to measure the severity of cholestasis is serum bilirubin concentrations [171]. Several studies have demonstrated that young infants with cholestatic liver disease have a marked depletion of LCPUFAs, particularly those of the n-6 series, whereas levels of nonessential fatty acids, such as OA and palmitoleic acid (POA, 16:1n-7) are increased [171]. Bilirubin concentrations have been shown to be unrelated to values for LA and ALA, but correlated inversely with values for their long-chain metabolites [171], which could suggest that patients with cholestatic liver disease have decreased conversion of EFAs to their LCPUFAs. These findings have implications for early neurodevelopment and growth. Reduced availability of these LCPUFAs may contribute to a disturbed eicosanoid balance and immunologic response.

Supplementation of EFA-rich (40 g/100 g) powder plus taurocholate to cholestatic children with EFAD has been successful in normalizing serum LA levels [172]. However, despite surgery (hepatoportoenterostomy) or oral treatment with LA and/or ursodeoxycholate, decreased levels of LCPUFAs, especially AA, continue to persist [162,172,173]. The mechanism for decreased LCPUFA levels in plasma in these patients may be related to a combination of factors, namely malabsorption of dietary lipid, increased oxidation of stored LCPUFAs to meet their high energy requirements, decreased conversion to LCPUFAs and/or enhanced conversion of AA to eicosanoids, such as prostaglandins and proinflammatory leukotrienes.

Cystic fibrosis

Cystic fibrosis (CF), the most common autosomal recessive disorder in Caucasians, is characterized by metabolic, gastrointestinal, pulmonary, and nutritional disturbances [174,175]. Liver disease is increasingly recognized as a major cause of morbidity in CF. About 30% of CF patients at some stage of the disease have hepatobiliary abnormalities such as non-functioning gallbladder, cholelithiasis, liver steatosis, focal biliary fibrosis and/or multinodular cirrhosis [165,176,177]. It is known that CF patients encounter bile disturbances, such as altered bile composition, decreased bile salt secretion by the liver, bile salt precipitation, a decreased bile salt pool size, and/or bile salt inactivation at low intestinal pH [178-182]. CF patients usually have impaired EFA status, especially decreased LA, and increased OA, 16:1n-9, and MA relative to normal values [183,184], which has been related to impaired bile flow. EFAD often complicates the effects of CF in pediatric patients, and it can be difficult to correct [179]. Tissue and plasma fatty acid abnormalities, especially low levels of LA [185-188], are presumably related to malabsorption caused by the pancreatic insufficiency observed in 80 to 95% of CF patients [189-191]. However, it has been suggested that other factors also contribute to decreased plasma concentrations of LA in CF, including an increased utilization of LCPUFAs for energy production [192,193]. LA is absorbed as efficiently in patients with CF as in control patients, albeit with some delay [183]. The hypothesis of an elongation and desaturation defect of LA has been raised in CF [194].

Corn oil, safflower oil, LA-MAG and various lipid infusions have all been used [185,195-199] as a source of LA for long-term supplementation trials, usually lasting one year. Results have been variable, but most have shown minimal [195,197] or no [198,199] improvement in the LA status of the CF patients. However, improvements and even normalization of plasma LA levels have been observed in supplementation studies in which major efforts towards compliance were applied [200,201].

Essential fatty acid deficiency

Decreased bile flow and bile salt secretion rate have been demonstrated in rat models of EFAD. However, a species specificity exists with respect to the effects of EFAD on bile formation. Different animal species vary in biliary lipid output. EFAD rats have a decreased phospholipid and cholesterol secretion rate [202-204] whereas EFAD hamsters have significantly increased biliary cholesterol compared to controls [205,206] and an increased [205] or unchanged [206] biliary phospholipid secretion rate. No values for biliary lipid content have been reported for humans with EFAD. Upon analysis of acyl species composition of biliary PC, it is frequently noted that biliary PC EFA content is decreased in EFAD rats [207,208]. Biliary PC content of LA and AA has found to be decreased in EFAD rats to roughly 10% and 26% of that of controls, respectively [208]. The ability of other fatty acids to be incorporated into biliary phospholipids during EFAD has been noted [207,209-211]. In EFAD, decreasing levels of LA and AA in phospholipids were compensated for by increased amounts of OA and POA [207,208].

1.6 Scope of this thesis

EFAs and LCPUFAs derived thereof are needed for specific functions in the body and cannot be synthesized *de novo* from other lipids. Therefore, they have been referred to as essential components in the diet of humans and all higher animals. A suboptimal status of EFAs and LCPUFAs is frequently noted in patients with intestinal bile deficiency, such as hepatobiliary disease [162,171,212,213]. Despite treatment with EFA-rich TAGs, reduced EFA levels persist. Low plasma concentrations of EFAs and LCPUFAs could contribute to the morbidity and mortality of patients with hepatobiliary disease. Theoretically, impaired status of EFAs and LCPUFAs in plasma may be related to malabsorption, altered metabolism due to changes in desaturation and elongation enzymes and/or oxidation, and/or tissue redistribution of these fatty acids. The mechanism underlying decreased plasma levels of EFAs and LCPUFAs in patients with hepatobiliary disease is unknown. Improvement of the EFA status will prevent negative effects of this condition on morbidity and mortality, and may improve outcome of liver transplantation. Alterations in relative proportions and concentrations of fatty acids in tissues can be related to one or more consequences involved in hepatobiliary disease, including decreased bile secretion into the intestinal lumen, retention of biliary components in the body and hepatocellular injury. Mechanistic information regarding the role of bile in EFA absorption and metabolism could provide insight into designing optimal treatment strategies for improving EFA status.

The aim of this thesis is to obtain mechanistic information on the role of bile and specific biliary components in the absorption and metabolism of dietary lipids, and particularly of the major dietary EFA, LA, with the ultimate goal to refine rational approaches to prevent and treat EFAD in patients with hepatobiliary disease. Particularly, the following topics were addressed:

(1) **The importance of the quantity of bile available in the intestine for quantitative absorption of LA.** Due to the fact that biliary output in the intestinal lumen is negligible in patients with hepatobiliary disease, it has been hypothesized that their decreased EFA levels are due to lipid malabsorption. Previous studies in animal models and in humans have shown that a relatively high percentage (~50-80%) of lipid can be absorbed in conditions of intestinal bile deficiency [99,102,214,215]. However, it is not known to what extent the quantitative absorption, or net uptake, of LA is affected in these conditions. We studied LA absorption and status under two different conditions: bile diversion and bile duct ligation (**Chapter 2** and **Chapter 3,** respectively). The bile-diverted rat allowed for the investigation of intestinal bile deficiency and the bile duct-ligated rat provided these same conditions in addition to the retention of non-secreted, potentially toxic biliary components in the body.

(2) **The relevance of biliary phospholipids for quantitative and qualitative absorption and status of LA.** Biliary PC would seem to be important for maintaining adequate levels of EFAs in the body due to their role in dietary lipid absorption [100-103] and in providing a rich source of EFAs to the intestinal lipid membrane [111,112]. The relevance of biliary phospholipids for the quantitative and qualitative absorption and status of LA was investigated under different dietary conditions (low-fat, high-fat, EFA-deficient) in *mdr2* knockout mice which produce phospholipid-free bile (**Chapters 4-6**).

(3) **The contribution of impaired long-chain fatty acid uptake *per se* to malabsorption in CF patients treated with oral pancreatic enzymes.** CF patients on enzyme replacement therapy continue to experience a certain degree of steatorrhea [180,216,217], with lipid absorption reaching 80-90% of their dietary lipid intake. Additionally, it has been proposed that hepatobiliary dysfunction may be present in these patients [178-182]. In **Chapter 7**, we investigated whether lipid malabsorption in pancreatic enzyme-treated CF patients is caused by insufficient lipolysis of dietary TAGs or by defective intestinal uptake of long-chain fatty acids using LA.

1.7 Models of altered bile flow and secretion

In general, intestinal absorption of LA was investigated in models in which the quantity of bile or its qualitative composition entering the intestinal lumen was altered by surgical or dietary means, transgenic techniques, or by an inherited condition (Table 6).

Table 6. *Characteristics of experimental models*

Model	Retention of biliary compounds in body	Availability of biliary bile salts in intestinal lumen	Availability of biliary phospholipids in intestinal lumen	Species
Bile diversion	-	-	-	Rat
Bile duct-ligation	++	-	-	Rat
mdr2 knockout	+[a]	+	-	Mouse
EFAD				
-Control	(-[c])	(++[c])	+	Mouse
-mdr2(-/-)	(+[c])	(++[c])	-	Mouse
Cystic fibrosis	±[b]	+	+	Human

[a]Previous studies by Smit et al. [218] have demonstrated elevated bilirubin in serum of all *mdr*2 (-/-) mice, however the increase is rather variable, ranging from 3- to 20-fold in individual mice. Increases have also been noted in alkaline phosphatase (3-fold), aspartate aminotransferase (4-fold), alanine aminotransferase (5.8-fold).
[b]Abnormalities of liver function occur in 30% of patients with CF. These patients have significant increases in serum bile acids [176].
[c]To be determined in present thesis.

1. Bile-diverted rat (**Chapter 2**)

The effects of intestinal bile deficiency *per se,* without accumulation of non-secreted compounds in the body, on LA status and absorption were evaluated in permanently bile-diverted rats equipped with permanent catheters in bile duct, jugular vein and duodenum, as described by Kuipers et al. [155]. This experimental model allows for physiological studies in unanesthetized rats with long-term bile diversion without the interference of stress or restraint. After surgery, catheters in bile duct and duodenum can be either connected, to restore the enterohepatic circulation (control rats), or chronically interrupted (bile-diverted rats). Under these conditions, absorption of LA can be measured by administration of a lipid bolus via the duodenal catheter, followed by blood sampling via the jugular vein catheter.

2. Bile duct-ligated rat (**Chapter 3**)

It has not yet been determined whether the mechanism of decreased LA levels in pediatric patients with cholestatic liver disease is related to malabsorption of dietary LA (possibly due to intestinal bile deficiency), changes in metabolism of LA and/or a redistribution of LA in tissues (possibly due to the accumulation of non-secreted compounds in the body and its consequences). The classical rat model of bile duct-ligation lends itself ideally to address the

effect of short-term extrahepatic cholestasis on intestinal LA absorption and EFA status *in vivo*. Obstructive cholestasis can be experimentally induced in rats by ligation of the common bile duct. This procedure results in the disruption of the structural and functional integrity of hepatocellular tight junctions and in alterations of various intracellular processes. During the initial stages of cholestasis, there is an elevation in serum bile salts, bilirubin and liver transaminases [219]. For our studies, we used one-week bile duct-ligated rats to exclude the potential effects of advanced biliary obstruction. Both the rat model of bile diversion (see above) and bile duct-ligation are similar in that they result in the complete absence of bile in the intestine. However, bile diversion is different in that there is no influence of accumulation of non-secreted compounds in the body on the parameters studied.

3. Mdr2-P-glycoprotein-deficient mouse (**Chapters 4-6**)

The importance of the biliary secretion of PC in the intestinal lumen for the status, absorption and metabolism of dietary lipids in general, and specifically of LA, was investigated in knockout mice. Mice lacking the *mdr*2 gene product in the bile canalicular membrane, also known as *mdr*2 knockout (-/-) mice, have recently become available for studying the effects of normal bile salt secretion rates accompanied by the virtual absence of biliary phospholipid and cholesterol secretion [218]. The *mdr*2 (-/-) mice eventually develop cholangitis and liver-related abnormalities due to the damaging effects of phospholipid-free bile on the bile canaliculi and ducts [218].

4. Essential fatty acid-deficient mouse (**Chapter 6**)

In order to discern the roles of bile salt and phospholipid secretion in EFAD-induced lipid malabsorption, an animal model is necessary. Mice lacking *mdr*2 gene product in the bile canalicular membrane (see above), have recently become available for studying the effects of phospholipid-free bile. The relevance of absent biliary phospholipid secretion in EFAD was investigated using *mdr*2 knockout mice. Control mice were used to examine the effects of EFAD on bile salt and phospholipid secretion.

5. *Patients with cystic fibrosis (CF)* (**Chapter 7**)

Pediatric patients with CF often have impaired plasma levels of EFAs and LCPUFAs in addition to bile disturbances, such as altered bile composition, decreased bile salt secretion by the liver, bile salt precipitation, a decreased bile salt pool size, and/or bile salt inactivation at low intestinal pH [178-182]. About 30% of these patients have abnormalities of liver function, which leads to significant increases in serum bile salts. Patients with CF are also known to have hepatobiliary abnormalities such as non-functioning gallbladder, cholelithiasis, liver steatosis, focal biliary fibrosis and/or multinodular cirrhosis [164]. We questioned whether LA absorption was related to overall lipid absorption in these patients, or alternatively, whether specificity in LA absorption and/or metabolism would apply in CF patients.

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CHAPTER 2

Decreased linoleic acid concentration in the bile-diverted rat is not due to decreased uptake of dietary linoleic acid

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Abstract

Decreased bile secretion into the intestine has been associated with low plasma concentrations of essential fatty acids (EFA) in humans. We studied the mechanism behind this relationship by determining the status and absorption of the major dietary EFA, linoleic acid (LA), in control and 1 wk bile-diverted rats. The absorption of LA was quantified by a balance method and by measuring plasma concentrations of [^{13}C]-LA after its intraduodenal administration. Absolute and relative concentrations of LA in plasma were decreased in bile-diverted rats (P<0.01 and P<0.001, respectively). Fecal excretion of LA was increased at least 20-fold in bile-diverted rats (0.72 ± 0.11 mmol/d vs 0.03 ± 0.00 mmol/d; P<0.0001). Due to increased chow ingestion by bile-diverted rats, net intestinal absorption of LA was similar between bile-diverted and control rats (1.96 ± 0.14 mmol/d vs 1.91 ± 0.07 mmol/d, respectively; P>0.05). After intraduodenal administration of [^{13}C]-LA, plasma concentrations were approximately 3-4 fold lower in bile-diverted rats for at least 6 h (P<0.001). Plasma concentrations of both [^{12}C]- and [^{13}C]-arachidonic acid were increased in bile-diverted rats (P<0.05). We conclude that decreased plasma concentrations of LA in 1 wk bile-diverted rats are not due to decreased net intestinal absorption of LA, but may be related to increased metabolism of LA.

Introduction

Since essential fatty acids (EFA) cannot be synthesized *de novo* by mammals, their concentration in the body depends on adequate ingestion and efficient intestinal absorption of EFA from exogenous sources. In case of insufficient dietary supply, decreased absorption and/or increased metabolism of EFA, a deficiency may develop, eventually leading to serious clinical symptoms [1-3]. A decreased absorption of EFA has been implied to cause impaired EFA status when bile secretion into the intestine is decreased or absent, such as in cholestatic conditions [4-6]. To determine if indeed the lack of bile flow to the intestine can be *per se* responsible for changes in EFA status, we studied the status and absorption of linoleic acid (LA), the major dietary EFA, in a rat model of bile diversion, in the absence of cholestatic liver injury. This rat model allows absorption to be studied under physiological conditions (normal bile secretion into the duodenum) and under conditions of permanent biliary drainage (lack of bile secretion into the duodenum) [7]. The absorption of LA was quantified by a balance method and by measuring plasma concentrations of [^{13}C]-LA after its intraduodenal administration. Using this approach, we were able to demonstrate that the diversion of bile from the intestine in the absence of cholestatic liver injury does affect the plasma concentration of LA, but does not influence the net absorption of LA from the intestine.

Materials and methods

Animals and diets

Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands), weighing 300-350 g (mean ± SD: 327 ± 19 g), were kept in an environmentally controlled facility with diurnal light cycling and free access to chow, tap water, and, in the case of bile-diverted rats, saline. Experimental protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen.

Experimental procedures

Rats were individually-housed in metabolic cages and fed a high-fat chow (35 en% lipid; major long-chain fatty acid composition as measured by gas chromatography: 16:0, 31.9%; 18:0, 5.2%; 18:1n-9, 32.7%; 18:2n-6, 30.2%) (Hope Farms BV, Woerden, The Netherlands). After 1 week, rats were equipped with permanent catheters in the right jugular vein, bile duct and duodenum, as described by Kuipers et al. [7]. This experimental model allows for physiological studies in unanesthetized rats with long-term bile diversion without the interference of stress or restraint. After surgery, catheters in bile duct and duodenum were either connected, to restore the enterohepatic circulation (control rats) (n=13), or were chronically interrupted (bile-diverted rats) (n=9). Animals were allowed to recover from surgery for 6 days.

On day 7, 1.67 mL lipid/kg body weight was slowly administered as a bolus via the duodenal catheter. Medium chain triglyceride oil was included in the bolus, as a carrier oil in order to

have a sufficient amount to allow a reliable, reproducible delivery of the label. Specifically, the lipid bolus was composed of olive oil (25% v/v; fatty acid composition: 16:0, 14%; 18:1n-9, 79%; 18:2n-6, 8%) and medium chain triglyceride oil (75% v/v; composed of extracted coconut oil and synthetic triglycerides; fatty acid composition: 6:0, 2%; 8:0, 50-65% max.;10:0, 30-45%; 12:0, 3% max.) and contained 6.7 mg [U-^{13}C]-LA/kg body weight (Martek Biosciences Corporation, Columbia, MD, USA; [U-^{13}C]-LA was 99% enriched with a chemical purity exceeding 97%). The lipid bolus represented <10% of the daily lipid intake for control and bile-diverted rats. Blood samples (0.2 mL) were taken from the jugular cannula at baseline and hourly for 6 h after administration of the label, and were collected into tubes containing heparin. A blood sample for quantification of [^{13}C]-LA and [^{13}C]-arachidonic acid was taken at 24 h after label administration. 24 h was selected as a time point since absorption of the label was demonstrated to exceed 90% of the amount absorbed in 48 h. Plasma was separated by centrifugation (10 min, 2000 rpm, 4ºC) and stored at -20ºC until further analysis. Feces was collected in 24 h fractions starting 24 h before label administration and ending 48 h afterwards (72 h total). Feces samples were stored at -20ºC prior to analysis. During bile diversion, bile was collected in 6 h increments for 24 h using a fraction collector. Chow ingestion was documented for 72 h by daily weighing of the chow container. At the end of the experiment, rats were sacrificed and small intestines were removed (n=3 per group, at random) and stored at -80ºC for further histological analysis.

In order to determine whether the observed effects could be attributed to the charged character of the lipid molecules, such as in fatty acids, a set of control experiments was performed with an uncharged lipid, retinol. Water-soluble retinol (50,000 IU) in an olive oil bolus was administered via the duodenal catheter to control and bile-diverted rats (n=4 per group), after which blood samples (see above) were taken hourly up to 6 h.

Analytical techniques

Plasma and biliary lipids. Total plasma lipids (triacylglycerols, phospholipids, etc.) and biliary phospholipids were extracted, hydrolyzed and methylated according to Lepage and Roy [8]. In order to account for losses during lipid extraction, heptadecanoic acid (17:0) was added to all samples as an internal standard before the start of the extraction and methylation procedure. Resulting fatty acid methyl esters were analyzed by gas chromatography to measure total and individual amounts of major fatty acids. Cumulative fatty acid concentrations were calculated using the sum of the area of major fatty acid (>90%) peaks (16:0 + 18:0 + 18:1n-9 + 18:2n-6 + 20:4n-6). For plasma and bile, relative fatty acid concentrations (molar percentages) were calculated by expressing the area of each individual fatty acid as a percentage of the sum of the area of major fatty acids (16:0 + 18:0 + 18:1n-9 + 18:2n-6 + 20:4n-6).

Methylated plasma samples were analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to measure the [^{13}C]-enrichment of LA and arachidonic acid. The concentration of [^{13}C]-fatty acid in plasma at each time point was determined from the fatty acid concentration and [^{13}C] enrichment and expressed as a percentage of the dose administered per milliliter plasma (% dose/mL). Metabolism of [^{13}C]-LA to [^{13}C]-arachidonic

acid was expressed as a ratio between the % dose [^{13}C]-arachidonic acid/mL plasma and the % dose [^{13}C]-LA/mL plasma at 24 h after [^{13}C] bolus administration.

Chow and fecal lipids. After freeze-drying and mechanically homogenizing, aliquots of high-fat chow and feces were extracted, hydrolyzed and methylated [8]. Heptadecanoic acid (17:0) was added to all samples as an internal standard before the extraction and methylation procedure. Resulting fatty acid methyl esters were analyzed by gas chromatography to calculate ingestion and fecal excretion of major fatty acids, including LA. Fatty acid methyl esters were analyzed by GC-C-IRMS to calculate the enrichment of [^{13}C]-LA. Total fecal lipid excretion was calculated as the sum of excretion rates of the major long-chain fatty acids (16:0 + 18:0 + 18:1n-9 + 18:2n-6). Total fecal lipid (LA) excretion was expressed as mmol/day. The percentage of lipid (LA) absorption was calculated from daily lipid (LA) ingestion and daily lipid (LA) excretion and expressed as a percentage of the daily lipid (LA) ingestion.

% total dietary lipid (LA) absorbed =

$$\frac{\text{Daily lipid (LA) ingestion (mol) - Daily fecal lipid (LA) output (mol)}}{\text{Daily lipid (LA) ingestion (mol)}} \times 100\%$$

Net lipid (LA) absorption was calculated as the molar ingestion minus the molar excretion of lipid (LA). Using the ingestion and fecal excretion of [U-^{13}C]-LA, identical calculations were performed to measure the percentage and the net amount of [U-^{13}C]-LA absorbed by the intestine.

Gas liquid chromatography. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard gas chromatograph Model 6890 with a 50 m x 0.32 mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA, USA). The oven temperature was programmed from an initial temperature of 160°C to a final temperature of 290°C in 3 temperature steps (160°C held 2 min; 160-240°C, ramp 2°C/min, held 1 min; 240-290°C, ramp 10°C/min, held 10 min). Fatty acids were quantified using heptadecanoic acid (17:0) as internal standard.

[^{13}C]-enrichments of fatty acid methyl esters were determined by GC-C-IRMS (Delta S/GC Finnigan MAT, Bremen, Germany). Separation of the methyl esters was achieved on a 50 m x 0.32 mm CP-SIL 88 capillary column (Chrompack, Middelburg, The Netherlands). The gas chromatograph oven temperature was programmed from an initial temperature of 80°C to a final temperature of 225°C in three temperature steps (80°C held 1 min; 80-150°C, ramp 30°C min $^{-1}$; 150-190°C, ramp 5°C min $^{-1}$; 190-225°C, ramp 10°C min $^{-1}$, held 5 min).

$^{12}CO_2^+$ and $^{13}CO_2^+$ ions were measured at m/z 44 and 45. Correction for ^{17}O was achieved through measurement of ^{18}O abundance at m/z 46 [14]. [^{13}C] abundance was expressed as the $^{13}C_{PDB}$ value, i.e., the difference between the sample value and the reference compared to the

Pee Dee Belemnite limestone. $^{13}C_{PDB}$ values were converted to atom % [^{13}C] values. Enrichment was expressed by subtracting the baseline [^{13}C] abundance from all enriched values. The enrichment (atom % excess) was converted to mol % [^{13}C] fatty acid. The [^{13}C] fatty acid concentration was calculated from the fatty acid concentration and mol % [^{13}C] fatty acid. The concentration of [^{13}C] fatty acid in plasma was then expressed as the percentage of the dose administered per milliliter plasma (% dose/mL).

Retinyl palmitate. Retinyl palmitate concentration in 50 µL plasma samples was determined after two extractions with hexane [9]. Retinyl acetate was added to plasma samples before lipid extraction as an internal standard. Samples were resuspended in ethanol (15 µL) and analyzed by reverse-phase HPLC using a 150 x 4.6 mm Symmetry RP18 column (Waters Corp., Milford, MA, USA) [10]. Peak area of retinyl palmitate was normalized to that of retinyl acetate. At each time point, concentrations were expressed as µmol retinyl palmitate/mL plasma.

Intestinal lipids and histology. Intestinal mucosa fractions were obtained by scraping proximal and distal sections of the small intestine. Triglyceride concentrations in mucosal tissue were determined after lipid extraction [11] as described previously [12]. Specimens (~0.5 cm) of duodenum, jejunum, and ileum of control and bile-diverted rats were fixated in 4% paraformaldehyde (v:v). Cross-sections of the tissues were embedded in paraffin, microsectioned and processed in a routine manner for histologic examination with hematoxylin and eosin stain.

Statistics

The experimental data are reported as means ± S.E.M. for the indicated number of animals per group. Differences between control and bile-diverted groups were calculated using the two-tailed Student's t-test for unpaired data. Significance was considered at $P<0.05$.

Results

Body weight and food ingestion

During the bolus and fat balance experiments, there was no significant difference in body weight between bile-diverted and control rats (321.7 ± 7.8 g vs 335.8 ± 4.6 g; $P>0.05$). Mean values revealed that bile-diverted rats ingested considerably more chow than control rats (20.3 ± 1.2 g vs 14.7 ± 0.6 g; $P<0.001$; equal to 4283 kcal/kg chow).

Absolute and relative linoleic acid (LA) concentrations in plasma

Already after 1 week of bile diversion, the total concentration of major fatty acids was decreased compared to control rats (5.43 ± 0.30 mM vs 6.77 ± 0.47 mM; $P<0.05$). LA concentration was significantly lower in bile-diverted rats compared with control rats (0.90 ± 0.08 mM vs 1.45 ± 0.12 mM; $P<0.01$). Also, the molar percentage (Fig. 1) was decreased in bile-diverted rats compared with control rats (16.37 ± 0.79% vs 21.18 ± 0.44%; $P<0.001$).

Absolute concentrations of arachidonic acid were not significantly different between bile-diverted and control rats (1.57 ± 0.09 mM vs. 1.47 ± 0.12 mM, $P>0.05$), but its relative concentration was increased in bile-diverted rats (29.21 ± 1.40% vs 21.91 ± 1.40%; $P<0.01$) (Fig. 1). The triene:tetraene ratio (20:3n-9/20:4n-6, biochemical indicator of EFA deficiency) was significantly higher in the bile-diverted rats (0.020 ± 0.002 vs 0.011 ± 0.001; $P<0.01$), although values remained considerably below 0.2, the accepted threshold cutoff value for EFA deficiency in humans [13].

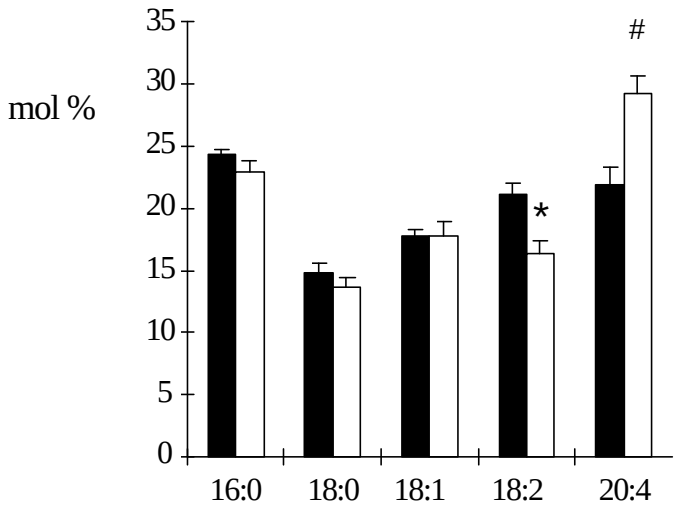

Figure 1. *Relative concentrations (mol%) of major fatty acids (16:0, 18:0, 18:1n-9, 18:2n-6, 20:4n-6) in plasma of control (closed squares) and bile-diverted (open squares) rats. Data are means ± S.E.M of 13 control and 9 bile-diverted rats. Symbols indicate significant difference between control and bile-diverted rats (*P<0.001, # P<0.01).*

Calculation of daily linoleic acid (LA) supply to the intestine via bile

For accurate fat balance measurements, we quantified LA in chow and in biliary phospholipid. The high-fat chow contained 0.15 µmol LA/mg chow, therefore providing on average (mean chow intake for control rats: 16.3 ± 0.5 g) about 2.4 millimoles LA/day. The LA content of bile from male Wistar rats fed high-fat chow was 21.8 ± 1.0 mol% (n=4). Using the biliary phospholipid secretion rate in rats under physiological conditions as determined by us previously [14] (569 nmol/min/kg), total daily LA input into the intestine via bile for a 300 g rat was calculated to be 0.11 millimoles. Using these values, the amount of LA in the intestine delivered via bile is <5% of that provided by the high-fat, LA-rich chow. Therefore, the contribution of biliary LA to the LA balance was relatively minor.

Lipid balance

In Table 1, dietary lipid absorption data of control and bile-diverted rats are shown. Bile-diverted rats excreted significantly increased amounts of lipid and of LA in feces compared to control rats ($P<0.0001$). Although the percentages of lipid and LA absorption were decreased in bile-diverted rats by 38 and 22%, respectively, when compared to control rats ($P<0.0001$), net lipid and net LA absorption values remained similar ($P>0.05$). In Table 2, the lipid balance data for [^{13}C]-LA are presented for control and bile-diverted rats. No quantitative differences in excretion or net absorption of the labeled substrate by the intestine were found between control and bile-diverted rats under the conditions studied.

Table 1. *Dietary lipid and linoleic acid (LA) balance data for control and bile-diverted rats*

	Ingestion (mmol/d)	Fecal excretion (mmol/d)	Absorption (%)	Absorption (mmol/d)
I. Total lipid [#]				
Control	6.59 ± 0.23	0.41 ± 0.04	93.8 ± 0.8	6.18 ± 0.25
Bile-diverted	9.05 ± 0.47*	3.72 ± 0.37**	58.6 ± 4.1**	5.33 ± 0.50
II. LA				
Control	1.94 ± 0.06	0.03 ± 0.00	98.5 ± 0.3	1.91 ± 0.07
Bile-diverted	2.68 ± 0.14*	0.72 ± 0.11**	73.3 ± 4.1	1.96 ± 0.14

Data are means ± S.E.M. of 13 control and 9 bile-diverted rats. Mean values represent the average of 3 days per rat. Included in these 3 days is the experimental day in which the lipid bolus was administered. [#] *Total lipid includes 16:0, 18:0, 18:1n-9 and 18:2n-6. * P<0.001, ** P<0.0001.*

Table 2. *^{13}C-linoleic acid (LA) balance data for control and bile-diverted rats*

	Amount administered (µmol)	Fecal excretion (µmol/48 h)	Absorption (%)	Absorption (µmol/48 h)
Control	7.44 ± 0.12	0.19 ± 0.05	97.3 ± 0.7	7.25 ± 0.15
Bile-diverted	0.57 ± 0.20	0.57 ± 0.20	92.5 ± 2.6	6.83 ± 0.26

Data are means ± S.E.M. of 13 control and 9 bile-diverted rats. No statistical significance noted in any category between groups.

Plasma [^{13}C]-linoleic acid (LA) concentrations

We studied absorption kinetics by determining the appearance of [^{13}C]-LA in plasma after its duodenal administration. Figure 2 shows the time course of [^{13}C]-LA appearance in plasma after intraduodenal administration of [^{13}C]-LA to control and bile-diverted rats, respectively. In control rats, plasma [^{13}C]-LA concentrations increased within 1 h, reaching a maximum value of 0.12 ± 0.02% dose/mL plasma at 6 h after bolus administration. Upon bile diversion, plasma [^{13}C]-LA concentrations were significantly lower than in controls (P<0.001). A maximum value of 0.05 ± 0.01% dose/mL plasma was obtained at 5 h.

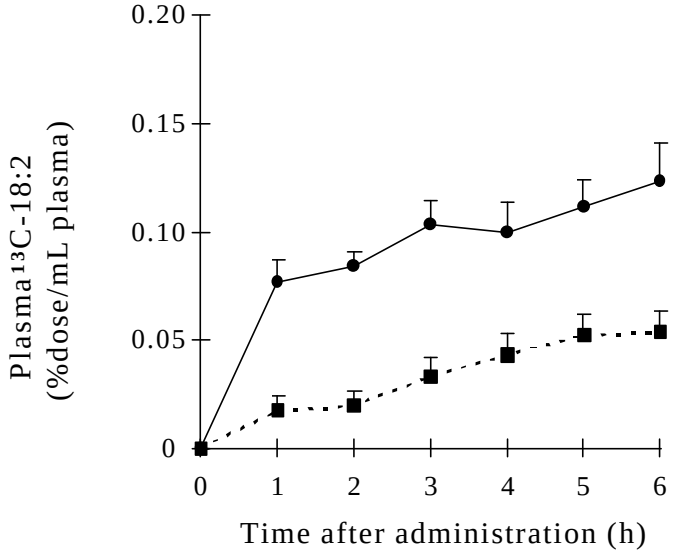

Figure 2. *Time course of [^{13}C]-linoleic acid concentration in plasma of control (circles) and bile-diverted (squares) rats after intraduodenal administration of [^{13}C]-linoleic acid (6.7 mg/kg body weight) at time zero. Data are means ± S.E.M of 14 control and 10 bile-diverted rats. Statistical significance was reached at all individual time points (t=1, 2, 3 h, P<0.001; t=5, 6 h, P<0.01; t=4, P<0.05) and at the area under the curve (0-6 h) (P<0.001).*

Plasma retinyl palmitate concentrations

To compare the plasma appearance of [^{13}C]-LA with that of an uncharged lipid molecule, we investigated the appearance of retinyl palmitate in plasma after intraduodenal retinol (vitamin A) administration. The metabolism of postprandial lipoproteins is commonly studied after the ingestion of a fat load supplemented with retinol. Figure 3 shows the time course of retinyl palmitate appearance in plasma after intraduodenal administration of retinol to control and bile-diverted rats. In control rats, plasma retinyl palmitate concentration reached a maximum of 21.1 ± 4.3 µmol retinyl palmitate/L plasma at 2 h after administration. Upon bile diversion, plasma concentrations of retinyl palmitate were significantly lower than in controls (P<0.001). Maximum values of 1.99 ± 0.62 µmol retinyl palmitate/L plasma were obtained at 4 h.

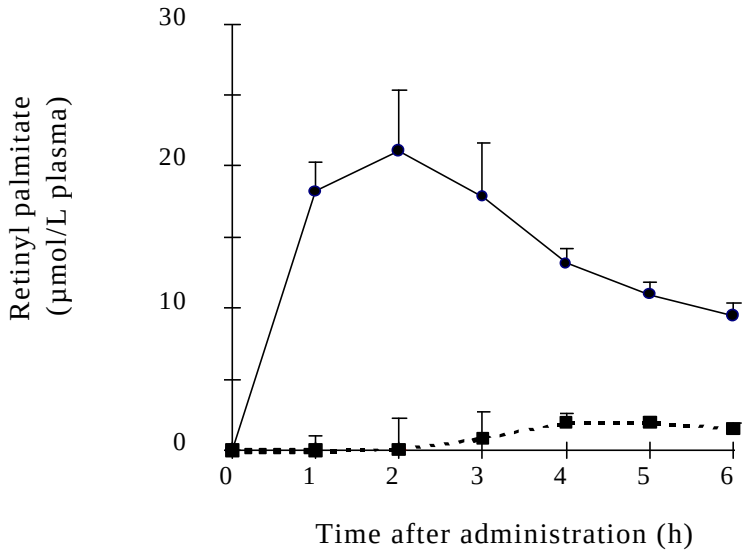

Figure 3. *Time course of retinyl palmitate concentration in plasma of control (circles) and bile-diverted (squares) rats after intraduodenal administration of 50,000 IU retinol at time zero. Data are means ± S.E.M of 4 rats per group. Statistical significance was reached at all individual time points (t=1, 4, 5, 6 h, P<0.001; t=3 h, P<0.01; t=2 h, P<0.05) and at the area under the curve (0-6 h) (P<0.001).*

Intestinal morphology

To investigate whether bile-diversion induced changes in intestinal morphology, we examined the morphology of the small intestine of control and bile-diverted rats. Villus length of duodenal mucosa was increased in bile-diverted rats compared with control rats (Fig. 4).

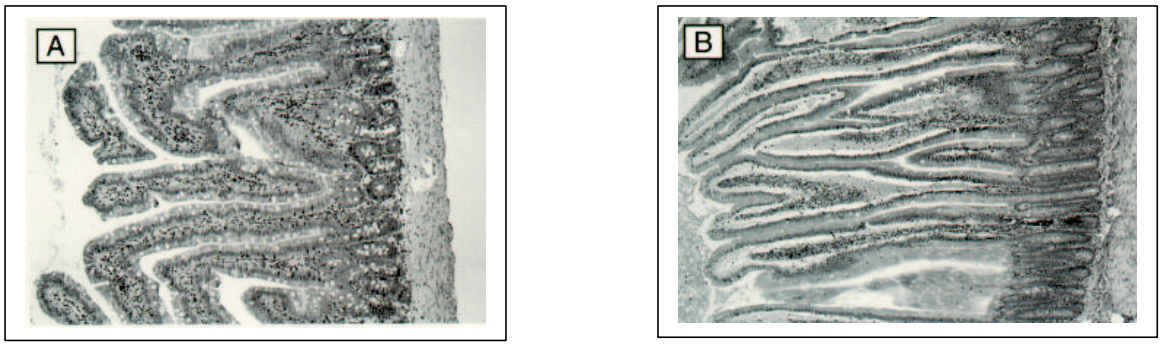

Figure 4. *Morphology of duodenal mucosa from control (A) and bile-diverted (B) rats as determined by light microscopy (10x) after fixation by paraformaldehyde and hematoxylin eosin staining.*

Metabolism of [^{13}C]-linoleic acid (LA) to [^{13}C]-arachidonic acid

The decreased plasma concentration of LA during bile diversion could be due to increased metabolic conversion to, for example, arachidonic acid (20:4n-6). At 24 h after [^{13}C]-LA

administration, bile-diverted rats had an increased ratio of plasma [^{13}C]-arachidonic acid to plasma [^{13}C]-LA concentration compared to controls (1.38 ± 0.31 vs. 0.47 ± 0.08; P<0.05).

Discussion

The relationship between lack of bile secretion into the intestine and lipid malabsorption is well known. Many investigators have suggested that the decreased LA status in cholestatic patients (~30% lower than control patients) is due to lipid malabsorption [15-18]. However, actual balance studies have not been performed. In addition, it has not been determined whether decreased levels in these patients are associated with the lack of bile secretion into the intestine or with other phenomena related to cholestasis, for example, cholestatic hepatocellular injury. In our present study, we found that plasma LA concentration was decreased in rats already 1 week after bile diversion. Yet, although the percentage dietary LA absorption was lower, the net absorption of dietary LA by the intestine was not altered by bile diversion. The results were compatible with an increased metabolism of LA to arachidonic acid, as a possible cause for the decreased LA concentration in plasma of bile-diverted rats.

We investigated lipid malabsorption as a possible cause of impaired LA status in bile-diverted rats. In control rats, the percentage of dietary lipid absorbed, as determined from lipid balance calculations, was ~94%, which is within the normal range of lipid absorption (92-95%) [19]. The percentage of dietary LA absorbed was even more efficient, averaging about 99%. In contrast, the percentage of dietary lipid and LA absorption in bile-diverted rats was decreased to 58% and 73%, respectively. Similarly, Demarne et al. demonstrated that bile-ligated rats fed chow containing 10 wt% lipid (22 en%) experienced a reduced percentage of dietary fatty acid absorption (56%) [20]. Percentage of LA absorbed as a free fatty acid ([^{13}C]-LA), as determined by the lipid balance, was more efficient than when LA was given in the diet as triacylglycerols. The higher percentage of [^{13}C]-LA absorbed may be due to the fact that the amount of [^{13}C]-LA administered to the rats was <1% of the daily LA intake (tracer effect) and that it was administered in a specific, relatively soluble form. Compared with the diet, the lipid bolus contained a majority of MCT along with the tracer, which could have potentially facilitated enteral and even portal transport of [^{13}C]-LA.

In spite of the decrease in the percentage of dietary lipid and LA absorption in bile-diverted rats, the rats managed to maintain a quantitatively similar lipid and LA absorption. This adaptive response can be due to a number of changes. An important compensatory effect for bile diversion seemed to be an increased food ingestion (~40%). It has been observed previously that, during bile diversion, rats ingest more chow in order to maintain an adequate energy balance [7]. Mechanisms underlying this adaptation of food intake are unclear, although it is tempting to speculate that a decreased concentration of apoprotein (apo) A-IV is involved. Apo A-IV, which is only synthesized in the intestine, has been identified as having characteristics of a ‘satiety factor’[21,22]. Intestinal synthesis of apo A-IV is stimulated upon transport of absorbed lipid via chylomicrons into lymph. Impaired chylomicron assembly in

bile-diverted rats would decrease concentrations of apo A-IV in plasma, which theoretically could result in enhanced chow ingestion [23].

Apart from increased ingestion of chow, present data indicate the presence of adaptive responses to bile diversion. Similar net absorption of dietary lipid by bile-diverted rats may be explained in part by altered morphological and/or functional characteristics of the bile-diverted rat intestine. We demonstrated that the villus length of enterocytes of bile-diverted rats was longer than those of the control rats. This observation is in accordance with those of Bloch et al. [24], who found that 12-day bile-diverted rats have increased villus and crypt height (40%) compared to control rats. The mechanism responsible for this adaptation has not been elucidated, but may be related to the absence of detergent bile salts in the intestinal lumen [25,26]. The increased surface area available for nutrient absorption, provided by the longer villi, may partially compensate for the lack of bile secretion into the intestine. Additionally, the functional area of absorption may also be increased by using the distal section of the intestine in conditions which interfere with the efficiency of lipid absorption, such as in bile diversion [27]. In support of this possibility, our analysis of the proximal and distal small intestine of bile-diverted rats revealed greater amounts of triglyceride in the distal compared to the proximal small intestine (0.16 ± 0.02 µmol triglyceride/mg protein vs. 0.08 ± 0.02 µmol triglyceride/mg protein for distal and proximal sections, respectively). In contrast, control rats had similar amounts of triglyceride in proximal and distal sections of the small intestine (0.17 ± 0.02 µmol triglyceride/mg protein vs. 0.17 ± 0.03 µmol triglyceride/mg protein, respectively).

Theoretically, the mechanism behind these morphological and/or functional changes may not be related (only) to the intestinal absence of bile salts, but rather to the lack of biliary phospholipid EFA input to the intestine. Depending on the quantity of EFA in the diet, the biliary supply of EFA can be a substantial source of EFA for structural (i.e., lipid membrane constituents) and/or functional (i.e., prostaglandin formation, energy) needs of the intestine. Since the small intestinal cells have a relatively rapid turnover (5-6 days in humans, 2-3 days in rodents), a constant supply of EFA are needed for continual cell renewal [28,29]. Using control and bile-diverted rats, Melin et al. [28] have estimated that absorbed bile arachidonic acid contributes significantly (30%) to the arachidonic acid pools of the absorptive villus cells. Yet, in the present study, biliary supply of LA contributed <5% to the total dietary LA intake, suggesting that the potential effects of the lack of biliary LA on either small intestinal function, or on plasma LA concentrations, were minimal.

Although net intestinal absorption was similar between control and bile-diverted rats, plasma concentrations of [^{13}C]-LA and retinyl palmitate were significantly lower in bile-diverted rats for 6 h after administration compared to control rats. This finding is compatible with the hypothesis that the intestine can compensate for the net amount of lipid absorbed, but is unable to meet the physiological rate of absorption, due to the lack of bile. The absorption of dietary lipids from more distal parts of the intestine compared with the physiological condition can certainly play a role. In addition, the bile-diverted intestine may be able to compensate for the

lack of biliary phospholipids by using alternate routes of transport. The concept that relatively hydrophilic, long-chain fatty acids such as LA are partially transported via the portal vein cannot be excluded. Since the intestinal lipoprotein production is partially regulated by biliary lipids [30], it would be interesting to study if bile-diversion leads to a different distribution of LA over lipid classes (phospholipids, triacylglycerols) or lipoproteins.

Based on our data, a decreased net absorption could be ruled out as a possible cause for the decreased LA concentration in bile-diverted rats. Yet, the present results suggest an alternative explanation, namely an increased conversion of LA to its long-chain polyunsaturated fatty acid metabolites, such as arachidonic acid. Relative concentrations of arachidonic acid were increased by ~25% in bile-diverted rats compared to control rats. Also, an increased conversion of [^{13}C]-LA to [^{13}C]-arachidonic acid was observed in bile-diverted rats at 24 h after [^{13}C] label administration. These two independent observations indicate that in bile diversion without cholestasis, there is an increased conversion of LA to its major metabolite, arachidonic acid. Although there may be other contributors to the decreased LA concentration in plasma of bile-diverted rats, it seems likely to suggest that an accelerated metabolism plays a role.

In summary, we conclude that absolute and relative concentrations of LA in plasma are decreased in bile-diverted rats. However, decreased levels are not due to decreased net absorption of dietary LA by the intestine. Our data are compatible with an accelerated metabolism of LA to arachidonic acid upon bile diversion, as indicated by increased relative concentrations of arachidonic acid in plasma and by increased conversion of [^{13}C]-LA to [^{13}C]-arachidonic acid after [^{13}C] label administration. It presently remains to be determined whether cholestasis, apart from its effects on bile secretion into the intestine, influences LA status and metabolism.

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CHAPTER 3

Decreased net uptake in the intestine but unchanged desaturation and elongation of linoleic acid in a rat model of acute cholestasis

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Submitted

Abstract

It remains unknown whether impaired linoleic acid status in plasma of patients with cholestatic liver disease is due to malabsorption, altered metabolism, or to both. Bile duct ligation was performed in rats to study the acute effects of the absence of bile in the intestinal lumen and the retention of non-secreted biliary components on linoleic acid absorption and metabolism. The absorption percentage and net uptake of dietary fatty acids and of [^{13}C]-labeled linoleic or palmitic acids were compared in 1 wk bile duct-ligated (BDL) and sham-operated control rats using balance techniques over a 3-day period. After their intraduodenal administration, plasma concentrations of [^{13}C]-labeled linoleic and palmitic acids were measured up to 6 h. Fatty acid desaturation and elongation was determined by quantifying hepatic delta-6 desaturase activity and by comparing [^{13}C]-arachidonic acid concentration to that of [^{13}C]-linoleic acid in plasma, respectively. BDL rats had decreased lipid absorption compared with control rats (53.7 ± 5.0% vs. 94.2 ± 0.6%; P<0.001). The balance study revealed that of all fatty acids studied, linoleic acid had the highest absorption percentage in BDL and control rats (linoleic>oleic>palmitic>stearic acid). Nevertheless, net uptake of linoleic acid was reduced in BDL rats compared with control rats (1.50 ± 0.16 mmol/d vs. 2.08 ± 0.07 mmol/d; P<0.001). Recovery of [^{13}C]-palmitic and -linoleic acids from feces after their intraduodenal administration also revealed significantly lower absorption of [^{13}C]-palmitic acid compared with [^{13}C]-linoleic acid in BDL rats (29.7 ± 13.4% vs. 91.1 ± 2.3%; P<0.05). For at least 6 h after administration, plasma concentrations of [^{13}C]-linoleic and palmitic acids were lower in BDL rats (P<0.05). Conversion of [^{13}C]-linoleic acid to [^{13}C]-arachidonic acid was not different between groups. Present data indicate that 1 wk bile duct ligation in rats is associated with relatively preserved linoleic acid absorption, but results in decreased net uptake of linoleic acid. Linoleic acid conversion to arachidonic acid is not changed in bile duct ligation. Based on these observations, we propose that decreased linoleic acid net uptake predominantly contributes to impaired linoleic acid status in patients with cholestatic liver disease.

Introduction

Essential fatty acids (EFAs) and their polyunsaturated fatty acid (PUFA) metabolites are major components of structural lipids in all tissues and modulate cell membrane fluidity and function. The availability of long-chain PUFA (>18 carbon atoms), such as arachidonic acid (20:4n-6), is important for early human growth and for the production of eicosanoids, which modulate various immunological and vascular functions [1,2]. A suboptimal status of EFAs and LCPUFAs is frequently noted in patients with impaired bile formation, such as cholestatic liver disease [3-6]. Low plasma concentrations of these fatty acids could contribute to the morbidity and mortality of these patients.

Theoretically, impaired status of EFAs and LCPUFAs in plasma may be related to malabsorption, altered metabolism (i.e., desaturation and elongation enzymes, oxidation) and/or to tissue redistribution of these fatty acids. Since patients with cholestatic liver disease have impaired bile secretion into the intestinal lumen, it would seem reasonable that they would malabsorb a substantial amount of their dietary lipids. Kobayashi et al. [8] have reported that dietary lipid absorption was reduced to 30% in patients with biliary atresia. However, the individual dietary intakes of these patients were not reported, so it is not known if they were compensating for increased fecal lipid losses. In a previous study [7], we found that bile-diverted rats had decreased percentages of linoleic acid absorption. However, they compensated for fecal losses of linoleic acid by ingesting more chow (~40%). As a result, their net uptake of dietary linoleic acid was not different from control rats. In addition to malabsorption, the toxic accumulation of non-secreted components in the body may potentially alter fatty acid absorption or metabolism. Retention of endogenous bile acids within the hepatocyte plays a key role in the hepatocyte necrosis occurring in patients with biliary obstruction [9]. The resulting hepatocellular damage may be responsible for changes in liver fatty acid metabolism.

Since it is not known which of these effects in the cholestatic condition are responsible for impaired EFA and LCPUFA status, we investigated the acute effects of the absence of bile in the intestinal lumen and of the retention of non-secreted biliary components in the body on the absorption and metabolism of the major dietary EFA, linoleic acid, using one-week bile duct-ligated rats. Our findings indicate that short-term (1 wk) bile duct ligation in rats is associated with decreased net uptake of linoleic acid, whereas linoleic acid desaturation and elongation of linoleic acid seem unaffected. Based on these observations, we propose that decreased linoleic acid net uptake contributes to impaired linoleic acid status in patients with cholestatic liver disease.

Materials and methods

Animals and diets

Male Wistar rats (Central Animal Laboratory, Groningen, The Netherlands), weighing 250-350 g (mean ± SD: 277 ± 37 g), were kept in an environmentally controlled facility with diurnal light cycling and free access to chow, tap water, and, in the case of bile duct-ligated (BDL) rats, saline (0.9% NaCl, w/v). Experimental protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen.

Labeled substrates

[1-^{13}C]-palmitic acid and [U-^{13}C]-linoleic acid were purchased from Isotec Inc. (Matheson, USA) and Campro Scientific (Veenendaal, The Netherlands), respectively. All stable isotopes were more than 99% enriched.

Experimental procedures

Rats were individually-housed in metabolic cages and fed a high-fat chow (35 en% lipid; major long-chain fatty acid composition as measured by GLC: 16:0, 31.5%; 18:0, 7.3%; 18:1n-9, 31.2%; 18:2n-6, 30.0%) (Hope Farms BV, Woerden, The Netherlands). After 1 week of feeding, rats were equipped with permanent catheters in jugular vein and duodenum, as described by Kuipers et al. [10]. Bile duct ligation was performed on one group of rats (n=6), and the other group was sham-operated (n=6). The experimental model allows for physiological studies in unanesthetized rats with bile duct ligation without the interference of stress or restraint. Animals were allowed to recover from surgery for 6 days.

On day 7, 1.67 mL lipid/kg body weight was slowly administered as a bolus via the duodenal catheter to half of the rats in each group. Medium chain triglyceride oil was included in the bolus, in order to have a sufficient amount to allow a reliable, reproducible delivery. Specifically, the lipid bolus was composed of olive oil (25% v/v; fatty acid composition: 16:0, 14%; 18:1n-9, 79%; 18:2n-6, 8%) and medium chain triglyceride oil (75% v/v; composed of extracted coconut oil and synthetic triglycerides; fatty acid composition: 6:0, 2%; 8:0, 50-65% max.;10:0, 30-45%; 12:0, 3% max.) and contained 6.7 mg [U-^{13}C]-linoleic acid and 6.7 mg [1-^{13}C]-palmitic acid/kg body weight. The lipid bolus represented <10% of the daily lipid intake for control and BDL rats. Blood samples (0.2 mL) were taken from the jugular cannula at baseline and hourly for 6 h after administration of the label, and were collected into tubes containing heparin. A blood sample for quantification of [^{13}C]-linoleic acid and [^{13}C]-arachidonic acid was taken at 24 h after label administration. Plasma was separated by centrifugation (10 min, 2000 rpm, 4ºC) and stored at -20ºC until further analysis. Feces was collected in 24 h fractions starting 24 h before label administration and ending 48 h afterwards (72 h total). Feces samples were stored at -20ºC prior to analysis. Chow ingestion was documented for 72 h by daily weighing of the chow container. On the third day after bolus administration, the heart was punctured to obtain a large blood sample from all rats (~2mL) for analysis of cholestatic markers (ALT, AST, bilirubin, bile salts). Livers were removed from the rats that participated in the bolus experiment, weighed and placed directly in -80ºC until further analysis. Livers from the rats that did not participate in the bolus experiment were removed and weighed, followed immediately by homogenization and microsome isolation.

Analytical techniques

Plasma ALT, AST and bilirubin were determined by routine clinical procedures. Plasma bile salts were measured enzymatically [10]. Lipids from bile or from plasma were extracted and methylated according to Lepage and Roy [11] (see Chapter 2). After freeze-drying and mechanical homogenization, aliquots of chow and feces were subject to the same procedure [11]. Duplicate aliquots of liver homogenate (n=3 per group) were extracted [12] and methylated [11] as described previously. Resulting fatty acid methyl esters from all biological samples were analyzed by gas chromatography to measure total and individual amounts of major fatty acids and, for plasma, liver and feces, by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to measure the [^{13}C] enrichment of palmitic and linoleic acids and linoleic acid metabolites.

Gas liquid chromatography. Fatty acid methyl esters in plasma were separated and quantified by gas liquid chromatography as detailed in [7,13] (see Chapter 2) using heptadecanoic acid (17:0) as internal standard.

Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). [^{13}C] enrichment of palmitic, linoleic and arachidonic methyl esters was determined using the procedure described in Chapter 2.

Delta-6 and delta-9 desaturase assay. Delta-6 desaturase activity was measured using a [^{13}C]-labeled and nonlabeled method. The metabolism of [^{13}C]-linoleic acid to [^{13}C]-arachidonic acid was measured using the ratio between [^{13}C]-arachidonic acid and [^{13}C]-linoleic acid in plasma at 24 h after [^{13}C]-linoleic acid bolus administration. After isolation of hepatic microsomes, the activity of delta-6 and delta-9 desaturase was determined by measuring the conversion of linoleic (18:2n-6) and palmitic (16:0) acids to gamma-linolenic (18:3n-6) and palmitoleic (16:1n-7) acids, respectively. Delta-6 and delta-9 desaturase activity was expressed as the change in mass of 18:3n-6 and 16:1n-7 was calculated directly from the quantitative GC results as the simple difference between the background value and the timed incubation. The conditions for the desaturation assay were described by Su and Brenna [15] using incubation media made according to de Antueno et al. [16].

Calculations

The triene:tetraene ratio was calculated for plasma and for liver by dividing the concentration of 20:3n-9 by that of 20:4n-6. In plasma and in liver, n-6 fatty acid status was calculated using the sum of the area of major fatty acids (>90%) (16:0 + 16:1n-7 + 18:0 + 18:1n-9 + 18:1n-7 + 18:2n-6 + 18:3n-6 + 20:3n-6 + 20:3n-9 + 20:4n-6) and then expressing the area of each individual n-6 fatty acid as a percentage of the total amount. Absorption of major dietary fatty acids (palmitic, stearic, oleic, linoleic acids) and of [^{13}C]-palmitic and linoleic acids was measured using balance techniques, which are described in Chapter 2.

Statistics

Values represent means ± S.E.M. for the indicated number of animals per group. Using SPSS

6.0 statistical software (Chicago, IL, USA), significance of differences was calculated using the two-tailed Student's t-test for normally distributed, unpaired data or a Mann-Whitney *U* test for data that were not normally distributed. Variance between data was determined using Levene's Test for Equality of Variances. $P \leq 0.05$ was considered significant.

Results

Body weight and food ingestion

There was no significant difference between BDL and control rats either in body weight (277.5 ± 14.9 g vs 290.3 ± 12.2 g; $P>0.05$) or in chow ingestion (14.2 ± 1.0 g vs 16.3 ± 0.6 g; $P>0.05$; equal to 4283 kcal/kg chow), respectively.

Cholestatic markers in plasma

Compared with control rats, BDL rats had strongly increased levels of liver enzymes (aspartate transaminase, alanine transaminase) ($P<0.05$), bilirubin ($P<0.01$) and bile salts ($P<0.01$) in plasma, in accordance with the presence of cholestasis (Table 1). Plasma cholesterol and triglyceride concentrations were similar between BDL and control rats (1.88 ± 0.06 mM vs. 1.90 ± 0.12 mM, and 0.62 ± 0.10 mM and 0.69 ± 0.08 mM, respectively; $P>0.05$). Liver weights were similar for BDL and control rats (13.9 ± 1.0 g vs. 11.3 ± 0.4 g; $P>0.05$).

Table 1. *Biochemical markers for cholestasis in plasma of control and 1 wk bile duct-ligated (BDL) rats*

	Control	BDL
Alanine transaminase (Units/L)	34.0 ± 3.0	74.5 ± 10.3*
Aspartate transaminase (Units/L)	98.0 ± 7.0	273.7 ± 59.0$^{\#}$
Total bilirubin (μmol/L)	4.2 ± 0.2	172.3 ± 10.7*
Bile salts (μmol/L)	9.4 ± 2.0	229.5 ± 20.8*

*Data are means ± S.E.M. of control and BDL rats (n=6 per group). *P<0.01; $^{\#}$P<0.05.*

Absolute and relative fatty acid concentrations in plasma and in liver

Total plasma lipid analysis revealed a similar cumulative concentration of major fatty acids in BDL and control rats (7.11 ± 0.18 mM vs 6.90 ± 0.33 mM; $P>0.05$). Plasma concentrations of palmitic, linoleic and arachidonic acids were similar between both groups (data not shown) ($P>0.05$). However, plasma concentrations of stearic and oleic acids were decreased and increased, respectively, in BDL rats compared with controls (0.79 ± 0.05 mM vs. 1.04 ± 0.04 mM and 1.39 ± 0.06 mM vs. 0.98 ± 0.07 mM, respectively; $P<0.01$). Accordingly, the molar percentages (Fig. 1) for stearic and oleic acids were decreased and increased, respectively, in plasma of BDL rats compared with that of control rats (11.08 ± 0.24% vs 15.13 ± 0.40%; $P<0.001$; 19.63 ± 0.71% vs. 14.22 ± 0.71%; $P<0.001$). In plasma, the triene:tetraene ratio (20:3n-9/20:4n-6, biochemical indicator of EFA deficiency [17]) was increased in BDL rats compared with control rats (0.024 ± 0.004 vs 0.013 ± 0.001; $P<0.05$). However, no evidence of EFA deficiency was present since both values remained considerably below 0.2, the accepted threshold cutoff value for EFA deficiency in humans [17].

Relative fatty acid concentrations in liver were similar between control and BDL rats for all major fatty acids (data not shown). No statistical difference was found in the triene:tetraene ratio in liver between both groups (0.015 ± 0.002 vs. 0.013 ± 0.002 for control and BDL rats, respectively; $P>0.05$).

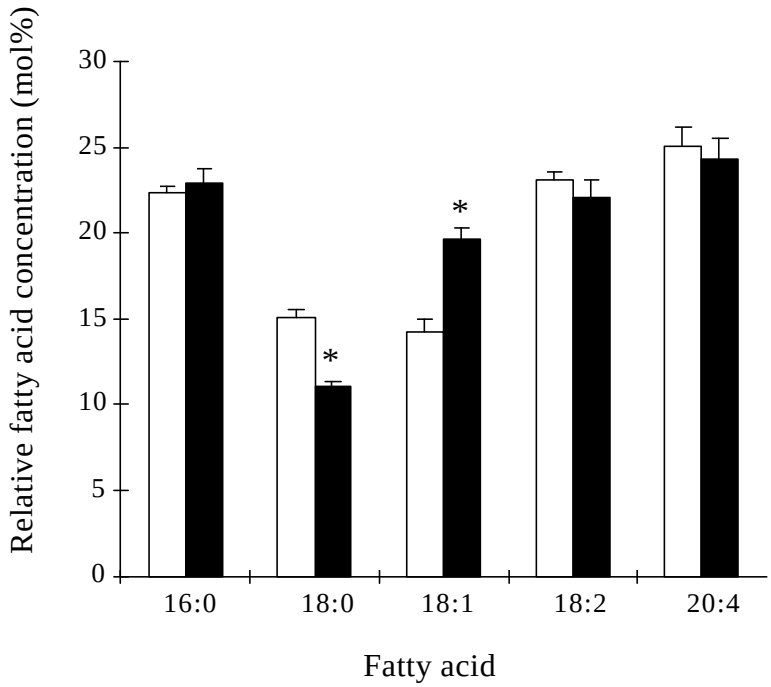


Figure 1. *Relative concentrations (mol%) of major fatty acids (16:0 + 18:0 + 18:1n-9 + 18:2n-6 + 20:4n-6) in plasma of control (open bars) and bile duct-ligated (closed bars) rats. Data are means ± S.E.M of 6 rats per group. Symbols indicate significant difference between control and bile duct-ligated rats (*P<0.001).*

Dietary fatty acid balance profile

In Table 2, dietary fatty acid balance data (ingestion, fecal excretion and net absorption) for control and BDL rats are shown. BDL rats excreted significantly more fatty acids into feces compared to control rats ($P<0.0001$). The overall lipid absorption percentage was significantly decreased in BDL rats compared with control rats (53.7 ± 5.0% vs. 94.2 ± 0.6%; $P<0.001$). Saturated fatty acids (palmitic and stearic acids) were less well absorbed than unsaturated species (oleic and linoleic acids) (Fig. 2). Since lipid intake was not altered, BDL rats had significantly decreased net fatty acid absorption (Table 2) ($P<0.001$).

Table 2*. Dietary fatty acid balance data for control (C) and 1 wk bile duct-ligated (BDL) rats*

	Ingestion (mmol/d)		Fecal excretion (mmol/d)		Net absorption (mmol/d)	
	C	BDL	C	BDL	C	BDL
16:0	2.21 ± 0.07	1.93 ± 0.17	0.25 ± 0.01	1.39 ± 0.06*	1.96 ± 0.08	0.53 ± 0.13#
18:0	0.51 ± 0.02	0.45 ± 0.04	0.09 ± 0.00	0.38 ± 0.01*	0.43 ± 0.02	0.07 ± 0.03#
18:1	2.28 ± 0.06	2.00 ± 0.18	0.04 ± 0.01	0.62 ± 0.05*	2.23 ± 0.07	1.37 ± 0.18#
18:2	2.10 ± 0.07	1.83 ± 0.16	0.02 ± 0.00	0.32 ± 0.04*	2.08 ± 0.07	1.50 ± 0.16#
Total	7.01 ± 0.23	6.11 ± 0.43	0.40 ± 0.03	2.72 ± 0.16*	6.61 ± 0.21	3.39 ± 0.38#

*Data are means ± S.E.M. of control and bile duct-ligated rats (n=6 per group). Mean values represent the average of 3 d per rat. Included in these 3 d is the experimental day in which the lipid bolus was administered (n=3 per group). *P<0.0001; #P<0.001.*

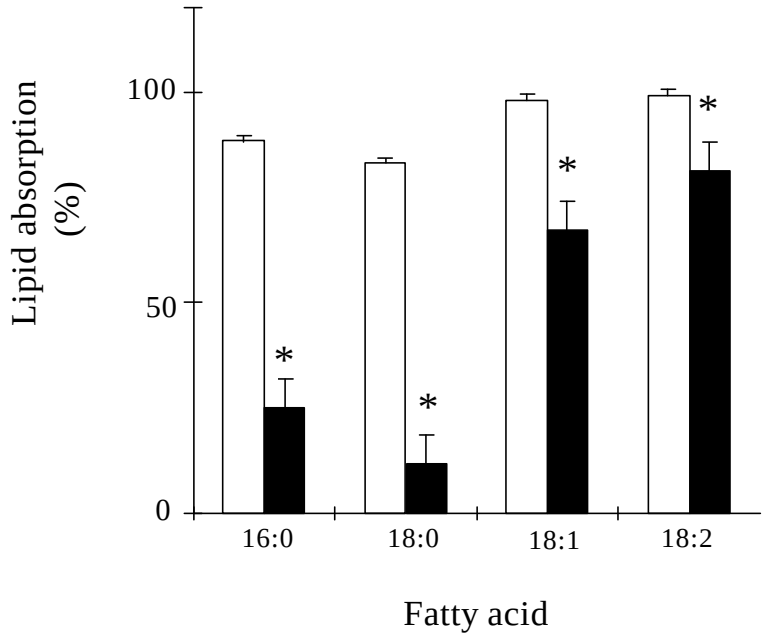


Figure 2. *Lipid absorption percentages for the major dietary fatty acids:palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9) and linoleic acid (C18:2n-6) as calculated from chow ingestion and fecal lipid excretion in control (open bars) and bile duct-ligated (closed bars) rats. Data are means ± S.E.M of 6 rats per group. Symbols indicate significant difference between control and bile duct-ligated rats (* P<0.001).*

Excretion of [^{13}C]-palmitic acid was significantly greater in BDL rats compared with control rats (4.67 ± 0.50 vs. 1.66 ± 0.50; P<0.05). In contrast, no quantitative differences in fecal excretion (0.52 ± 0.36 vs. 0.54 ± 0.19 for control and BDL rats; P>0.05) or net absorption (5.72 ± 0.05 vs. 5.35 ± 0.39 for control and BDL rats; P>0.05) of [^{13}C]-linoleic acid were found between control and BDL rats under the conditions studied. The percentage of [^{13}C]-palmitic acid absorbed was significantly decreased in BDL rats compared with control rats (P<0.05); however, there was no difference in the percentage of [^{13}C]-linoleic acid absorbed between both groups (P>0.05) (Fig. 3).

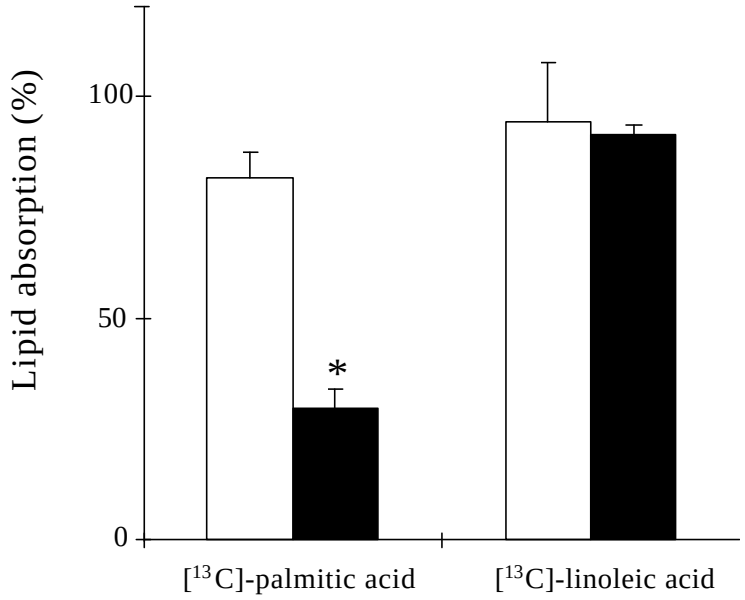
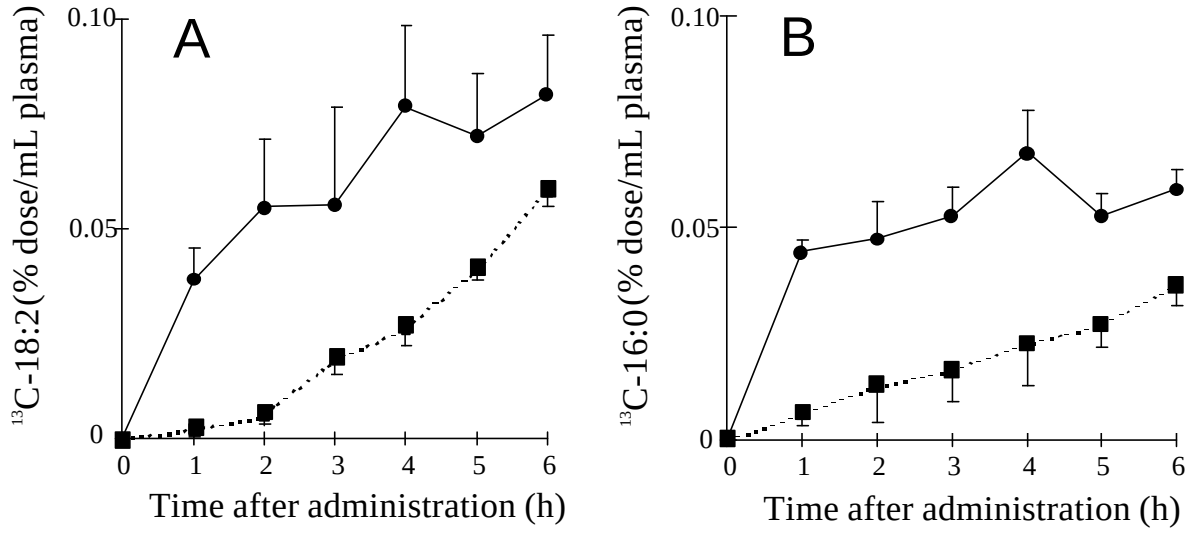


Figure 3. *Lipid absorption percentages for [^{13}C]-palmitic and [^{13}C]-linoleic acids as calculated from chow ingestion and fecal lipid excretion in control (open bars) and bile duct-ligated (closed bars) rats. Data are means ± S.E.M of 3 rats per group. Symbols indicate significant difference between control and bile duct-ligated rats (* P<0.05).*

Plasma [^{13}C]-fatty acid concentrations

Absorption kinetics of saturated and unsaturated fatty acids were studied by determining the appearance of [^{13}C]-fatty acids in plasma after their duodenal administration. Fig. 4a and 4b show the time course of [^{13}C]-linoleic acid and [^{13}C]-palmitic acid appearance in plasma after their intraduodenal administration to control and BDL rats, respectively. In control rats, plasma [^{13}C]-linoleic acid and [^{13}C]-palmitic acid concentrations increased within 1 h, reaching apparent maximum values of 0.082 ± 0.014% dose/mL plasma and 0.068 ± 0.009% dose/mL plasma at 6 h and 4 h after bolus administration, respectively. Upon bile duct ligation, plasma [^{13}C]-fatty acid concentrations were significantly lower than in controls (P<0.001). Maximum values of 0.059 ± 0.003% dose/mL plasma and 0.036 ± 0.005% dose/mL plasma were obtained at 6 h for [^{13}C]-linoleic and palmitic acids, respectively.



Figs. 4a and 4b. *Time course of [^{13}C]-linoleic acid (A) and [^{13}C]-palmitic acid (B) concentration in plasma of control (circles) and bile duct-ligated (squares) rats after intraduodenal administration of [^{13}C]-fatty acids (6.7 mg/kg body weight for each fatty acid) at time zero. Data are means ± S.E.M of*

3 rats per group. Statistical significance was reached at the following individual time points for [^{13}C]-linoleic acid: t=1 and 2 h; P<0.05 and for [^{13}C]-palmitic acid: t=1, 3, 4 and 5 h; P<0.05.

Metabolism of [^{13}C]-linoleic acid (LA) to [^{13}C]-arachidonic acid

The metabolism of linoleic acid was determined by measuring conversion of [^{13}C]-linoleic acid to [^{13}C]-arachidonic acid in plasma and by measuring delta-6 and delta-9 desaturase enzymes in hepatic microsomes which are responsible for the conversion of linoleic acid (18:2n-6) to gamma-linolenic acid (18:3n-6) and palmitic acid (16:0) to palmitoleic acid (16:1n-7), respectively. At 24 h after [^{13}C]-linoleic acid administration, BDL rats had a similar ratio of plasma [^{13}C]-arachidonic acid to plasma [^{13}C]-linoleic acid concentration compared to control rats (0.53 ± 0.19 vs. 0.60 ± 0.13; P>0.05). In accordance with these data, delta-6 desaturase activity *in vitro* was not significantly different between control and BDL rats (30.8 ± 10.2 pmol/min/mg protein vs. 35.7 ± 19.5 pmol/min/mg protein). Since the relative increase in plasma oleic acid in BDL rats could be due to increased activity of delta-9 desaturase, we measured delta-9 desaturase activity in hepatic microsomes. Although the number of animals used is too low to draw a definite conclusion (n=3 per group), there was an indication that the activity of delta-9 desaturase was enhanced in BDL rats (61.1 ± 24.2 pmol/min/mg protein vs. 41.5 ± 19.5 pmol/min/mg protein for BDL and control rats, respectively).

Discussion

In the present study, we used a rat model of acute cholestasis to investigate the absorption and desaturation/elongation of linoleic acid. The relationship between intestinal bile deficiency and fatty acid malabsorption is well known. Yet, malabsorption in patients with cholestatic disease has only been expressed as a percentage (~30%) and not in terms of net uptake, or the total amount of lipid ingested minus the total amount of lipid excreted in the feces. We recently found that the mere absence of bile in the rat intestine leads to a decreased absorption percentage (59%); however, the rats managed to maintain a quantitatively similar net fatty acid and linoleic acid absorption by a compensatory increase in chow ingestion (~40%) [7]. In analogy, net uptake may be similar in control and cholestatic patients if intake is increased. In this case, the actual malabsorption would not significantly contribute. Similar to bile-diverted rats, bile duct-ligated rats had a significantly decreased fatty acid absorption percentage (54%). However, bile duct-ligated rats did not ingest more chow to compensate for their increased fecal lipid excretion. As a result, the net uptake of dietary linoleic acid was decreased by ~28% in bile-ligated rats compared with control rats. The fatty acids differed in their absorption percentage. Of all dietary fatty acids studied, absorption percentage and net uptake decreased in bile duct-ligated rats in the following order: linoleic acid>oleic acid>palmitic acid>stearic acid. These results are in accordance with those of Demarne et al. [18] in a study using 10-day bile duct-ligated rats fed chow containing 10% peanut oil. They reported a decrease in dietary saturated fatty acid (16:0 and 18:0) absorption percentages compared with dietary unsaturated fatty acids (18:1 and 18:2). However, no net uptakes of these fatty acids were reported. In support of the selectivity of fatty acid uptake in bile duct

ligation, we found intraduodenally administered [^{13}C]-linoleic acid to be excreted to a lesser extent in feces of bile duct-ligated rats compared with [^{13}C]-palmitic acid. Accordingly, net uptake of [^{13}C]-linoleic acid was greater than that of [^{13}C]-palmitic acid. In contrast to the dietary linoleic acid balance, bile duct-ligated rats had minimal excretion of [^{13}C]-linoleic acid into feces. This discrepancy could be due to the fact that tracer doses of [^{13}C]-linoleic acid were used.

In addition to fecal balance data, we measured lipid absorption indirectly by measuring the appearance of [^{13}C]-fatty acids in plasma after their intraduodenal administration. The appearance of either [^{13}C]-linoleic acid or [^{13}C]-palmitic acid was significantly decreased in bile duct-ligated rats compared with controls. When the two [^{13}C]-fatty acids were used, [^{13}C]-linoleic acid appeared in plasma to a slightly greater extent than [^{13}C]-palmitic acid in bile duct-ligated and in control rats. The difference in the absorption of linoleic and palmitic acids has been documented in *in vivo* [19] and *in vitro* [20,21] models. After intraduodenal infusion into rats as mixed micelles or emulsions containing [^{14}C]-fatty acid, monoolein, and taurocholate, it was found that palmitic acid consistently required a greater length of intestine for its absorption than linoleic acid did [19]. Studies by Sallee and Dietschy [20] have focused attention on the potential importance of an "unstirred water layer" lining the intestinal mucosa as a rate-limiting factor for fatty acid uptake. According to Ockner et al. [19], it is possible that with higher concentrations of lipid, the corresponding increase in micelle size and low solubility of saturated fatty acids could result in a progressive decrease in rate of diffusion across the unstirred layer. With regard to fatty acid solubility in mixed micelles, it has been shown previously that a given concentration of bile salt will maintain less palmitic acid than linoleic acid in micellar solution [21]. The solubility of palmitic acid in the absence of bile salts *in vitro* is less than that of linoleic acid [22]. In addition to changes in uptake of saturated fatty acids compared with that of unsaturated fatty acids, these fatty acids may be handled differently within the enterocyte. Specifically, in short term studies using everted sacs incubated in mixed micelles, Ockner et al. [19] noted that even when uptake of the two fatty acids by the intestinal mucosa was similar, palmitic acid esterification occurred less rapidly [19]. This finding could explain the apparent decrease in [^{13}C]-palmitic acid in plasma compared with [^{13}C]-linoleic acid.

These results lead us to conclude that in acute conditions of cholestasis, linoleic acid absorption is preserved to a greater extent than that of saturated fatty acids like palmitic acid. This phenomenon may be related in general to the differences in the physiochemical nature of fatty acids, including melting point [22] and molecular configuration [23]. Despite the fact that linoleic acid absorption is *relatively* well-preserved, its uptake is nevertheless reduced in bile duct-ligated rats compared with controls. This finding could suggest that patients with chronic cholestatic disease experience long-term decreased net uptake of dietary linoleic acid, which would eventually result in lowered linoleic acid levels. In the short course of our study (1 week), linoleic acid status was not impaired but the triene:tetraene ratio in plasma was already significantly increased, indicating the tendency to develop essential fatty acid deficiency. An

especially high risk group would be pediatric patients since their fat stores of linoleic acid are minimal.

The possibility that increased metabolism (i.e., desaturation/elongation and oxidation) contributes to impaired EFA and LCPUFA status in cholestatic patients cannot be excluded. Yamashiro et al. [24] demonstrated that ursodeoxycholic acid treatment alone or in combination with artificial bile corrected impaired linoleic acid levels in patients with extrahepatic biliary atresia, but failed to correct levels of the LCPUFA, arachidonic acid. Socha et al. [3] suggested that the hepatic microsomal desaturase/elongase system used for LCPUFA synthesis is altered in patients with cholestatic liver disease. In our rat model of acute cholestasis, conversion of [^{13}C]-linoleic acid to [^{13}C]-arachidonic acid was not different between bile duct-ligated and control rats. Also, delta-6 desaturase activity as measured in liver microsomes was not different between groups. A majority of LCPUFA synthesis occurs in the liver [25]. Since relative concentrations of oleic acid were increased in plasma of bile duct-ligated rats, we investigated delta-9 desaturase activity in liver microsomes and found that it was increased, although not significantly, compared with control rats. However, due to the limited number of animals used in that experiment, we are unable to draw any definite conclusion. Socha et al. [3] have indirect indications that delta-9 desaturase activity is enhanced in pediatric patients with cholestasis. Compared with control patients, cholestatic patients have lower levels of stearic acid and higher levels of oleic acid. Finally, although not examined in this study, it would be helpful to measure the oxidation of individual fatty acids in cholestatic liver disease patients to determine if indeed their metabolism of EFA is increased, thus resulting in lower concentrations of EFA in plasma. Our present study points to a preservation of linoleic acid absorption compared with other dietary fatty acids in 1 week bile duct-ligated rats compared with control rats. It would be interesting to know whether EFAs and LCPUFAs were spared from other physiological processes such as oxidation, in order to maintain constant levels in the body.

In summary, as determined from balance techniques, we conclude that one-week bile duct ligation in rats is associated with decreased net uptake of linoleic acid. Elongation/desaturation activity as measured indirectly by measuring the formation of [^{13}C]-arachidonic acid from administered [^{13}C]-linoleic acid) or directly by measuring the conversion of linoleic acid to dihomogamma-linolenic acid in liver microsomes was not significantly different between bile duct-ligated and control rats. Based on these observations, we speculate that decreased linoleic acid net uptake contributes to impaired linoleic acid status in patients with cholestatic liver disease.

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CHAPTER 4

Biliary phospholipid secretion is not required for intestinal absorption and plasma status of linoleic acid in mice

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Abstract

Biliary phospholipids have been hypothesized to be important for essential fatty acid homeostasis. We tested this hypothesis by investigating the intestinal absorption and the status of linoleic acid in *mdr*2 Pgp-deficient mice which secrete phospholipid-free bile. In mice homozygous (-/-) for disruption of the *mdr*2 gene and wildtype (+/+) mice, dietary linoleic acid absorption was determined by 72-h balance techniques. After enteral administration, [^{13}C]-linoleic acid absorption was determined by measuring [^{13}C]-linoleic acid concentrations in feces and in plasma. The status of linoleic acid was determined in plasma and in liver by calculating the molar percentage of linoleic acid and the triene:tetraene ratio. Although plasma concentration of [^{13}C]-linoleic acid at 2 h after enteral administration was significantly lower in (-/-) compared to (+/+) mice (P 0.05), net intestinal absorption of dietary linoleic acid or of [^{13}C]-linoleic acid was similar in (+/+) and (-/-) mice. Molar percentage of linoleic acid and the triene:tetraene ratio were not different in whole plasma or in liver of (-/-) compared to (+/+) mice. Present data indicate that biliary phospholipids are involved in the rate of appearance in plasma of enterally administered linoleic acid, but are not required for net intestinal absorption or plasma status of linoleic acid.

Introduction

Since essential fatty acids (EFAs) cannot be synthesized *de novo* by mammals, their concentration in the body depends on adequate dietary supply and subsequent efficient absorption. The classical indicator of overall EFA-deficiency is an increased ratio of plasma concentration of eicosatrienoic acid (20:3n-9) compared to that of arachidonic acid (20:4n-6). A triene:tetraene (20:3n-9/20:4n-6) ratio above 0.2 would justify classification as EFA-deficient in humans [1]. Specifically, the status of linoleic acid (LA;18:2n-6), the major dietary EFA, is conventionally determined by measuring its concentration in relation to that of other fatty acids (mol %) [1,2]. Theoretically, decreased plasma concentrations of LA can be the result of decreased absorption, increased catabolism, or a redistribution of LA over the various body compartments, including plasma. Severe LA deficiency has been associated with numerous clinical symptoms [3].

Biliary phospholipids could be important for the maintenance of EFA status, based on their well-documented role in the lymphatic transport of dietary lipid under physiological conditions [4-9]. In bile-diverted rats, the lymphatic triglyceride content has been demonstrated to increase during intestinal infusion of triolein and bile salts and can be enhanced further by the inclusion of phosphatidylcholine (PC) into the perfusate [5,6,10-13]. Also, the supply of biliary phospholipids to the bile-diverted rat intestine resulted in an increase in the synthesis of apolipoprotein B-48 (apo B-48), the apolipoprotein needed for chylomicron formation [14,15]. Finally, although it has not been demonstrated, the specific EFA-rich acyl chain composition of the biliary PC molecule may be relevant for chylomicron production and/or for maintenance of the intestinal mucosa membrane. This speculation is based on studies which have shown that under physiologic conditions, phospholipids of lymph chylomicrons are derived predominantly from biliary rather than from dietary origin [8,9].

In this study, we investigated if biliary phospholipids play a role in the absorption of LA and in the maintenance of physiological plasma concentrations of LA. Mice lacking the *mdr*2 gene product in the bile canalicular membrane, also known as *mdr*2 knockout (-/-) mice, lack biliary phospholipids and have a strongly reduced (~97%) cholesterol secretion into bile, in spite of normal bile salt secretion rates [16]. The availability of *mdr*2 (-/-) mice allow the opportunity to investigate the role of biliary phospholipid secretion for LA absorption and homeostasis *in vivo* [16-18]. In the present study, we determined the absorption of dietary and [^{13}C]-LA and the status of LA in wildtype (+/+) and *mdr*2 knockout (-/-) male mice. Our results indicate that biliary phospholipids are not required for intestinal absorption and plasma status of linoleic acid in mice.

Materials and Methods

Animals

Two-month old male wildtype (+/+) and *mdr2* knockout (-/-) mice from a free virus breed

(FVB) strain were used [19]. Animals were obtained from the colony at the Central Animal Facility, Academic Medical Center, Amsterdam, The Netherlands. Experimental protocols were approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, Groningen, The Netherlands. Two weeks before the experiment, the mice (~ 25 g) were individually-housed and allowed tap water and chow (Hope Farms B.V., Woerden, The Netherlands) (Fig. 1a) ad libitum. Animals were housed in a light-controlled (lights on 6 AM - 6 PM) environment.

Experimental procedures

After an overnight fast, mice of both genotypes (n=7 per group) were anesthetized with halothane and a baseline blood sample was obtained by tail bleeding. Blood was collected in micro-hematocrit tubes containing heparin and centrifuged to obtain plasma (<40 µL). Directly after taking the blood sample, 100 µL lipid, containing [^{13}C]-linoleic acid, was slowly administered by intragastric gavage. This amount of lipid provided enough volume for reliable, reproducible administration of the labeled compound. The lipid bolus was composed of olive oil (25% v/v; fatty acid composition: 16:0, 14%; 18:1n-9, 79%; 18:2n-6, 8%) and medium chain triglyceride oil (75% v/v; composed of extracted coconut oil and synthetic triglycerides; fatty acid composition: 6:0, 2%; 8:0, 50-65% max.;10:0, 30-45%; 12:0, 3% max.), and contained a tracer amount of [U-^{13}C]-LA (0.90 µmol) (Martek Biosciences Corporation, Columbia, MD, USA); [U-^{13}C]-LA was 99% enriched with a chemical purity exceeding 97%. The bolus represented ~25% and ~4% of the daily molar lipid intake and the caloric intake, respectively. Blood samples were collected by tail bleeding 2 and 4 h after bolus administration. Feces samples were collected in 24 h fractions beginning 24 h before bolus administration and ending 72 h afterwards. Chow ingestion was measured by daily weighing of chow containers. At 72 h, a large blood sample (0.6-1.0 mL) was obtained by heart puncture, and liver was removed. In a separate experiment, mice (n=6) were anaesthetized by intraperitoneal injection of Hypnorm (fentanyl/fluanisone) and diazepam and their gallbladders were cannulated for collection of bile for 1 h as described previously [20].

Analytical techniques

Lipid extraction and methylation. Lipids from bile or from plasma were extracted and methylated according to Lepage and Roy [21] (see Chapter 2). After freeze-drying and mechanical homogenization, aliquots of chow and feces were subject to the same procedure [21]. Duplicate aliquots of liver homogenate (n=3 per group) were extracted [22] and methylated [21] as described previously. Resulting fatty acid methyl esters from all biological samples were analyzed by gas chromatography to measure total and individual amounts of major fatty acids and, for plasma, liver, and feces, by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to measure the [^{13}C]-enrichment of LA and LA metabolites. Biliary phospholipid content was determined in bile samples collected for one hour using a commercially available kit (WAKO Chemicals GmbH, Neuss, Germany).

Gas liquid chromatography. Fatty acid methyl esters in plasma were separated and quantified

by gas liquid chromatography as detailed in [23] (see Chapter 2) using heptadecanoic acid (17:0) as internal standard.

Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). [^{13}C]-enrichment of the LA methyl esters was determined using the procedure described in Chapter 2.

Calculations

Linoleic acid (LA) absorption using 72-h balance techniques. Absorption of dietary LA and of [^{13}C]-LA was measured using balance techniques, which are described in Chapter 2.

Essential fatty acid (EFA) and linoleic acid (LA) status. The triene:tetraene ratio and relative concentrations of major fatty acids were calculated for plasma and for liver as described in Chapter 2.

Biliary fatty acid molar percentages. Relative concentrations (molar percentages) of major fatty acids in bile were calculated using the sum of the area of major fatty acid (>90%) peaks (16:0 + 18:0 + 18:1n-9 + 18:2n-6 + 20:4n-6) and then expressing the area of each individual fatty acid as a percentage of the total amount.

Statistics

Values represent means ± S.E.M. for the indicated number of animals per group. Using SPSS 6.0 statistical software (Chicago, IL, USA), significance of differences was calculated using the two-tailed Student's t-test for normally distributed, unpaired data or a Mann-Whitney *U* test for data that were not normally distributed. Variance between data was determined using Levene's Test for Equality of Variances. P 0.05 was considered significant.

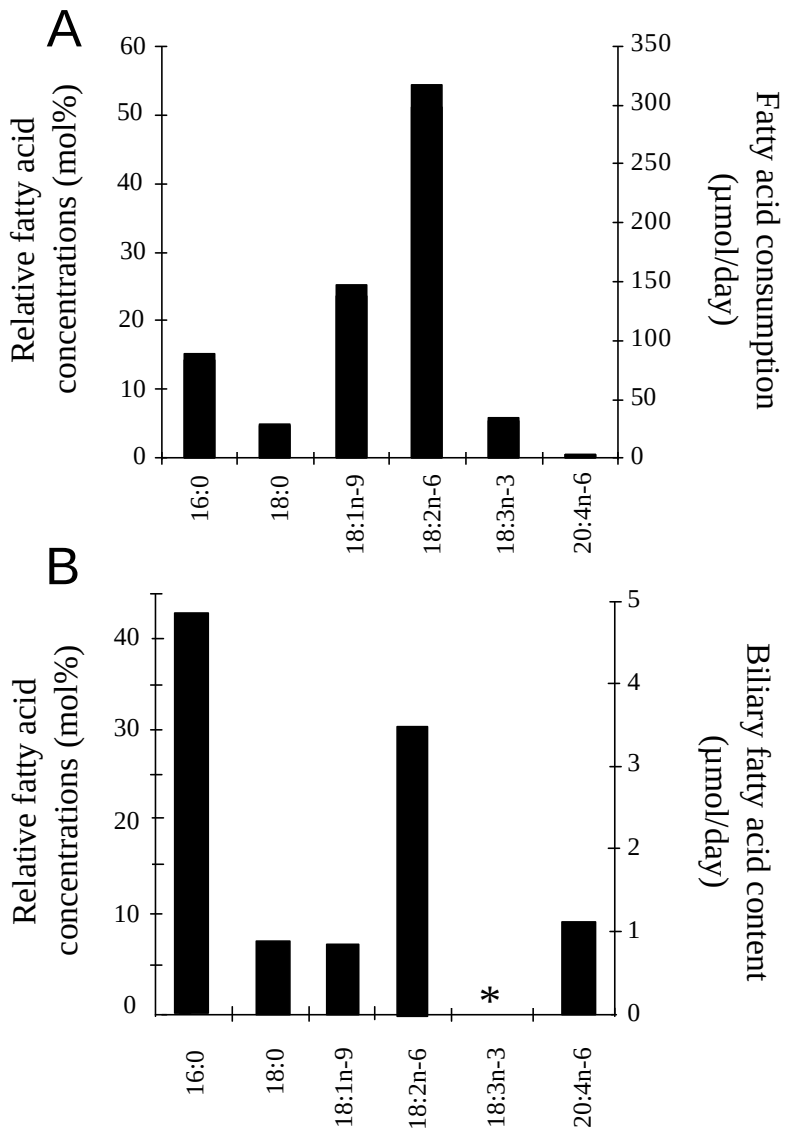
Results

Body weight and chow ingestion

During the experiment, there was no significant difference in body weight (30.4 ± 0.5 g vs 30.8 ± 0.6 g; P>0.05) or in chow ingestion for 72 h (11.8 ± 0.4 g/72 h vs 12.5 ± 0.4 g/72 h; equal to 3748 kcal/kg chow) between (+/+) and (-/-) mice.

Linoleic acid (LA) quantification in chow and in bile

For accurate balance measurements, we quantified LA in chow and in bile. The chow contained 0.155 µmol lipid/mg and the chow fatty acids consisted of 51.2 mol% LA (Fig. 1a). The LA content of biliary fatty acids from male FVB (+/+) mice was 31.4 ± 0.9 mol% (mean ± S.E.M.) (Fig. 1b). Based on the phospholipid secretion rate in these mice (15.7 ± 0.7 nmol phospholipids/min/100g BW, n=6), total daily biliary LA input to the intestine for a 25 g mouse was calculated to be 3.6 µmol/day (Fig. 1b). Using the average daily ingestion of chow for both (+/+) and (-/-) mice (4.05 ± 0.01 g/day), the average amount of LA ingested daily was estimated to be 317 µmoles (Fig. 1a). Thus, biliary LA secretion amounted only to approximately 1% of LA ingested per day (3.6 µmol/d vs 317 µmol/d) in (+/+) mice.



Figures 1a and 1b. *Relative long-chain fatty acid concentration (mol%) in chow (**a**) and bile (**b**), and daily input of these fatty acids (µmol/day) via chow and bile in male FVB wildtype (+/+) mice. Molar percentage (mol%) is based on the total amount of major fatty acids: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9), linoleic acid (18:2n-6), linolenic acid (18:3n-3), and arachidonic acid (20:4). Chow samples ground to fineness and measured in duplicate. Chow provided 0.155 µmol lipid/mg chow (average daily chow ingestion: 4.05 ± 0.01 g). Bile samples are the mean of 3 wildtype (+/+) male mice. Phospholipid input (15.7 ± 0.7/min/100 g, n=6) based on bile samples that were obtained during the first hour after cannulation of the gallbladder. *=not detectable.*

Linoleic acid (LA) absorption

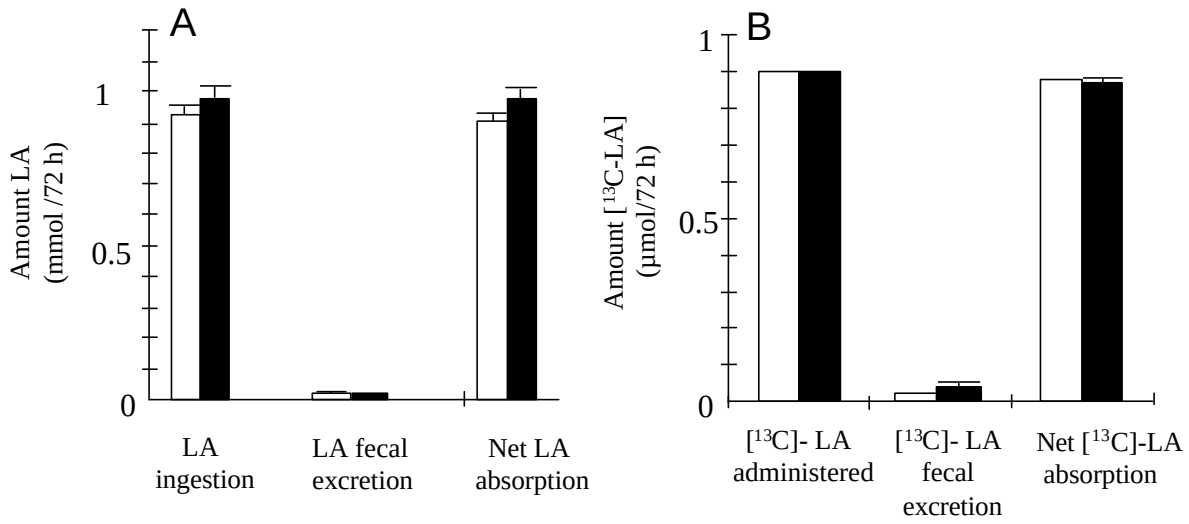
LA absorption was determined by balance techniques (i.e., ingestion and fecal excretion) and by measuring plasma appearance of [^{13}C]-LA after enteral lipid bolus administration.

Dietary linoleic acid (LA) and [^{13}C]-LA balances

In Fig. 2a and 2b, dietary LA and [^{13}C]-LA balance data of (+/+) and (-/-) mice are shown. No differences between groups were found for ingestion, excretion or net intestinal absorption. No [^{13}C]-enrichments of other fecal fatty acids were noted, indicating that the minor amount of unabsorbed [^{13}C]-LA was not metabolized in the large intestinal lumen. Also, the percentage of dietary LA or of [^{13}C]-LA was not different between (+/+) and (-/-) mice, and in each group exceeded 96%.

Plasma [^{13}C]-linoleic acid (LA)

Fig. 3 shows the time course pattern of [^{13}C]-LA appearance in plasma after enteral administration of µmol [^{13}C]-LA/mL plasma. In (-/-) mice, maximum values were measured at 4 h after bolus administration (0.016 ± 0.003 µmol [^{13}C]-LA/mL plasma). At 4 h, a plateau phase seemed not yet to have been reached, although it is difficult to reach such a conclusion with only three data points. In (-/-) mice, the concentration of [^{13}C]-LA at 2 h was significantly decreased (0.009 ± 0.001 µmol [^{13}C]-LA/mL plasma) compared to (+/+) mice ($P \leq 0.05$).



Figures 2a and 2b. *Dietary linoleic acid (**a**) and [^{13}C]-LA (**b**) balance data for wildtype (+/+) (open bars) and mdr2 knockout (-/-) (closed bars) mice. Values are the mean ± S.E.M. of data from six to seven individual mice per group. None of the parameters studied was significantly different between (+/+) and (-/-) mice. *Biliary LA in mdr2 (-/-) mice was 20% of wildtype (+/+) values.*

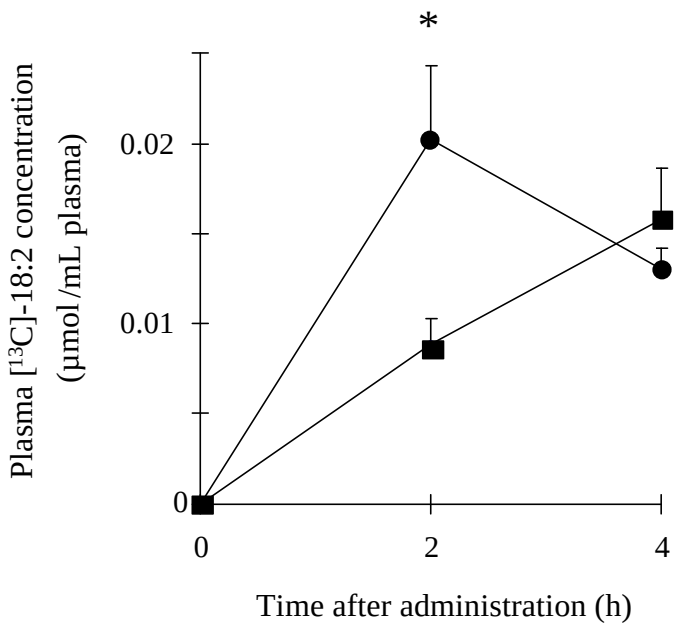


Figure 3. *Time course of [^{13}C]-linoleic acid concentrations in plasma in wildtype (+/+)(circles) (n=7) and mdr2 knockout (-/-)(squares) (n=6) mice after enteral administration of [^{13}C]-linoleic acid (0.90 µmol) at t=0. *P≤0.05.*

Linoleic acid (LA) status

Plasma

Total fatty acid and LA concentrations were considerably reduced in plasma from (-/-) mice compared to (+/+) mice, in accordance with the reduced triglyceride levels which have been described before [25] (total fatty acids: 7.96 ± 0.36 mM vs 13.04 ± 0.81 mM; LA: 2.08 ± 0.13 mM vs 3.47 ± 0.25 mM; for -/- and +/+ mice, respectively; $P<0.001$). The biochemical parameter used to describe EFA status, the triene:tetraene ratio (20:3n-9/20:4n-6), was similar in (+/+) and (-/-) mice (Fig. 4). The triene:tetraene ratio did not reach the cutoff value for deficiency in either (+/+) or (-/-) mice. As shown in Figure 5a, the molar percentage of LA was not significantly different between groups; yet, molar percentages of LA metabolites, gamma-linolenic acid (18:3n-6), dihomo-gamma-linolenic acid (20:3n-6), and arachidonic acid (20:4n-6) were significantly decreased in (-/-) mice.

Liver

Molar percentages of LA and LA metabolites and the triene:tetraene ratio were similar in livers of (+/+) and (-/-) mice (Figs. 4 and 5b). In contrast to the observations in plasma, no difference in long-chain polyunsaturated metabolites of LA were noted between (+/+) and (-/-) mice (Fig. 5b).

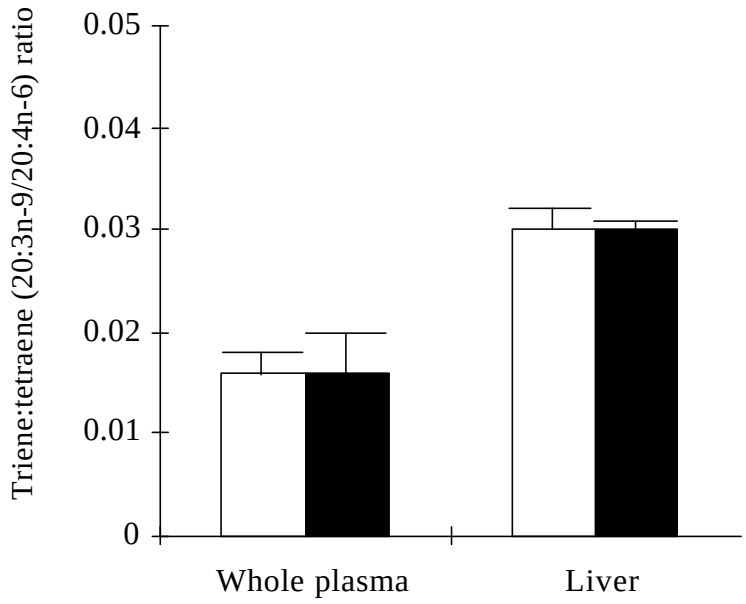
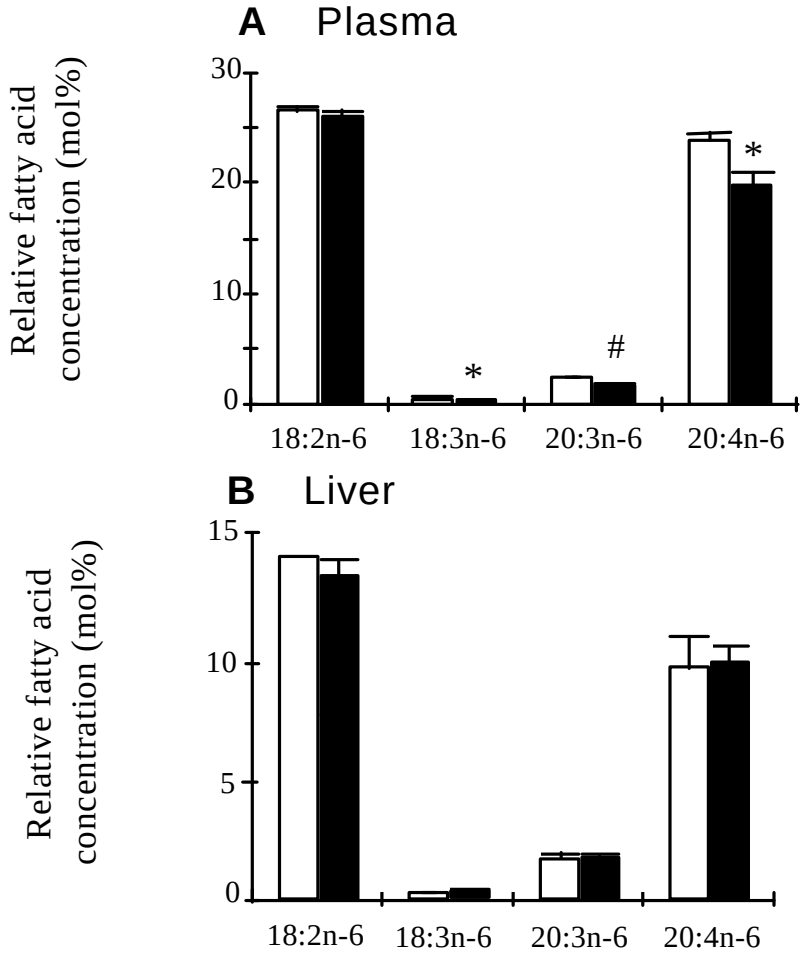


Figure 4. *Triene:tetraene (20:3n-9/20:4n-6) ratio in whole plasma and in liver of wildtype (+/+)* (*open bars*) *and mdr2 knockout (-/-)* (*closed bars*) *mice. Individual concentrations were determined by gas chromatography, as detailed in the Methods section. No significant difference was noted between groups.*

Metabolism of [^{13}C]-linoleic acid (LA) to [^{13}C]-arachidonic acid

Since the decreased concentration of LCPUFA in plasma (Fig. 5a) could be due to an impaired desaturation and/or elongation of dietary LA in (-/-) mice, we analyzed [^{13}C]-arachidonic acid in plasma and in liver of (+/+) and (-/-) mice. At 72 h after enteral [^{13}C]-LA administration, plasma concentrations of [^{13}C]-LA were lower in (-/-) mice compared to (+/+) mice (0.15 ± 0.02 µmol/L plasma vs. 0.30 ± 0.04 µmol/L plasma for (-/-) (n=4) and (+/+) (n=7) mice, respectively; $P<0.05$). However, no significant difference was noted in [^{13}C]-arachidonic acid

concentrations in plasma between both groups (0.64 ± 0.20 µmol/L plasma vs. 0.61 ± 0.08 µmol/L plasma for (+/+) and (-/-) mice (n=7 per group), respectively; $P>0.05$). There was no significant difference between (+/+) and (-/-) mice in the ratio of [^{13}C]-arachidonic acid concentration compared to [^{13}C]-LA concentration in liver (0.75 ± 0.10 vs. 0.82 ± 0.09 for (+/+) and (-/-) mice, respectively; $P>0.05$).



Figures 5a and 5b. *N-6 fatty acid status measurements in whole plasma (**a**) and in liver (**b**) of wildtype (+/+)(open bars) and mdr2 knockout (-/-) (closed bars) mice. Individual concentrations were determined by gas chromatography, as detailed in the Methods section. Plasma and liver values represent the mean ± S.E.M. of seven and three mice per group, respectively. *P<0.01, #P<0.001.*

Discussion

Biliary phospholipids would seem to be crucial for maintaining essential fatty acid (EFA) levels in the body due to their well-documented role in the transport of dietary lipid from the enterocyte into the lymph. In this study, we investigated whether biliary phospholipid secretion into the intestine is important for the intestinal absorption and for the status of LA *in vivo*. We used the *mdr*2 Pgp-deficient mouse model, in which bile salt secretion into bile remains normal while phospholipid secretion is absent and cholesterol secretion is strongly reduced [16]. Due to its unique characteristics, this model was able to provide an answer to the hypothesis as to whether biliary phospholipids are important for LA absorption and status [25]. The results indicated that the absorption of LA from the intestine is delayed in *mdr*2 Pgp-deficient mice. However, the total net intestinal absorption and the plasma status of LA

appeared highly preserved, even under the condition of complete absence of biliary phospholipids.

Plasma [^{13}C]-LA concentrations at 2 h after its enteral administration were significantly lower in (-/-) compared with (+/+) mice (Fig. 3). [^{13}C]-LA concentrations at 4 h were no longer significantly different between groups; however, they were increased in (-/-) mice compared to their 2 h values. Thus, it seemed that the appearance in plasma of enterally administered [^{13}C]-LA is delayed in *mdr*2 knockout (-/-) mice. Present data do not indicate what mechanism(s) can explain this apparently delayed appearance in plasma of (-/-) mice. In a previous study, we did not find indications for a difference in gastric emptying rate between control and *mdr*2 knockout (-/-) mice: the disappearance rate of radioactivity from the gastric lumen was virtually identical [26]. After intragastric administration of radiolabeled triglycerides, control and knockout mice had no difference in the radioactive content in the stomach and intestinal lumen [26]. Since there are strong indications that absorbed biliary (lyso)-PC provides the main source of PC for chylomicron coating [5,8,9,27], a lack of biliary PC could lead to a delay in one or more intracellular events in the lipid absorption process (i.e., reesterification, chylomicron assembly and/or secretion). It would be helpful to study if LA was distributed differently within lipid classes (i.e., triacylglycerols, phospholipids) or within the lipoproteins. These analyses were not possible in our study as we were limited by the amount of blood that could be drawn and the amount of lipid needed for accurate analysis by the GC-C-IRMS. Preliminary data indicate that the size of chylomicrons is similar between both control and (-/-) mice after intraduodenal administration of a lipid bolus, but that their number was decreased [26]. An alternative explanation for the apparently delayed absorption of [^{13}C-LA] in (-/-) mice involves impaired solubilization of lipolytic products in the intestinal lumen after dietary triacylglycerol ingestion. It is conceivable that a relatively low ratio of phospholipid to bile acids results in a disturbed formation of mixed micelles [28]. Upon conditions of impaired solubilization, (-/-) mice may absorb enteral lipids at a slower rate, using both proximal and distal sections of the intestine, in analogy to studies in bile-diverted rats [29]. Finally, another factor which could theoretically influence the appearance of [^{13}C]-LA in the blood is the relatively large amount of MCT oil included in the [^{13}C]-LA bolus. Apart from the fact that we found no indications favoring this possibility, it is not likely that it would explain the differences observed between control and (-/-) mice.

Although the presence of phospholipids in bile appeared to influence the kinetics of [^{13}C]-LA absorption from the intestine, the 72-h net absorption of either [^{13}C]-LA or of dietary LA was not impaired in *mdr*2 Pgp-deficient mice. As detailed in Figure 2, (+/+) and (-/-) mice absorbed similar amounts of LA. It was demonstrated that the contribution of LA from biliary phospholipids to total intestinal input of LA only amounted to ~1% (Figs. 1a and 1b). Since biliary phospholipid LA is part of ingested LA and accounts for a negligible fraction of the total LA in the intestinal lumen, biliary LA was not taken into consideration in the fecal balance calculations. Based on these observations, we conclude that the apparent delay in LA absorption does not influence its net absorption. From the present data, it cannot be derived whether the preserved efficiency of LA absorption upon absence of biliary phospholipids is

due to the high reserve capacity for lipid absorption present in the intestine [29], to an increased involvement of an alternative pathway, for example, transport via the portal vein [30,31], or both.

*Mdr*2 knockout (-/-) mice did not have biochemical indications for an impaired LA status in plasma or liver lipids (Figs. 4 and 5). Both the relative concentrations of LA (mol%) and the triene:tetraene ratio in plasma or in liver lipids were similar in (+/+) and (-/-) mice. This observation is in good agreement with the unaffected net LA absorption in (-/-) mice. Given the similar relative LA concentrations, remarkably, the relative concentrations of gamma-linolenic acid (18:3n-6), dihomo-gamma-linolenic acid (20:3n-6), and arachidonic acid (20:4n-6) were decreased in plasma of (-/-) mice, but not in liver. Low concentrations of LCPUFA in plasma can be due to one or more of the following: decreased absorption of the LCPUFA, decreased synthesis of LCPUFA from their precursors (including LA), increased catabolism of LCPUFA, or a redistribution of LCPUFA over the various compartments in the body. Since we have demonstrated that net intestinal absorption of LA is not decreased in (-/-) mice, it would seem unlikely that the absorption of the (physiochemically very similar) LCPUFA would specifically be affected. Moreover, except for minute amounts of arachidonic acid (20:4n-6), the administered chow did not contain LCPUFA. The supply of arachidonic acid from biliary phospholipids was ~65% of the amount provided by the chow on a daily basis, which could imply that (-/-) mice have lower levels of this LCPUFA due to their absence in bile. In rats fed chow containing virtually no arachidonic acid, Melin et al. [32] reported that up to 30% of the arachidonic acid in intestinal lipid could originate from bile. Also, the desaturation and elongation of LA to LCPUFA could be impaired in (-/-) mice due to a somehow decreased activity of the desaturation and elongation process. Yet, two observations do not support this possibility. No differences in LCPUFA concentrations of (-/-) livers were observed, compared with (+/+) livers, although the liver plays the main regulating role in LCPUFA homeostasis [33]. Additionally, the ratio of [^{13}C]-arachidonic acid to [^{13}C]-LA was not different in livers of (-/-) mice compared with (+/+) mice, suggestive of similar conversion rates of the enterally administered [^{13}C]-LA. Finally, the concentration of [^{13}C]-arachidonic acid 72 h after [^{13}C]-LA administration was similar in both groups. Present data do not allow us to conclude whether either the distribution of LCPUFA over the body compartments, including plasma, or the catabolism of LCPUFA (prostaglandin biosynthesis, oxidation) is different in (-/-) compared with (+/+) mice.

In summary, we have shown in a mouse model that the net intestinal absorption of dietary LA and of ^{13}C-LA is quantitatively similar, independent of biliary phospholipid secretion. However, [^{13}C]-LA appearance into plasma after its enteral administration is delayed in *mdr*2 knockout (-/-) mice, suggesting that biliary phospholipids are important for the rate of lipids from the intestinal lumen to the plasma compartment. Although specific effects on LCPUFA concentration in plasma cannot be excluded, biliary phospholipids are not required to maintain net intestinal absorption and status of LA in mice.

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CHAPTER 5

Impaired postprandial chylomicron formation but quantitatively unaffected fat absorption in multidrug resistance gene-2 P-glycoprotein-deficient mice

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Submitted

Abstract

It has been proposed that biliary phospholipids fulfill specific functions in the absorption of dietary fat from the intestine, but the physiological significance hereof has not been established. To evaluate the role of biliary phospholipids in dietary fat absorption *in vivo*, we used mice homozygous or heterozygous for disruption of the *mdr2* gene (*mdr2*$^{(-/-)}$, *mdr2*$^{(+/-)}$) and control (*mdr2*$^{(+/+)}$) mice. *Mdr2*$^{(-/-)}$ mice do not secrete phospholipids and cholesterol into bile, while bile salt secretion is not impaired. *Mdr2*$^{(+/-)}$ mice only show impaired (-40%) phospholipid secretion. Analysis of time-dependency of intestinal uptake and plasma appearance of intragastrically administered (radiolabeled) triglycerides and measurement of three-days fecal fat balance with low- and high-fat diets. Intragastric administration of olive oil resulted in a rapid increase in plasma triglycerides in *mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ but not in *mdr2*$^{(-/-)}$ mice. The "postprandial response" of plasma triglycerides could be partially restored in *mdr2*$^{(-/-)}$ mice by intraduodenal infusion of whole rat bile. After intragastric [^{3}H]-triolein administration in Triton WR1339 pre-treated animals, the appearance of [^{3}H]-triglycerides in plasma was reduced by 70% in *mdr2*$^{(-/-)}$ compared to *mdr2*$^{(+/+)}$ mice, excluding accelerated lipolysis as the cause of defective triglyceride response in *mdr2*$^{(-/-)}$ mice. [^{3}H]-triglycerides accumulated in enterocytes in *mdr2*$^{(-/-)}$ mice. Surprisingly, the efficacy of fat absorption as derived from balance studies was not or only minimally affected in *mdr2*$^{(-/-)}$ mice fed either low (14 en%) or high (35 en%) fat diets, respectively (all > 95%). These results demonstrate that biliary lipid secretion is necessary for postprandial appearance in plasma of chylomicrons *in vivo*, but not for quantitative absorption of dietary lipids.

Introduction

Bile has an important function in the absorption of dietary fat from the intestine. According to the classical Hofmann-Borgström hypothesis [1], biliary bile salts, phospholipids and cholesterol form mixed micelles that function as transport vehicles for fatty acids and monoglycerides, the products of triglyceride lipolysis, across the unstirred water layer towards the enterocytes. More recently, it has been suggested that vesicular structures, to a large extent composed of phosphatidylcholine and cholesterol, may have a function in the solubilization of dietary fat in the intestinal lumen [2-4]. Biliary phosphatidylcholine may have an additional role in fat absorption, namely in production of chylomicrons by the intestinal cells [5-7]. Studies in bile-diverted rats by Tso et al. [6,8-11] and others [12,13] have shown that the lymphatic triglyceride content increases during intestinal infusion of triolein and bile salts and can be enhanced further by the inclusion of phosphatidylcholine into the perfusate. Davidson et al. [14,15] demonstrated that the intestinal delivery of biliary phospholipids stimulates the synthesis of apolipoprotein B-48 (apoB-48), the apolipoprotein essential for chylomicron formation, by the intestine of bile diverted rats [14,15]. Furthermore, secretion of triglyceride-rich, apolipoprotein B-containing lipoproteins by Caco-2 cells incubated with taurocholate is strongly enhanced by co-incubation of the cells with bile-type phospholipids [16,17]. Collectively, these results indicate that biliary phospholipids may have a specific role in intestinal lipid absorption. However, because biliary phospholipid secretion is tightly coupled to that of bile salts under physiological conditions [18], it has sofar not been possible to evaluate the physiological importance of biliary lipids in lipid absorption independent from that of bile salts in the intact animal.

To elucidate the specific role of biliary phospholipids in intestinal fat absorption *in vivo* we used *mdr*2 P-glycoprotein knockout mice (*mdr2*$^{(-/-)}$) [19]. *Mdr*2 P-glycoprotein functions as a phospholipid flippase at the canalicular pole of hepatocytes and appears to be essential for biliary phospholipid secretion [19]: biliary secretion of phospholipids is absent in *mdr2*$^{(-/-)}$ mice [20]. Since biliary cholesterol secretion is linked to that of phospholipids, *mdr2*$^{(-/-)}$ mice also have a strongly reduced biliary cholesterol secretion [19,20]. On the other hand, biliary bile salt secretion is not impaired in the *mdr*2 Pgp-deficient mice [19-21]. Recently, we demonstrated that *mdr2*$^{(-/-)}$ mice have strongly reduced plasma High Density Lipoprotein (HDL) cholesterol levels, increased fecal neutral sterol loss and a reduced intestinal cholesterol absorption [22]. In the present study, we have compared plasma appearance of triglycerides and triglyceride uptake by intestinal cells after an intragastric (radiolabeled) fat load in *mdr2*$^{(-/-)}$, *mdr2*$^{(+/-)}$ and control *mdr2*$^{(+/+)}$ mice. Total lipid absorption was determined by a three day fecal fat balance. The results of these studies show that biliary lipids have a specific role in postprandial chylomicron production by the intestine. However, since total dietary fat absorption is virtually unaffected in *mdr2*$^{(-/-)}$ mice, alternative, chylomicron-independent routes for fat absorption are apparently operational that compensate for impaired chylomicron formation.

Materials and Methods

Animals

Mice homozygous (*mdr2*$^{(-/-)}$) or heterozygous (*mdr2*$^{(+/-)}$) for disruption of the multidrug resistance gene-2 (*mdr2*) and control (*mdr2*$^{(+/+)}$) mice of the same FVB (Free Virus Breed)-background were obtained from the breeding colony at the Animal Facility of the Academic Medical Center, Amsterdam. All mice were 2-4 months old and weighed 25-30 grams. Mice were housed in a light- and temperature-controlled facility and fed standard lab-chow for one week to adapt to their new environment. Mice were then put on a semi-purified low-fat diet containing 14 en% fat or on a high-fat diet containing 35 en% fat (Hope Farms, Woerden, The Netherlands) for two weeks. The composition of the diets is given in Table 1. Food and water were available *ad libitum*. All experiments were approved by the ethical committee on animal testing, University of Groningen, The Netherlands.

***Table 1**: Composition of the semi-purified low-fat (14 en%) and high-fat (35 en%) diets **

Compound	Low-fat diet % (wt/wt)	High-fat diet % (wt/wt)
Cerelose	54.3	37.6
Casein	20.0	22.3
Corn starch	10.0	11.4
Cellulose	5.0	5.7
Soy oil	5.0	-
Sunflower oil	-	6.0
Palm oil	-	10.0
Choline	0.4	0.5
Vitamins/minerals etc.	5.3	6.5

** As provided by the manufacturer (Hope Farms, Woerden, The Netherlands)*

Experimental procedures

Postprandial triglyceride response. After two weeks of feeding the semi-purified low-fat diet, *mdr2*$^{(-/-)}$, *mdr2*$^{(+/-)}$ and *mdr2*$^{(+/+)}$ mice (n = 6 per group) were fasted overnight. After taking a basal blood sample by tail bleeding, the animals received an intragastric load of 200 µL olive oil. Subsequently, hourly blood samples (75 µL) were drawn by tail bleeding up to four hours. Plasma was obtained by centrifugation at 9000 rpm for 10 minutes (Eppendorf centrifuge, Hamburg, Germany) and stored at -20^{o}C until analysis. Four hours after olive oil administration the small intestines of the mice were removed, washed in ice-cold phosphate buffered saline (PBS), immediately frozen in liquid nitrogen and stored at -80^{o}C until mRNA analysis.

In a separate experiment, eight male *mdr2*$^{(-/-)}$ mice were equipped with a permanent catheter in the duodenum that was tunelled subcutaneously to the head and fixed with acrylic glue to the skull of the mouse, as described previously for rats [23]. Three days after surgery, native rat bile collected from a rat with chronic bile diversion [23] (bile salts, 19.5 mmol/L; phospholipids, 3.6 mmol/L; cholesterol, 0.6 mmol/L) or a 0.9% NaCl solution was infused at a rate of 300 µL/hour via the intestinal catheter. At this infusion rate ~400 nmol bile

salts/min.100g, ~80 nmol phospholipids/min.100g and ~12 nmol cholesterol/min.100g are delivered to the intestine, which equals approximately two times the physiological biliary secretion rates of these components in normal FVB mice [19]. Two hours after infusion, a basal blood sample (75 µL) was taken from the tail vein and 200 µL olive oil was administered via the duodenal catheter. Subsequently, infusion of either rat bile or saline was continued for an other three hours. Blood samples (75 µL) were drawn at time points 0.5, 1, 2 and 3 hours after olive oil administration by tail bleeding. Plasma was obtained and handled as described above.

Distribution of intragastrically administered [^{3}H]-triglycerides in Triton WR1339-treated mice. Mice received an intragastric load consisting of 10 µCi [^{3}H]-labeled triolein (glycerol tri [9, 10(n)-^{3}H] oleate, 21.0 Ci/mmol, Amersham, Buckinghamshire, UK) in 200 µL olive oil directly after an intravenous injection of 12.5 mg Triton WR1339 (12.5 mg/100 µL PBS) to block lipoprotein lipase-mediated lipolysis [24-26]. Mice were bled at one, two and four hours after the intragastric load by cardiac punction and the small intestine was collected and flushed with 5 mL taurocholate (0.5 mmol/L) in saline, to remove excess luminal oil, and tissue was homogenized. The content of [^{3}H]-triglyceride in the luminal wash and tissue homogenates were measured by scintillation counting (Packard Instruments, Dowers Grove, IL, USA). Plasma was obtained as described above and 25 µL was used for scintillation counting. Small segments of the small intestine were immediately frozen in liquid isopentane and stored at -80^{o}C. Subsequently, 4 µm sections were cut for fat staining using Oil-Red-O (ORO).

In an independent series of experiments, the time course of plasma [^{3}H]-triglyceride appearance was determined in individual mice. Mice received a intragastric load of 10 µCi [^{3}H]-labeled triolein in 200µL olive oil directly after an intravenous injection of 12.5 mg Triton W1339 to block lipolysis. Blood samples (75 µL) were collected before and at one, two, three and four hours after label administration by tail bleeding and plasma was obtained as described above. Lipids were extracted following a modified Bligh & Dyer method [27] and triglycerides were separated by thin layer chromatography (Kieselgel 60 F_{254}, Merck, Darmstadt, Germany), using hexane: diethyl ether: acetic acid, 80: 20: 1 as eluens [28]. Spots containing [^{3}H]-triglycerides were scraped and the content of radioactivity was measured by scintillation counting (Packard Instruments, Dowers Grove, IL, USA). At four hours after oil administration, the mice were bled by cardiac punction and the chylomicron fraction was immediately isolated from 500 µL plasma by ultracentrifugation (Optima^{TM} TLX Ultracentrifuge, Beckman Instruments, Palo Alto, CA, USA) according to Pietzsch *et al*. [29].

Fat balance. Mice (n = 6 per group) were kept on the low- or high-fat diets. At two weeks after starting the experimental diets, food intake was recorded and feces was collected during a period of three days. Subsequently, animals were anesthetized with halothane and blood was collected by cardiac punction for lipid analysis, and liver and intestine were removed for further analysis. Analysis of fat intake and fecal fat output was determined as described previously in our laboratory by gas liquid chromatography [30] (see Chapter 2).

Northern Blotting

Total RNA of intestine was isolated according to Chromczynski and Sacchi [31], separated on agarose formaldehyde gel and transferred to a nylon membrane, (Hybond N, Amersham, Little Chalfont, UK), by overnight blotting. cDNA probes for apolipoprotein B were labeled using a random primed labeling kit to a specific activity of 10^8-10^9 cpm/µg [22]. Blots were prehybridized in hybridization solution (0.5 M $NaHPO_4$, 1 mM Na_2EDTA and 7% SDS, pH 7.2) and 100 µg herring sperm DNA per mL and hybridized at 65°C overnight at 1-2 *10^6 cpm/mL in hybridization solution. They were washed twice for 15 minutes in 2* SSC washing Buffer (0.3 M NaCl, 15 mM Na-citrate and 1 % SDS, pH 7.0) at 65°C. Activities were corrected for RNA concentration differences, using 28S rRNA as an internal control.

Analyses

Triglycerides, total cholesterol and cholesterol esters were determined using commercially available kits (Boehringer Mannheim, Mannhein, Germany). Phospholipids were determined using a commercially available kit based on phospholipase D hydrolysis and choline determination (WAKO Chemicals GmbH, Neuss, Germany). Total protein of tissue homogenates was determined using the method described by Lowry *et al*. [32].

Statistical analysis

All results are presented as means ± S.D. for the number of animals indicated. Differences between the three genotypes and differences between the different time points were determined by one-way ANOVA analysis [33], with posthoc comparison by Newmann Keuls t-test [33]. Differences between control and knockout mice was determined by Mann-Whitney *U* test [33]. Level of significance for all statistical analyses was set at $p < 0.05$. Analyses was performed using SPSS for Windows software (SPSS, Chicago, IL, USA)

Results

Plasma triglycerides after an intragastric load of olive oil

Control *mdr2*$^{(+/+)}$, heterozygous *mdr2*$^{(+/-)}$ and homozygous *mdr2*$^{(-/-)}$ mice received an intragastric olive oil load after which plasma triglycerides levels were determined during four hours. *Mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ mice showed a “postprandial” increase in plasma triglycerides, peaking at one hour after oil administration (Fig. 1). The postprandial response of plasma triglycerides was completely absent in the *mdr2*$^{(-/-)}$ mice.

The absence of a response in the *mdr2*$^{(-/-)}$ mice could theoretically be related to decreased expression of intestinal apolipoprotein B or to a general dysfunction of intestinal cells caused by chronic exposure to toxic, lipid-free bile. Intestinal apoB mRNA levels relative to 28S RNA levels were similar in knockouts and controls, i.e., 1.2 ± 0.4 and 1.0 ± 0.4, respectively. To check the second possibility, we tested whether infusion of native rat bile into the intestinal lumen would restore the postprandial plasma triglyceride appearance in *mdr2*$^{(-/-)}$ mice. Figure 2 shows that in *mdr2*$^{(-/-)}$ mice, that were infused with rat bile via a permanent duodenal

cathether, a significant increase in plasma triglycerides occurred, which peaked at two hours after olive oil administration. In contrast, *mdr2*$^{(-/-)}$ mice infused with saline did not show any response.

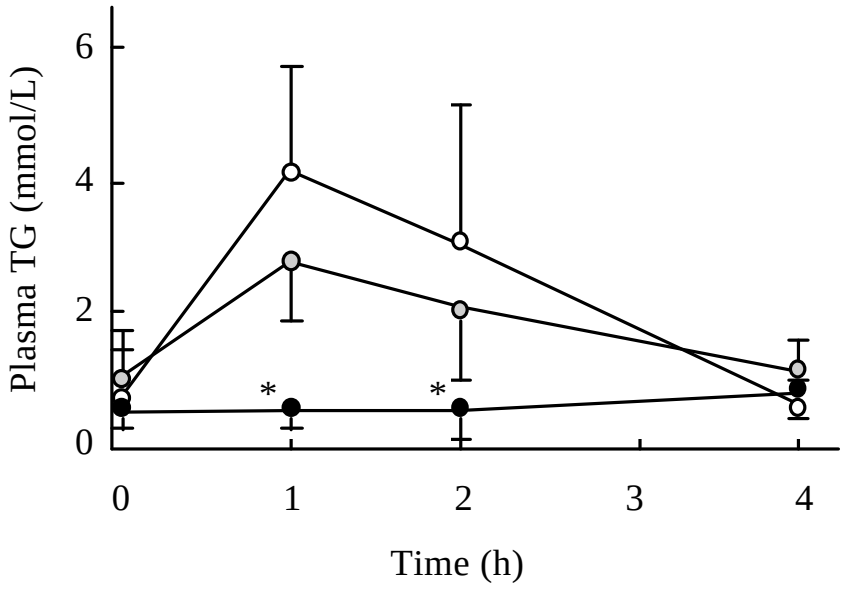

Figure 1. *Plasma triglyceride concentrations in mice after an intragastric lipid load (200 µL olive oil) at time point zero. Values represent mean ± SD, n = 6 per group. Symbols: mdr2*$^{(+/+)}$ *mice, open circles; mdr2*$^{(+/-)}$ *mice, grey circles; mdr2*$^{(-/-)}$ *mice, closed circles. *Differences between mdr2*$^{(-/-)}$ *and mdr2*$^{(+/-)}$*, mdr2*$^{(+/+)}$ *mice at time points one and two hours were significant as determined by ANOVA analysis and Newman-Keuls t-test posthoc analysis, P < 0.05.*

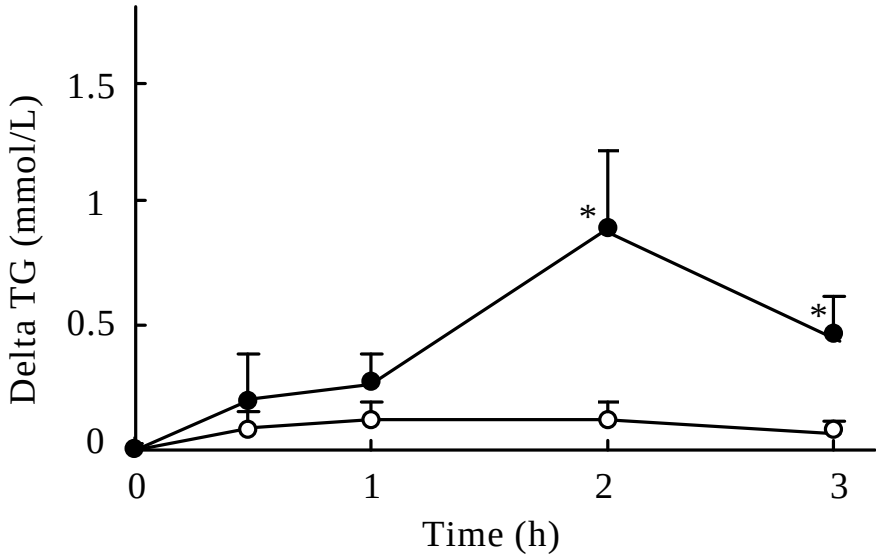

Figure 2*. Absolute increase in postprandial plasma triglyceride concentrations in mdr2*$^{(-/-)}$ *mice after an intraduodenal lipid load (200 µL olive oil) at time point zero during infusion of either whole rat bile or saline. The infused rat bile was collected overnight from a rat with chronic bile diversion [23]. Values represent delta values at the different time point compared to time point zero (mean ± SD), n = 4 per group. Symbols: saline, open circles; rat bile, closed circles. *Differences between saline*

and bile infused mice at time points two and three hours were significant as determined by Mann-Whitney U-test,P<0.05.

Distribution of intragastrically administered ^{3}H-triglycerides in Triton WR1339-treated mice

The absence of a postprandial triglyceride response in *mdr2*$^{(-/-)}$ mice could theoretically be due to 1. impaired uptake of "dietary" fat by intestinal cells; 2. impaired secretion from the intestinal cells into the lymph, i.e., chylomicron production; 3. strongly accelerated lipolysis of chylomicrons in the circulation. To differentiate between these possibilities, we injected mice with Triton WR1339 after which an intragastric load of olive oil containing ^{3}H-triolein was given. Figure 3 shows the time course of plasma appearance of radioactivity after an intragastric olive oil load with ^{3}H-triolein in individual Triton WR1339-injected *mdr2*$^{(+/+)}$, *mdr2*$^{(+/-)}$ and *mdr2*$^{(-/-)}$ mice. Thin-layer chromatographical analysis revealed that > 95% of plasma radioactivity was present in the triglyceride fraction. The plasma [^{3}H]-triglyceride content was reduced by 70% in *mdr2*$^{(-/-)}$ mice compared to *mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ mice at four hours after administration. No differences between *mdr2*$^{(+/-)}$ and *mdr2*$^{(+/+)}$ were observed.

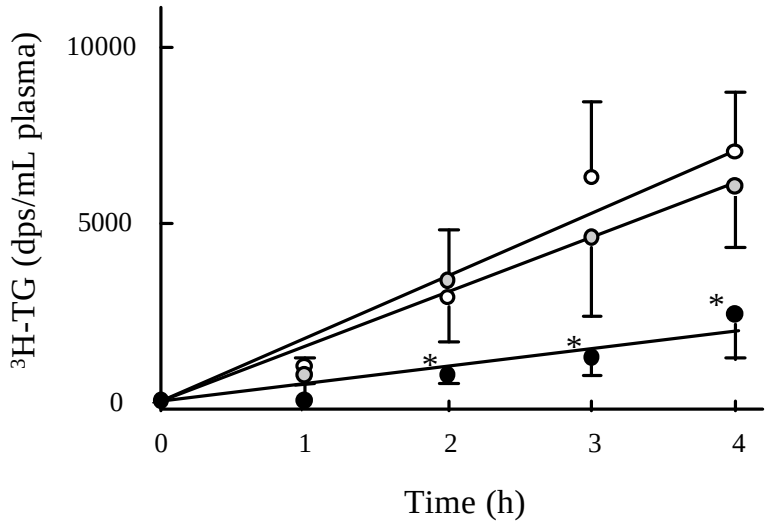

***Figure 3** Plasma appearance of [^{3}H]-triglycerides after an intragastric load of olive oil containing [^{3}H]-triolein and i.v. injection of Triton WR1339 at time point zero. Scanning of the thin layer plates indicated that essentially all of the [^{3}H]-label was in the triglyceride fraction. Values represent dps per milliliter plasma as determined by scintilation counting (mean ± SD), n = 5 per group. Symbols: mdr2$^{(+/+)}$ mice, open circles; mdr2$^{(+/-)}$ mice, grey circles; mdr2$^{(-/-)}$ mice, closed circles. *Differences between mdr2$^{(-/-)}$ and mdr2$^{(+/-)}$, mdr2$^{(+/+)}$ mice at time points two, three and four hours were significant as determined by ANOVA analysis and Newman-Keuls t-test posthoc analysis, p < 0.05.*

In a second experiment, Triton WR1339-pretreated mice were sacrified at one, two or four hours after [^{3}H]-triolein administration and radioactivity in the intestinal lumen, the intestinal wall and plasma was determined. The amount of radioactivity remaining in the stomach was similar in *mdr2*$^{(-/-)}$ and *mdr2*$^{(+/+)}$ mice at all time points studied (data not shown). Figure 4 shows that the [^{3}H]-triglyceride content of the intestinal lumen (top panel) did not differ between *mdr2*$^{(-/-)}$ and *mdr2*$^{(+/+)}$ mice at any of the time points. Radiolabeled-triglycerides accumulated in the intestinal wall (mid panel) of *mdr2*$^{(-/-)}$ and *mdr2*$^{(+/+)}$ mice during the course

of the experiment: this accumulation was significantly higher in the *mdr2*$^{(-/-)}$ as compared to *mdr2*$^{(+/+)}$ animals at four hours after administration (41.8 ± 4.8 vs. 26.8 ± 5.6 % dose, $P < 0.05$). These results show that administered fat is taken up by the intestinal cells of *mdr2*$^{(-/-)}$ mice, excluding possibility 1. As shown in the lower panel of Figure 4, appearance of [^{3}H]-triglycerides in plasma was reduced in the *mdr2*$^{(-/-)}$ compared to the *mdr2*$^{(+/+)}$ mice. Because lipolysis was prevented by Triton WR1339, excluding possibility 3, these data collectively imply that enterally administrered fat is taken up at normal rate by the intestinal cells of *mdr2*$^{(-/-)}$ mice and that their secretion from the intestinal wall into the blood compartment is delayed.

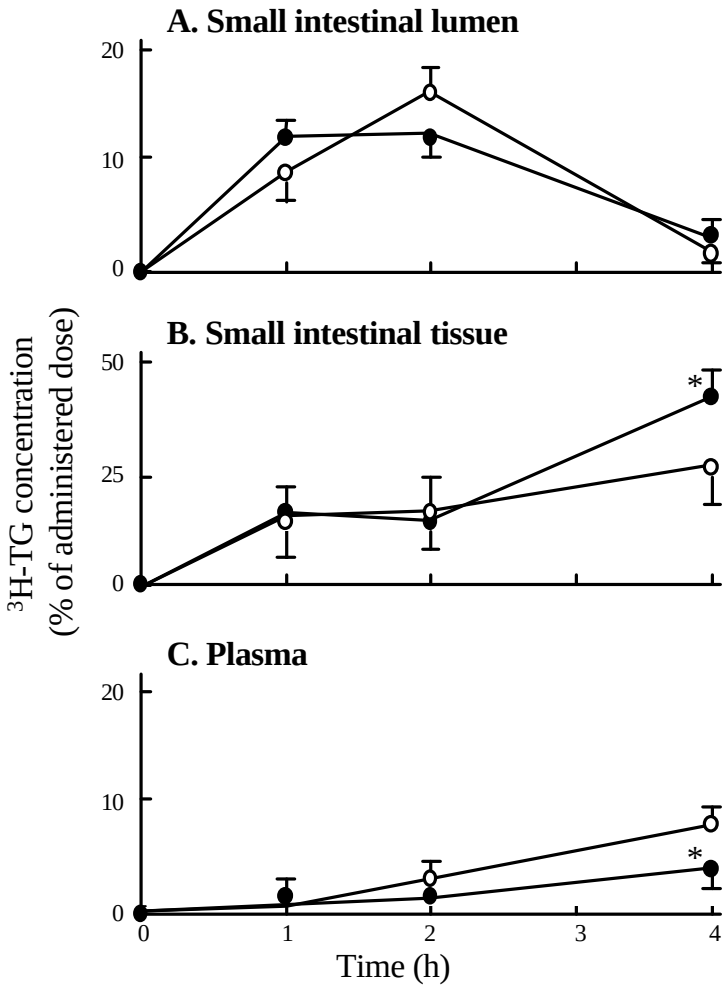

***Figure 4**. [^{3}H]-triglyceride content of the small intestinal lumen (A), the small intestinal wall (B) and plasma appearance (C) at different time points after intragastric installation of [^{3}H]-triolein labeled olive oil in mdr2$^{(+/+)}$ and mdr2$^{(-/-)}$ mice. Values represent percentage of the dose in the intestinal lumen wash, tissue homogenates and plasma at one, two and four hours after intragastric administration, n = 4 per group. Symbols: mdr2$^{(+/+)}$ mice, open circles; mdr2$^{(-/-)}$ mice, closed circles. *Differences between mdr2$^{(-/-)}$ and mdr2$^{(+/+)}$ mice were significant as determined by Mann-Whitney U test analysis, p < 0.05.*

At four hours after the fat load, intestinal tissue was collected for microscopical examination after staining for neutral fat by ORO (Fig. 5). Fat droplets in the intestine of control mice were mainly localized to the interstitium of the villi, probably in the lymph ducts (Fig. 5a). In *mdr2*$^{(-/-)}$ mice (Fig. 5b), on the other hand, there was intense staining of large fat droplets within the enterocytes and less staining in the interstitium.

Chylomicron formation and composition

At four hours after an intragastric fat load in Triton WR1339-pretreated mice, chylomicrons were isolated by ultracentrifugation and their composition was determined. As shown in Table 2, no significant differences were observed in the relative contribution of free cholesterol,

triglycerides or phospholipids to the total lipid content of the isolated chylomicron fractions between the three groups. A somewhat larger contribution of cholesterol esters was found in particles isolated from *mdr2*$^{(+/-)}$ and *mdr2*$^{(-/-)}$ mice when compared to those of *mdr2*$^{(+/+)}$ mice. The chylomicron fraction isolated from *mdr2*$^{(-/-)}$ mice contained approximately 70% less lipid mass (sum of total cholesterol, phospholipids and triglycerides) than those of *mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ mice, in accordance with the data obtained with radiolabeled triolein.

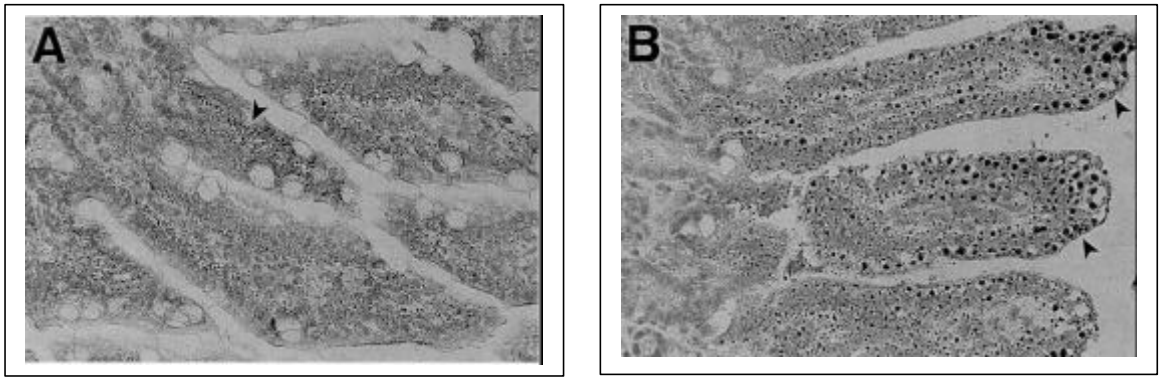

***Figure 5**. Representative small intestinal sections of mdr2$^{(+/+)}$ (A) and mdr2$^{(-/-)}$ mice (B), collected at four hours after an intragastric olive oil load. The small intestine was flushed with ice-cold PBS and immediately frozen at -80^{o}C. The 4 µm sections were stained with ORO and counterstained with haematoxilin (magnification 120 x). Arrowheads indicate the small fat droplets in the intestinal interstitium of mdr2$^{(+/+)}$ mice (A) and the large fat droplets in the enterocytes of mdr2$^{(-/-)}$ mice (B).*

***Table 2.** Lipid composition of chylomicron fractions of mdr2$^{(+/+)}$, mdr2$^{(+/-)}$ and mdr2$^{(-/-)}$ mice, isolated by ultracentrifugation of 500 µL plasma collected at four hours after an intragastric olive oil load and intravenous injection of 12.5 mg Triton WR1339. Values represent percentage of the lipid class of the total lipid mass and are given as mean ± SD, n = 3.*

Mdr2	Diet	Protein (%)	Lipid mass (mg)	Free cholesterol (%)	CE (%)	TG (%)	PL (%)
+/+	LF	2.8 ± 0.8	3.8 ± 0.9	3.9 ± 0.5	1.6 ± 0.01[a]	80.9 ± 5.8	14.1 ± 4.4
+/-	LF	3.3 ± 0.8	3.9 ± 0.4	2.5 ± 0.5	4.6 ± 1.0	76.5 ± 3.2	16.4 ± 3.2
-/-	LF	4.2 ± 0.4	1.1 ± 0.5[a]	2.3 ± 0.4	5.3 ± 0.3	78.1 ± 0.4	14.3 ± 0.3

[a] *significant difference between mdr2$^{(-/-)}$ and mdr2$^{(+/-)}$/mdr2$^{(+/+)}$ mice, $p < 0.05$. CE=cholesterol ester; TG=triglyceride; PL=phospholipid.*

Fat balance studies

To explore the physiological consequence of impaired chylomicron release into the plasma compartment, total fat absorption was measured using a three day fat balance in mice fed either a low-fat (14 en%) or a high-fat (35 en%) diet for two weeks. No differences were observed between the absorption coefficients in the three groups of mice on the low-fat diet (Table 3): all groups were able to absorb more than 98% of their dietary fat in this situation. On a high-fat diet, the *mdr2*$^{(-/-)}$ mice showed a marginal decrease in absorption coefficient compared to *mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ mice, but were still able to absorb ~95% of their dietary intake.

***Table 3**. Total daily dietary fat intake (µmol), fecal fat output (µmol) and fat absorption coefficient (%) in mdr2$^{(+/+)}$, mdr2$^{(+/-)}$ and mdr2$^{(-/-)}$ mice fed low-fat (LF, 14% en) or high-fat (HF, 35% en) diets. Dietary fat intake and fecal fat output were determined over a period of three days. Values represent the dietary intake and fecal output over the whole three-day period. The apparent absorption coefficient was calculated as input-output/input x 100%. Values are mean ± SD, n = 7.*

*Mdr*2	Diet	Lipid intake (µmol)	Fecal output (µmol)	Absorption (%)
+/+	LF	1710 ± 140	40 ± 80	97.7 ± 4.3
+/-	LF	1680 ± 900	30 ± 40	97.8 ± 2.5
-/-	LF	1810 ± 170	30 ± 30	98.4 ± 1.5
+/+	HF	4640 ± 1060 [b]	50 ± 30	98.8 ± 0.7
+/-	HF	5430 ± 640 [b]	130 ± 50 [b]	97.7 ± 1.2
-/-	HF	4980 ± 550 [b]	250 ± 90 [b,c]	94.9 ± 2.5 [a]

[a] *significant difference between mdr2$^{(-/-)}$ and mdr2$^{(+/+)}$ mice on same diet, p < 0.05.*
[b] *significant difference between high- and low-fat diets per mouse strain, p < 0.05.*
[c] *significant difference between mdr2$^{(-/-)}$ and mdr2$^{(+/-)}$/mdr2$^{(+/+)}$ mice on same diet, p < 0.05.*

Plasma cholesterol and triglycerides levels showed similar absolute increases in reponse to feeding of the high-fat diet in all groups (Table 4). In contrast, livers of *mdr2*$^{(-/-)}$ mice accumulated significantly more triglycerides than those of *mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ mice under these conditions, indicative for altered hepatic handling of dietary fat by *mdr2*$^{(-/-)}$ mice.

***Table 4**. Plasma cholesterol and triglyceride concentrations and hepatic triglyceride content of mdr2$^{(-/-)}$, mdr2$^{(+/-)}$ and mdr2$^{(+/+)}$ mice on low-fat and high-fat diets. Values represent mean ± SD, n = 7. Hepatic triglyceride levels were measured in liver homogenates after a modified Bligh & Dyer lipid extraction (27), and expressed as total amount per liver, mean ± SD, n = 4. Values in parentheses indicate percentage change induced by the high-fat diet compared to the low-fat diet.*

Mdr2	Diet	Plasma cholesterol (mmol/L) (%LF)	Plasma triglycerides (mmol/L) (%LF)	Hepatic triglycerides (µmol/liver) (%LF)
+/+	LF	5.3 ± 0.5	0.7 ± 0.5	27.3 ± 9.6
+/-	LF	5.1 ± 0.4	1.0 ± 0.6	31.5 ± 7.3
-/-	LF	2.0 ± 0.5[b]	0.5 ± 0.2	22.4 ± 8.7
+/+	HF	6.4 ± 1.0[a] (+ 20%)	1.1 ± 0.4 (+ 57%)	35.9 ± 3.7 (+ 32%)
+/-	HF	6.3 ± 0.9[a] (+ 44%)	1.6 ± 0.5 (+ 60%)	37.3 ± 3.2 (+ 18%)
-/-	HF	3.7 ± 0.8[ab] (+ 85%)	1.1 ± 0.4[a] (+ 120%)	55.5 ± 13.1[ab] (+ 148%)

[a] *significant difference between high- and low-fat diets per mouse genotype, p < 0.05.*
[b] *significant difference between mdr2$^{(-/-)}$ and mdr2$^{(+/-)}$/mdr2$^{(+/+)}$ mice, p < 0.05.*

Discussion

In this study we have addressed the role of biliary lipids in postprandial triglyceride secretion by the intestine and overall fat absorption *in vivo* in intact mice. Studies in bile diverted rats and cultured cells have indicated that biliary phospholipids may fulfill an independent role in the process of intestinal fat absorption [8,10,16] but the physiological consequences hereof for

the intact animal have remained unclear sofar. The present study shows that the characteristic plasma triglyceride response found in control animals after an intragastric fat load is completely abolished in mdr2 P-glycoprotein-deficient mice, that lack biliary phospholipids and cholesterol but do have normal bile salt secretion [19]. Under normal conditions dietary fats are incorporated into chylomicrons and subsequently transported via the lymph into the bloodstream. ApoB-48 is essential for chylomicron formation [24]. Yet, the absence of a postprandial response in *mdr2*$^{(-/-)}$ mice is unlikely related to (relative) apoB-48 deficiency in their intestine. First, we were able to demonstrate that apoB mRNA levels in the intestine of *mdr2*$^{(-/-)}$ mice are similar to those in *mdr2*$^{(+/+)}$ mice. This is in agreement with studies from Davidson et al. [14], showing no significant changes in apoB mRNA levels in the absence of bile or during infusion of bile components in rats. Second, infusion of rat bile into the intestine partly restored the capacity to elicit a postprandial triglyceride response in *mdr2*$^{(-/-)}$ mice. Since bile salts are already abundant in the intestinal lumen of *mdr2*$^{(-/-)}$ mice, these results indicate that the presence of biliary lipids *per se* is required for this response to occur. The postprandial plasma triglyceride response in *mdr2*$^{(-/-)}$ mice infused with rat bile was less pronounced and delayed when compared to that in control mice (compare Figs. 1 and 2). The volume of bile infused into the intestinal lumen was approximately two times the physiological bile flow in FVB mice [20]. This result could be possibly explained by the fact that the oil load was administered directly into the duodenum, which is not a physiological condition. A different response may have been obtained if the bolus was intragastrically administered, thus allowing time for the sequential steps involved in lipid absorption. As a consequence, the actual uptake of administered fat may have been delayed when compared to that of the intragastric fat load in the experiments shown in Fig. 1. In addition, the intestine of *mdr2*$^{(-/-)}$ mice has been deprived of biliary lipids from birth and it can be speculated that this may have physiological consequences for triglyceride handling by intestinal cells. Despite these limitations, the bile infusion experiment clearly shows that the intestine of *mdr2*$^{(-/-)}$ mice is able to evoke a postprandial triglyceride response when the appropriate bile components are available.

To differentiate directly between the potential causes underlying the defective postprandial triglyceride response in *mdr2*$^{(-/-)}$ mice, i.e., decreased uptake of fat by intestinal cells [34,35], impaired delivery from the intestine to the plasma compartment [11], or accellerated lipolysis of chylomicrons in the circulation [26], the fate of intragastrically installed radiolabeled triglycerides was followed in Triton WR1339-injected animals. Since chylomicron lipolysis was eliminated in these experiments, accellerated lipolysis could be excluded as the cause of the impaired [^{3}H]-triglyceride appearance in *mdr2*$^{(-/-)}$ mice observed in this experiment. Because the luminal content of radioactivity did not differ between *mdr2*$^{(+/+)}$ and *mdr2*$^{(-/-)}$ mice and the amount of radioactivity present in the intestinal wall was actually higher in the latter group, impaired uptake of fat by the intestinal epithelium is unlikely responsible for the decreased plasma appearence of [^{3}H]-triglycerides in *mdr2*$^{(-/-)}$ mice. It should be noted that the plasma appearance of labeled triglycerides in Triton WR1339-injected *mdr2*$^{(+/+)}$ (figure 3) was slower than that of mass triglycerides in untreated *mdr2*$^{(+/+)}$ mice (figure 1), although in both cases a similar load of fat was administered. This is probably explained by rapid

incorporation into postprandial lipoproteins of unlabeled triglycerides from endogenous sources present in the enterocytes [36]. Collectively, the data strongly indicate that intragastrically administered fat is able to enter the intestinal cells of *mdr2*$^{(-/-)}$ mice but is subsequently less efficiently transported into the lymph in the absence of biliary lipids. This is supported by the observation that, at four hours after intragastric fat administration, ORO-positive material is mainly retained in the enterocytes of *mdr2*$^{(-/-)}$ mice while in *mdr2*$^{(+/+)}$ mice there is a clear shift towards the interstitium of the intestinal villi.

Both from the labeling experiments and from measurement of chylomicron lipid mass, it appeared that plasma lipid accumulation after intragastric fat loading was ~70% lower in *mdr2*$^{(-/-)}$ mice compared to *mdr2*$^{(+/+)}$ mice treated with Triton WR1339. This indicates that chylomicrons of similar composition and size as in *mdr2*$^{(+/+)}$ mice are secreted by the intestine of *mdr2*$^{(-/-)}$ mice but at a lower rate. The total absence of a postprandial response in untreated *mdr2*$^{(-/-)}$ mice (Fig. 1) is therefore most likely explained by rapid hydrolysis of the few particles that are formed. Since biliary phospholipid content in *mdr2*$^{(+/-)}$ mice is about 60% of that of *mdr2*$^{(+/+)}$ mice, this observation indicates that 60% of the normal biliary phospholipid output is sufficient to maintain adequate chylomicron formation in this experimental set-up. Mathur et al. [17] showed *in vitro* that a certain threshold concentration of phosphatidylcholine is necessary for stimulation of chylomicron secretion by CaCo-2 cells. Apparently, these requirements are fulfilled in the *mdr2*$^{(+/-)}$ mice.

Since chylomicron surface materials, i.e., phospholipids and cholesterol, provide an important source for HDL lipids [37,38] it is tempting to speculate that decreased formation and secretion of chylomicrons in *mdr2*$^{(-/-)}$ mice contributes to the low HDL-cholesterol levels found in these mice [22].

Surprisingly, a three day fecal fat balance revealed that *mdr2*$^{(-/-)}$ mice were able to absorb more than 98% of dietary fat when fed a low-fat diet. Even on a high-fat diet, *mdr2*$^{(-/-)}$ mice absorbed ~95% of their dietary fat, although in this case the absorption coefficient was slightly decreased when compared to *mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ mice. Similar absolute increases in plasma cholesterol and triglyceride levels were induced by high-fat feeding in *mdr2*$^{(-/-)}$, *mdr2*$^{(+/-)}$ and *mdr2*$^{(+/+)}$ mice. The changes in plasma lipid concentrations were of the same order of magnitude as reported by others [39,40] in mice fed similar high-fat/low-cholesterol diets and demonstrate that the metabolic actions of dietary fats leading to elevation of plasma lipid levels, which are in all likelihood mainly exerted after their absorption, are not affected by *mdr*2 Pgp-deficiency. Several factors may contribute to the efficient overall lipid absorption in *mdr2*$^{(-/-)}$ mice. Experiments with bile-diverted rats have shown that fat absorption is delayed in time [41] and that it shifts from proximal to more distal parts of the intestine [41,42] in the absence of bile in the intestine. Absence of biliary lipids alone may have similar effects. In addition, absorption of dietary fat in the form of free fatty acids via the portal vein may occur, as proposed by Mansbach et al. [43,44]. Mansbach et al. [44] found that portal absorption of long acyl chain lipids in bile-diverted rats depends on the lipid load and is greatly reduced when phosphatidylcholine is included in a lipid infusion mixture. Thus, unchanged overall fat

absorption may in part be associated with increased portal long-chain fatty acid absorption in *mdr2*$^{(-/-)}$ mice. We found a significant increase in hepatic triglyceride content in *mdr2*$^{(-/-)}$ mice on the high-fat diet compared to the low-fat diet, while no significant increase was observed in *mdr2*$^{(+/+)}$ and *mdr2*$^{(+/-)}$ mice. This may be a consequence of direct entry of intestinally-derived fatty acids into the liver, followed by their incorporation in hepatic triglycerides, in the *mdr2*$^{(-/-)}$ mice. Portal concentrations of free fatty acids at four hours after an intragastric olive oil bolus were not different between *mdr2*$^{(+/+)}$ and *mdr2*$^{(-/-)}$ mice, i.e., 1.0 ± 0.3 and 0.8 ± 0.1 mmol/L, respectively. However, since portal blood flow has not been measured in these mice, these data cannot be interpreted with respect to the actual fatty acid delivery to the liver.

In conclusion, we have shown that the "postprandial" entry of triglycerides into plasma is decreased in *mdr2*$^{(-/-)}$ mice *in vivo*, due to delayed chylomicron production by the enterocytes. Based on the present results and on literature data [5,6,8,12,14,16], we propose that the absence of biliary phospholipids is responsible for this phenomenon, since biliary cholesterol does not seem to have a major role in intestinal chylomicron secretion [16]. Because chylomicron surface lipids provide an important source of HDL lipids, impaired postprandial chylomicron production may, at least in part, explain the low HDL levels found in the *mdr2*$^{(-/-)}$ mice [22]. In spite of impaired chylomicron formation, overall fat absorption is not affected in *mdr2*$^{(-/-)}$ mice. In contrast, cholesterol absorption as determined by a dual isotope procedure is reduced by ~50% in these animals [22], indicative for separate mechanisms of absorption for dietary long-chain fatty acids and for cholesterol. Our data support the existence of alternative, chylomicron-independent mechanisms of dietary long-chain fatty acid uptake that ensure maintenance of efficient fat absorption in situations in which chylomicron formation is disturbed.

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CHAPTER 6

Lipid malabsorption in essential fatty acid-deficient mice is not due to impaired bile secretion

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Submitted

Abstract

Essential fatty acid (EFA) deficiency results in lipid malabsorption. Biliary bile salts and phospholipids serve specific functions in the lipid absorption process, particularly in solubilization of dietary fat and in chylomicron formation, respectively. We investigated whether alterations in biliary bile salts and/or phospholipid secretion are involved in EFA-deficiency-induced lipid malabsorption in mice. Mice that secrete normal amounts of bile salts and phospholipid into bile (*mdr*2$^{(+/+)}$) and mice that produce phospholipid-free bile (*mdr*2$^{(-/-)}$) were fed either EFA-containing (EFA^{+}) or EFA-deficient (EFA^{-}) chow for 8 weeks. Then, dietary lipid absorption was quantified and bile flow and composition were measured. Lipid absorption, particularly of saturated, long-chain fatty acids, was significantly reduced in EFA-deficient mice compared with mice fed the EFA^{+} chow. The absence of biliary phospholipid secretion in EFA-deficient *mdr*2$^{(-/-)}$ mice was associated with a more pronounced decrease in lipid absorption compared to EFA-deficient *mdr*2$^{(+/+)}$ mice (60.4 ± 2.1% vs. 70.1 ± 1.6%; $P<0.01$). Bile flow and biliary bile salt secretion were more than 2-fold increased in EFA-deficient *mdr*2$^{(+/+)}$ and *mdr*2$^{(-/-)}$ mice compared with mice fed the EFA^{+} chow ($P<0.01$). Bile salt composition was virtually identical between EFA^{+} and EFA^{-} -fed mice of either strain. Present data indicate that lipid malabsorption in EFA-deficient mice is not due to decreased secretion rate of bile salts into bile or to alterations in bile salt composition. Under EFA-deficient conditions, the biliary secretion of phospholipids partially counteracts the extent of

lipid malabsorption. We hypothesize that EFA deficiency affects intracellular processing (i.e., chylomicron production) of dietary lipid by enterocytes.

Introduction

Essential fatty acid (EFA) deficiency has been associated with a number of pathological consequences in rats, including lipid malabsorption [1-5]. The pathophysiology of lipid malabsorption in this condition has not yet been elucidated. In an attempt to address this question, the various intraluminal and intracellular stages of the absorption process (i.e., dietary triglyceride lipolysis by pancreatic lipase, solubilization of lipolytic products by bile, uptake by the brush border membrane of the enterocyte, reesterification to triglycerides, and chylomicron assembly and secretion into the lymph) have been investigated in control and EFA-deficient rats [3,4]. Indirect indications support the view that neither pancreatic lipolysis nor uptake of fatty acids by the enterocyte is responsible for the lipid malabsorption encountered in EFA-deficient rats [3,4]. Rather, the most likely mechanisms by which EFA deficiency induces lipid malabsorption seem to involve its effects on bile formation and on intracellular processing of lipids by the enterocyte. Indeed, EFA-deficient rats had significantly decreased bile flow and secretion of bile salts, phospholipids and cholesterol [3,6]. Analysis of acyl chain composition of biliary phosphatidylcholine (PC) from EFA-deficient rats revealed that biliary PC content of the parent EFA, linoleic acid (18:2n-6), and its metabolite, arachidonic acid (20:4n-6), were decreased to approximately 10% and 26% of control values, respectively, and replaced with the nonessential fatty acid, oleic acid (18:1n-9) [4]. These changes may influence the solubilization of dietary lipids in the lumen. In addition to changes in bile, intracellular events, such as fatty acid reesterification and chylomicron secretion into the lymph, were severely impaired in EFA deficiency [3,4]. However, it has not been elucidated if either altered bile formation or intracellular changes are primarily involved in the development of lipid malabsorption, or represent secondary events.

Our hypothesis is that lipid malabsorption in EFA deficiency could be related in part to the effects of the deficiency on bile composition in general or, in particular, to altered biliary PC secretion. The role of bile formation *per se* in the intestinal absorption of dietary lipid is well established. Biliary bile salts, phospholipids, and cholesterol form mixed micelles that function as transport vehicles for fatty acids and monoacylglycerols, the products of triglyceride lipolysis, across the unstirred water layer towards the enterocytes [7]. PC may also serve other functions in intestinal lipid absorption, i.e., supplying the surface component required by chylomicrons before their secretion from the enterocyte into the lymph [8-11]. Also, although it has not been demonstrated, the EFA-rich acyl chain composition of the PC molecule may be relevant for dietary lipid uptake and/or for maintenance of the intestinal mucosa membrane.

In order to discern the roles of bile salt and phospholipid secretion in EFA-deficiency-induced lipid malabsorption, the availability of an animal model is necessary. Mice lacking *mdr*2 gene product in the bile canalicular membrane, also known as *mdr*2 knockout mice (*mdr*2$^{(-/-)}$), have

recently become available for studying the effects of phospholipid-free bile [12,13]. In the present study, we compared lipid absorption and bile formation in control mice and *mdr*2$^{(-/-)}$ mice after feeding EFA-containing or EFA-deficient chow.

Materials and Methods

Animals

Mice homozygous (-/-) for disruption of the multidrug resistance gene-2 (*mdr*2$^{(-/-)}$) and control (*mdr*2$^{(+/+)}$) mice with a FVB (free virus breed) background were obtained from the breeding colony at the Central Animal Facility, Academic Medical Center, Amsterdam, The Netherlands [14]. All mice were 2-4 months old and weighed 25-30 g. Mice were housed in a light- (lights on 6 AM - 6 PM) and temperature-controlled (21ºC) facility and allowed tap water and chow (Hope Farms B.V., Woerden, The Netherlands) *ad libitum*. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, Groningen, The Netherlands.

Experimental procedures

Female *mdr*2$^{(+/+)}$ and *mdr*2$^{(-/-)}$ mice (n=14 per group, 28 mice total) were fed low-fat (6 wt% lipid, 14 en% lipid) chow for standardization. One month after low-fat chow feeding, mice were anesthetized with halothane and a baseline blood sample for EFA status was obtained by tail bleeding. Blood was collected in micro-hematocrit tubes containing heparin, after which plasma was obtained by centrifugation at 9000 rpm for 10 min (Eppendorf centrifuge, Eppendorf, Germany) and stored at -20°C until analysis. After obtaining this baseline sample, mice were randomly assigned to be fed either EFA-enriched (EFA^{+}) or EFA-deficient (EFA^{-}) chow. The EFA^{+} and EFA^{-} chow were isocaloric and contained 16 wt% lipid. The EFA^{+} chow contained 20 en%, 37en% and 43en% from protein, lipid and carbohydrate, respectively, and had the following fatty acid profile: 32.1% palmitic acid (16:0), 5.5% stearic acid (18:0), 32.2% oleic acid (18:1n-9), and 30.2% linoleic acid (18:2n-6). The EFA^{-} chow contained 20en%, 34en% and 46en% from protein, lipid and carbohydrate, respectively, and had the following fatty acid profile: 41.4% 16:0, 47.9% 18:0, 7.7% 18:1n-9, and 3% 18:2n-6. After eight weeks of feeding, blood samples were taken in the same manner as described above. A 72 h fecal lipid balance was performed to measure lipid absorption. During the 72 h period, feces was collected in one fraction and chow intake was determined by weighing the chow container. After one week, mice were anaesthetized by intraperitoneal injection of Hypnorm (fentanyl/fluanisone) and diazepam and their gallbladders were cannulated for collection of bile for 1 h as described previously [15]. At the end of bile collection, a large blood sample (0.6 - 1.0 mL) was obtained by heart puncture. From three mice out of every group, the liver was removed, weighed and stored at -80ºC until further analysis.

Plasma accumulation of oral [^{3}H]-triolein and [^{14}C]-oleic acid after Triton W1339 injection

In a separate experiment, male *mdr2*$^{(+/+)}$ mice fed EFA^{+} or EFA^{-} chow for eight weeks (n=5 per group) were intravenously injected with 12.5 mg Triton W1339 (12.5 mg/100 µL PBS) to block lipolysis of circulating lipids in blood. Mice were intragastrically administered a lipid bolus containing 200 µL olive oil, in which 10 µCi [^{3}H]-triolein (glycerol tri [9, 10(n)-[^{3}H]] oleate) (Amersham, Buckinghamshire, UK) and 2 µCi [^{14}C]-oleic acid (NEN Laboratories, Boston, MA, USA) was dispersed. Blood samples (75 µL) were collected before (t=0) and at one, two, three and four hours after label administration by tail bleeding (as described above). The content of [^{3}H] and [^{14}C] in plasma (25 µL) was measured by scintillation counting (Packard Instruments, Downers Grove, IL, USA).

Analytical techniques

Lipid analyses. Fatty acid status was analyzed by extracting, hydrolyzing and methylating total plasma lipids (triglycerides, phospholipids, etc.), liver homogenate and biliary lipids according to Lepage and Roy [15] (see Chapter 2). Plasma lipids (cholesterol, HDL-cholesterol, triglycerides, free fatty acids and phospholipids) were measured using commercially available kits (Boehringer Mannheim, Mannheim, Germany) according to the instructions provided. Contents of cholesterol, cholesterol ester and triglycerides in liver tissue were determined after lipid extraction as described previously [16]. Biliary bile salt composition was measured as previously described [16]. Total protein of liver homogenates was determined using the method described by Lowry et al. [17]. Feces and chow pellets were first freeze-dried and then mechanically homogenized. Aliquots of each were extracted, hydrolyzed and methylated [15]. Resulting fatty acid methyl esters were analyzed by gas chromatography for their fatty acid content as detailed previously in [18] (see Chapter 2).

Calculations

Fatty acid status in plasma and in bile. Relative concentrations (mol%) of plasma and biliary phospholipid fatty acids were calculated using the sum of major fatty acid peaks and then expressing the area of each individual fatty acid as a percentage of this amount (see Chapter 4). EFA status was determined by calculating the triene:tetraene ratio (20:3n-9/20:4n-6) in plasma of mice. A ratio of >0.2 was considered deficient [19].

Fatty acid absorption using 72 h balance techniques. Absorption of major dietary fatty acids (palmitic, stearic, oleic, linoleic acids) was determined by subtracting the amount of an individual fatty acid excreted in feces over 72 h from the amount of dietary fatty acids ingested during 72 h (net molar absorption), and expressed as a percentage of the amount of fatty acid ingested during 72 h (percentage of absorption). Total lipid absorption was calculated by subtracting the sum of the excretion of these four fatty acids from the intake (see Chapter 2).

Statistics

All results are presented as means ± S.E.M. for the number of animals indicated. Data were statistically analyzed by ANOVA and Student's two-tailed *t* test. Level of significance for all statistical analysis was set at $P<0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL, USA).

Results

Body weight and chow ingestion

Body weight was monitored at the start of experimental feeding, and then every two weeks for eight weeks. No differences in weight for up to eight weeks were found for *mdr*2$^{(+/+)}$ mice fed either EFA^{+} or EFA^{-} chow (P<0.05). However, compared with the *mdr*2$^{(-/-)}$ mice fed EFA^{+} chow, body weights of *mdr*2$^{(-/-)}$ mice fed EFA^{-} chow gradually decreased and by eight weeks, the difference in weight between both groups was significant (25.3 ± 1.1g vs. 20.2 ± 1.0g for EFA^{+} and EFA^{-} chow-fed mice, respectively; P<0.01). Chow ingestion was measured after one and two months of experimental diet feeding. No significant differences were found between diet groups (data not shown).

Essential fatty acid status

Triene:tetraene ratio in plasma and in liver

The classical biochemical parameter used to describe EFA status, the triene:tetraene ratio (20:3n-9/20:4n-6), was measured in plasma and in liver of *mdr*2$^{(+/+)}$ and *mdr*2$^{(-/-)}$ mice at baseline and after eight weeks of EFA-deficient chow feeding. At baseline, *mdr*2$^{(-/-)}$ mice had significantly higher triene:tetraene ratios compared with *mdr*2$^{(+/+)}$ mice (0.035 ± 0.003 vs. 0.018 ± 0.001, P<0.0001), although this value was well below the cutoff value for EFA-deficiency (0.2). We determined whether biliary phospholipid availability was important for the extent of EFA-deficiency by comparing triene:tetraene ratios between *mdr*2$^{(-/-)}$ and *mdr*2$^{(+/+)}$ mice. After eight weeks, *mdr*2$^{(-/-)}$ and *mdr*2$^{(+/+)}$ mice fed EFA^{-} chow had EFA-deficiency according to triene:tetraene ratios in plasma and in liver (0.46 ± 0.03 vs. 0.66 ± 0.05 for plasma; P<0.01 and 0.38 ± 0.02 vs. 0.56 ± 0.04 for liver; P<0.05, respectively).

Characterization of EFA-deficiency in mice

To characterize our EFA-deficient mouse model, plasma and liver lipids were measured. No differences were found in cholesterol, HDL-cholesterol, free fatty acid and phospholipid concentrations in plasma between *mdr*2$^{(+/+)}$ mice fed different diets (Table 1). However, plasma triglyceride concentrations were decreased in EFA-deficient *mdr*2$^{(+/+)}$ mice compared with mice fed EFA^{+} chow (P<0.001). Cholesterol and phospholipid concentrations were increased and triglyceride concentrations were decreased in EFA-deficient *mdr*2$^{(-/-)}$ compared with mice fed EFA^{+} chow (P<0.001 and P<0.05, respectively). Liver lipid analysis revealed a significant lipid accumulation (triglyceride, unesterified cholesterol, cholesterol ester) in EFA-deficient groups compared with their EFA^{+} fed counterparts (Table 2).

Table 1. *Plasma lipid concentrations in mdr2$^{(+/+)}$ and mdr2$^{(-/-)}$ mice fed EFA-enriched (EFA^{+}) and EFA-deficient (EFA^{-}) chow for 8 weeks*

	mdr2$^{(+/+)}$		mdr2$^{(-/-)}$	
	EFA^{+}	EFA^{-}	EFA^{+}	EFA^{-}
Cholesterol (mM)	2.04 ± 0.09	2.21 ± 0.05	1.39 ± 0.06	1.82 ± 0.09*
HDL-cholesterol (mM)	1.44 ± 0.07	1.41 ± 0.03	1.04 ± 0.05	1.30 ± 0.08#

Triglyceride (mM)	0.48 ± 0.03	0.27 ± 0.02*	0.46 ± 0.04	0.34 ± 0.05†
Free fatty acids (mM)	0.58 ± 0.04	0.60 ± 0.02	0.61 ± 0.03	0.66 ± 0.05
Phospholipids (mM)	2.84 ± 0.18	3.11 ± 0.07	2.18 ± 0.07	2.82 ± 0.11*

**P<0.001; #P<0.01; †P<0.05 n=7 mice per group.*

Table 2. *Hepatic lipid concentrations in mdr2$^{(+/+)}$ and mdr2$^{(-/-)}$ mice fed EFA-enriched (EFA+) and EFA-deficient (EFA-) chow for 8 weeks*

	mdr2$^{(+/+)}$		mdr2$^{(-/-)}$	
	EFA$^+$	EFA$^-$	EFA$^+$	EFA$^-$
Triglyceride	152.0 ± 8.3	219.7 ± 10.4*	101.0 ± 8.4	226.0 ± 36.3†
Total cholesterol	34.9 ± 1.0	75.6 ± 2.0*	45.8 ± 0.8	81.4 ± 4.5#
Cholesterol esters	8.0 ± 0.8	35.8 ± 3.6*	11.6 ± 0.1	41.7 ± 2.9*

Concentrations are given in nmol/mg protein. P<0.001; #P<0.01; †P<0.05;n=7 mice per group.

Lipid absorption

Fecal fatty acid balance

The fecal balance revealed a decreased absorption of dietary lipid in both EFA-deficient groups compared with their EFA$^+$ fed counterparts (P<0.001) (Fig. 1). The absorption percentage for EFA-deficient *mdr*2$^{(-/-)}$ mice was lower than for EFA-deficient *mdr*2$^{(+/+)}$ mice (60.4 ± 2.1% vs. 70.1 ± 1.6%; P<0.01). Individual fatty acid balances for 16:0, 18:0, 18:1n-9 and 18:2n-6 were calculated (Fig. 2). EFA-deficient groups had lower absorption percentages for saturated fatty acids (palmitic and stearic acids) compared with unsaturated fatty acids (oleic and linoleic acids). EFA$^+$ chow fed mice had fatty acid absorption percentages above 85%. Absorption of all individual fatty acids was slightly lower in *mdr*2$^{(-/-)}$ than in *mdr*2$^{(+/+)}$ mice.

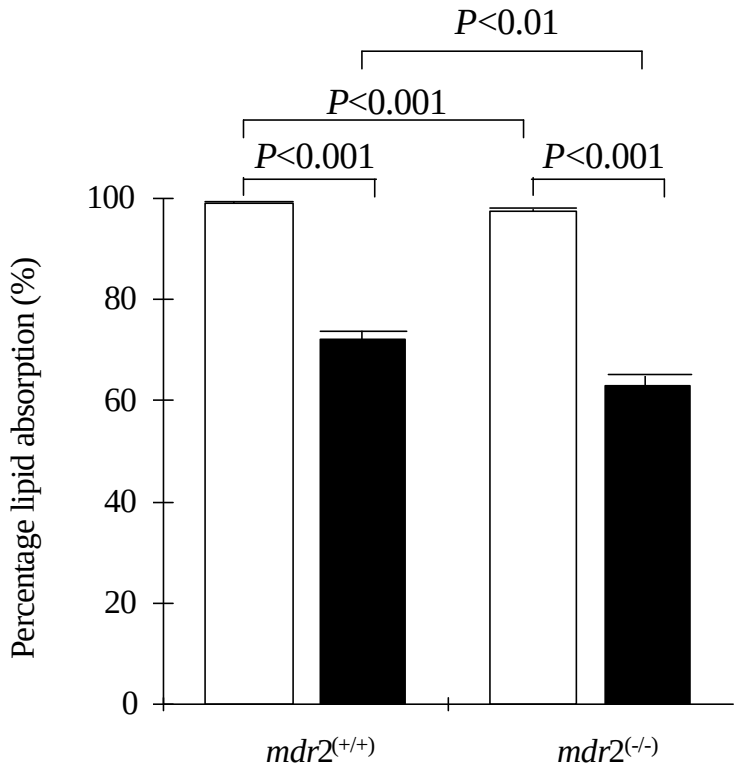


Figure 1. *Lipid absorption percentages of major dietary fatty acids (16:0+18:0+18:1n-9+18:2n-6) in mdr2$^{(+/+)}$ and mdr2$^{(-/-)}$ mice fed EFA^{+} (open bars) and EFA^{-} (closed bars) chow for 8 weeks. Feces was collected after a 72 h period in which chow intake was monitored by weighing of the chow container. Data are means ± S.E.M. of 7 mice per group. Statistical significance between groups is indicated by brackets.*

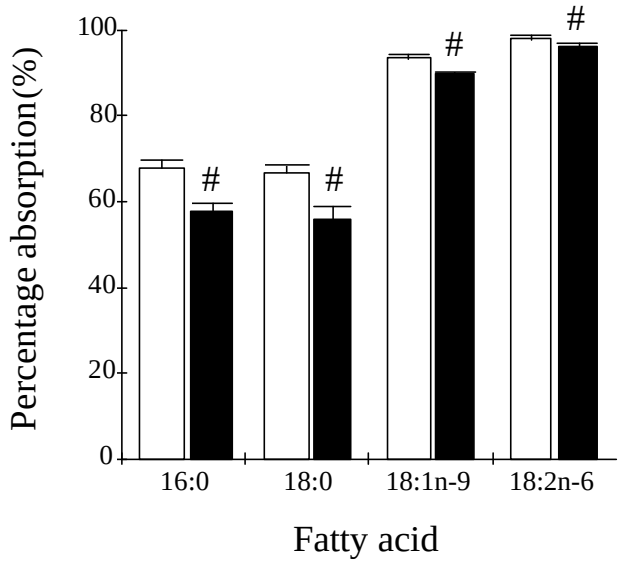
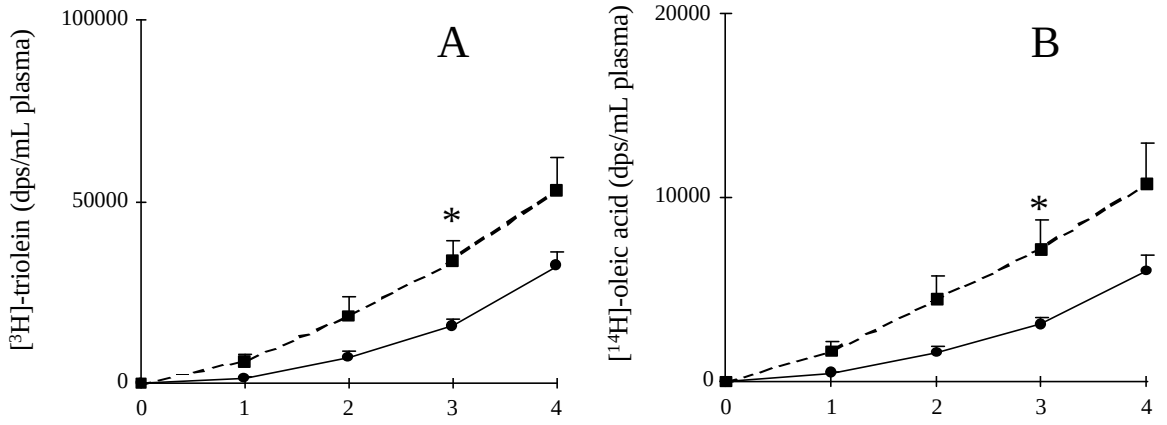


Figure 2. *Absorption percentages for the major dietary fatty acids (16:0, 18:0, 18:1n-9, 18:2n-6) for EFA-deficient mdr2$^{(+/+)}$ (open bars) and mdr2$^{(-/-)}$ (closed bars) mice after EFA-deficient chow feeding for 8 weeks. Absorption was calculated by subtracting fecal excretion of these fatty acids after 72 h from their dietary intake in 72 h and then multiplying the result by 100, in order to obtain a percentage (as detailed in the Materials and Methods section). Data are means ± S.E.M. of 7 mice per group. # indicates that statistical significance was reached (P<0.01).*

Plasma accumulation of [^{3}H]-triolein and [^{14}C]-oleic acid after Triton W1339 administration

To test the possibility that lipid malabsorption in EFA-deficiency is due to impaired lipolysis of triglyceride in the intestinal lumen, a double label test with intravenous administration of Triton W1339 and subsequent intragastric bolus administration of [^{3}H]-triolein and [^{14}C]-oleic acid was performed in *mdr*2$^{(+/+)}$ mice. Figures 3a and 3b show the time course of plasma appearance of radioactivity for [^{3}H]-triolein and [^{14}C]-oleic acid, respectively, hourly for four hours. EFA-deficient mice had a slightly increased concentration of both radioactive labels in plasma in the time frame studied. Values reached statistical significance at the 3 h time point.

For both EFA$^+$ and EFA$^-$ fed mice, thin layer chromatography revealed that both [^{3}H] and [^{14}C] radioactivity was exclusively (>95%) present in the triglyceride fraction. When expressed as the ratio between [^{3}H] and [^{14}C] in plasma, no significant difference was found between EFA$^+$ and EFA$^-$ fed *mdr*2 $^{(+/+)}$ mice (Fig. 3c).



Figures 3a and 3b. *Plasma appearance of [^{3}H]-triolein (**A**) and [^{14}C]-oleic acid (**B**) in mdr2$^{(+/+)}$ mice after an intragastric load of olive oil containing these lipids and after intravenous injection of Triton WR1339 at time point zero. Mice were fed either EFA$^+$ (circles, solid line) or EFA$^-$ (squares, dashed line) chow for 8 weeks. Data represent mean ± S.E.M., n=5 per group. Statistical significance was reached for [^{3}H]-triolein and [^{14}C]-oleic acid at 3 h after administration (P<0.05).*

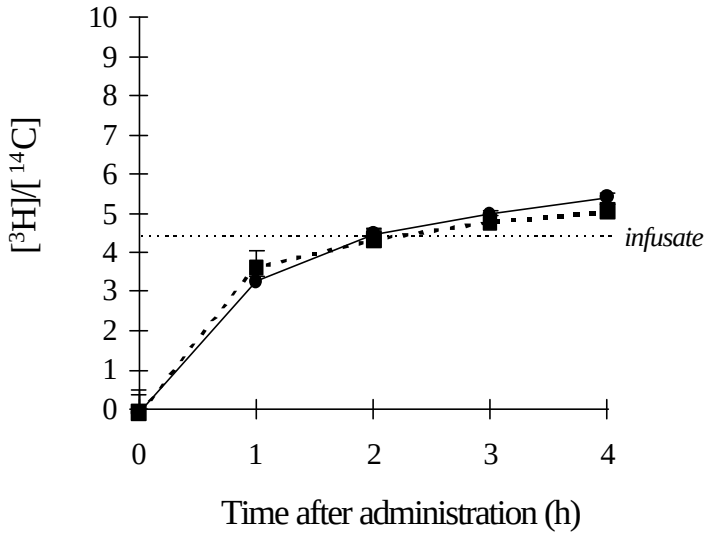


Figure 3c. *The ratio of [^{3}H]/[^{14}C] in plasma of in mdr2$^{(+/+)}$ mice fed either EFA$^+$ (circles) or EFA$^-$ (squares) chow for 8 weeks after an intragastric load of olive oil containing [^{3}H]-triolein and [^{14}C]-oleic acid and after intravenous injection of Triton WR1339 at time point zero. The [^{3}H]/[^{14}C] in the infusate was 4.43 ± 0.05. Data represent mean ± S.E.M., n=5 per group. No significant differences were found between groups at any time point (P>0.05).*

Bile secretion and composition

Bile flow and bile salt, cholesterol and phospholipid secretion were measured after bile duct cannulation for 1 h (Table 3). In contrast to the results described in EFA-deficient rats [3,4], bile flow was increased in EFA-deficient *mdr*2$^{(+/+)}$ and *mdr*2$^{(-/-)}$ mice compared with their EFA$^+$ fed counterparts, respectively (P<0.001 and P<0.05, respectively). Biliary secretion of bile salts, cholesterol and phospholipid were higher in EFA-deficient *mdr*2$^{(+/+)}$ mice compared with EFA$^+$ fed *mdr*2$^{(+/+)}$ mice (P<0.001). Bile flow and bile salt secretion were higher in

*mdr*2$^{(-/-)}$ compared with *mdr*2$^{(+/+)}$ mice (P<0.01). In order to investigate whether changes in bile salt hydrophobicity could be responsible for lipid malabsorption in EFA-deficiency, we analyzed bile salt composition. Bile salt composition was virtually identical between both dietary groups (Table 4). Relative concentrations of major fatty acids (mol%) were measured in bile of *mdr*2$^{(+/+)}$ mice fed EFA$^+$ and EFA$^-$ chow (Fig. 4). Compared with mice fed EFA$^+$ chow, EFA-deficient mice had increased 16:1n-7, 18:1n-9 and 18:1n-7 (P<0.001) and decreased 16:0, 18:3n-6, 18:2n-6, 18:0 and 20:4n-6 (P<0.05) in bile.

***Table 3**. Biliary flow and biliary secretion rates in mdr2$^{(+/+)}$ and mdr2$^{(-/-)}$ mice fed EFA-enriched (EFA+) and EFA-deficient (EFA$^-$) chow for 8 weeks*

	mdr2$^{(+/+)}$		mdr2$^{(-/-)}$	
	EFA$^+$	EFA$^-$	EFA$^+$	EFA$^-$
Bile flow[a]	2.87 ± 0.29	4.87 ± 0.36*	6.57 ± 0.64	9.27 ± 0.97†
Bile salts[b]	145 ± 20	353 ± 30*	260 ± 22	540 ± 82#
Cholesterol[b]	2.23 ± 0.19	4.96 ± 0.32*	0.97 ± 0.11	1.31 ± 0.15
Phospholipids[b]	30.11 ± 3.15	55.13 ± 3.77*	0.10 ± 0.10	0.07 ± 0.07

Biliary output rates are given in [a]µL/min/100 g body weight or [b]nmol/min/100 g body weight. P<0.001; #P<0.01; †P<0.05 for comparisons between EFA$^+$ and EFA$^-$ chow-fed groups; n=5-7 animals per group.

***Table 4**. Biliary bile salt* composition in mdr2$^{(+/+)}$ and mdr2$^{(-/-)}$ mice fed EFA-enriched (EFA+) and EFA-deficient (EFA-) chow for 8 weeks*

	mdr2$^{(+/+)}$		mdr2$^{(-/-)}$	
Bile salt	EFA$^+$	EFA$^-$	EFA$^+$	EFA$^-$
Cholate	49.0 ± 1.3	58.9 ± 1.9$^{\#}$	63.7 ± 3.0	56.2 ± 1.5
ß-Muricholate	39.0 ± 0.5	28.1 ± 1.9$^{\#}$	28.0 ± 3.3	35.9 ± 1.8
ω-Muricholate	6.5 ± 1.1	7.4 ± 0.6	4.8 ± 0.7	5.2 ± 0.5
α-Muricholate	2.7 ± 0.2	2.5 ± 0.2	1.0 ± 0.2	1.1 ± 0.1
Deoxycholate	1.6 ± 0.3	2.1 ± 0.4	1.6 ± 0.4	0.8 ± 0.2
Chenodeoxycholate	1.1 ± 0.3	1.0 ± 0.2	0.9 ± 0.2	0.7 ± 0.2

**>90% of all bile salts represented. Minor metabolites (<1% of total area) have been excluded. Values expressed as a percentage of the total amount. Data represent means ± S.E.M. $^{\#}P$<0.01. n=5-7 animals per group.*

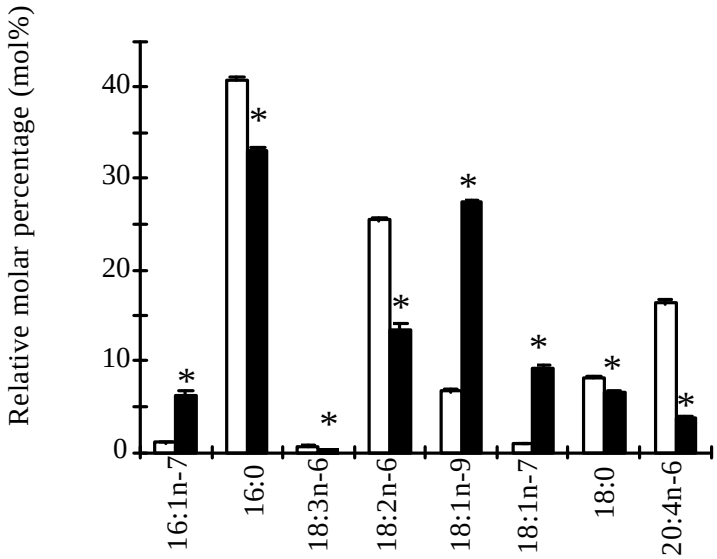


Figure 4. *Relative concentration (mol%) of major biliary fatty acids in mdr2$^{(+/+)}$ mice fed EFA^{+} (open bars) and EFA^{-} (closed bars) chow for 8 weeks. Data are means ± S.E.M. of 7 mice per group. * indicates statistical significance (P<0.001) between groups.*

Discussion

In the present study, EFA deficiency was characterized in control FVB and *mdr*2 knockout mice. EFA deficiency, as defined by a triene:tetraene ratio in plasma greater than 0.2 [19], was already appoximated after just 2 weeks of EFA-deficient diet feeding, which makes the mouse an attractive and versatile model for studying EFA deficiency. Similar to EFA-deficient rats [20,21], EFA-deficient mice experience changes in plasma and liver lipids. Specifically, EFA-deficient mice have decreased plasma triglycerides and increased hepatic triglycerides and cholesterol levels.

Under physiological conditions, lipid absorption in *mdr*2$^{(-/-)}$ mice (95-98%) is very similar to that in control mice (95-98%), supporting the concept that biliary phospholipids are not crucial for overall lipid absorption in mice (Voshol et al., unpublished results, 1999). In the present study, EFA-deficient mice had reduced dietary lipid absorption ranging between 60-70% of the amount ingested. In EFA-deficient rats, dietary lipid absorption has been reported to be between 80-90% [2,4,5]. The slight difference in absorption percentages between EFA-deficient mice and rats can be due to differences in the administered diets in these studies. For our experiments with mice, we used chow that contained more than twice the amount of lipid than that used by Hjelte et al. in rats [2] (16 wt% vs. 7 wt%, respectively). Both our EFA-deficient chow and the chow used in the study of Hjelte et al. [2] consisted almost entirely of saturated fatty acids. It is known that the less hydrophobic structure of unsaturated fatty acids relies less upon bile for solubilization in the intestine compared with saturated long-chain fatty acids [7,22-25]. This point is supported by the low absorption percentages that we obtained for saturated fatty acids (palmitic and stearic acids) compared with unsaturated fatty acids (oleic and linoleic acids) from EFA-deficient mice.

The primary aim of this study was to investigate the mechanism of lipid malabsorption in EFA deficiency. In particular, we investigated whether intraluminal events, namely impaired lipolysis and alterations in bile secretion and composition, were likely contributors to EFA-deficiency lipid malabsorption. Levy et al. [3] reported that lipolytic activity in EFA-deficient rats was unchanged compared with control rats. In accordance with these results, we found that the rate of intestinal lipolysis of triglyceride did not seem to be a rate-limiting factor for lipid absorption in EFA-deficient *mdr*2$^{(+/+)}$ mice. This interpretation is based on two results: 1) the appearance of [^{3}H]-triolein in plasma after its intragastric administration was similar in control and EFA-deficient mice and 2) the ratio of [^{3}H]/[^{14}C] in plasma between both groups was not different. Although not aimed for, the [^{3}H]/[^{14}C] experiment resulted in an intriguing observation; both the [^{3}H]-label (from [^{3}H]-triolein) and the [^{14}C]-label (from [^{14}C]-oleic acid) were recovered in plasma at similar or higher concentrations (3 h after administration) in EFA^{-} fed mice. This result is in contrast to our expectation, given the overall *decreased* absorption of dietary lipid in EFA-deficient mice. It is most likely, in our opinion, that the discrepancy relates to a tracer effect of the radiolabeled lipids. Yet, other explanations cannot be excluded.

EFA-deficient mice had significantly increased rates of bile flow and biliary bile salt, cholesterol and phospholipid secretion compared with control mice, in contrast to reported decreases in these parameters in EFA-deficient rats [3]. It may very well be that the effects of EFA-deficiency on bile formation are species specific. Robins and Fasulo [26] reported that EFA-deficient hamsters have increases in hepatic bile flow and bile salt and cholesterol secretion compared to controls. Unfortunately, the extent of lipid malabsorption in these animals has not been determined. It thus remains a matter of speculation whether the mechanism of EFA-deficiency lipid malabsorption is also species specific.

Originally, we had hypothesized that EFA deficiency lipid malabsorption in mice could be due to quantitative and/or qualitative changes in biliary components such as bile salts and PC, in accordance with previous data in rats. However, as shown above, the quantity of bile flow and of biliary bile salt and phospholipid secretion was increased, rather than decreased. Theoretically, an increase in the contribution of hydrophilic bile salts (to total bile salts) could contribute to impaired solubilization of dietary lipids. Yet, the biliary bile salt composition was virtually unchanged between control and EFA-deficient mice.

The quantity of biliary PC may also be important for lipid absorption due to their role in providing a surface coat for chylomicron particles. The availability of *mdr*2 knockout mice has allowed the possibility to study the effect of phospholipid-free bile on EFA deficiency-induced lipid malabsorption. On an EFA-adequate diet, *mdr*2$^{(-/-)}$ mice have increased bile flow and biliary bile salt secretion, which supports our use of this model to study the effects of bile salt secretion on EFA deficiency-induced lipid malabsorption. Despite similar increases in bile salt secretion due to EFA deficiency in both control and knockout mice, the knockout mice have a further reduced lipid malabsorption, suggesting that bile salts are not responsible for this disturbance and that biliary PC may partially counteract the lipid malabsorption in EFA deficiency.

It is not known to what extent changes in the biliary PC acyl chain composition can influence dietary lipid absorption. In accordance with findings by Bennett Clark et al. in EFA-deficient rats [4], the acyl chain composition of biliary PC in EFA-deficient mice contained less EFAs (i.e., 18:2n-6 and 20:4n-6) and more nonessential fatty acids (i.e., 18:1n-9, 18:1n-7, 16:1n-7). We can assume that the effect is minor since the EFAs normally found in biliary PC were replaced with relatively hydrophilic, monounsaturated fatty acids.

Instead of bile secretion, lipid malabsorption in EFA deficiency may be due to other steps involved in lipid absorption. The effects on mucosal uptake of lipids may occur due to the fact that intestinal mucosal phospholipids contain large amounts of 18:2n-6 and 20:4n-6 [27,28] and in EFA deficiency, the levels of these fatty acids markedly decrease [27-29]. The resulting structural changes and the increased cellular turnover in the intestinal mucosa reported in both EFA-deficient rats and mice [5] could be responsible for decreased dietary lipid absorption. Yet, uptake in everted jejunal slices of [^{14}C]-oleic acid from micelles was similar in control and EFA-deficient rats. Based on our study and previous studies in EFA-deficient rats [3,4], intraluminal events (i.e., dietary triglyceride lipolysis by pancreatic lipase, solubilization of lipolyzed triglyceride and uptake of lipids by the enterocyte) involved in lipid absorption seem to be relatively undisturbed in EFA deficiency. By inference, it is therefore more likely that defects in one of the several intracellular events (i.e., reesterification, chylomicron assembly and/or secretion) are involved in lipid malabsorption. Specific activities of the microsomal esterifying enzyme, monoglyceride acyltransferase, in jejunal mucosa have been demonstrated to be 30% lower in EFA-deficient rats [4]. The activity of esterification enzymes in EFA-deficient mice has not been investigated so far. Chylomicron production may also be implied in lipid malabsorption in EFA-deficient mice. Levy et al. [3] found that chylomicron output after a lipid load was significantly decreased in EFA-deficient rats. However, an EFA-related impairment in chylomicron production does not seem to explain the fatty acid specificity of the (mal)absorption, which mostly affects long-chain saturated fatty acids. In future studies, we would like to obtain better insight in the actual mucosal events in EFA-deficient mice by developing lymph cannulation techniques.

In summary, data from the present study shows that lipid malabsorption in EFA-deficient mice is not due to impaired lipolysis, decreased secretion rate of bile salts into bile, or to alterations in bile salt composition. Based on the present results and on literature data [3,4], we speculate that the mechanism of lipid malabsorption in EFA deficiency is related to other steps involved in dietary lipid absorption. For example, the fact that biliary phospholipid secretion partially counteracts the extent of lipid malabsorption in EFA deficiency may suggest that chylomicron production is disturbed.

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CHAPTER 7

Lipid malabsorption in cystic fibrosis patients on enzyme replacement therapy is due to impaired intestinal uptake of long-chain fatty acids

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Abstract

Pancreatic enzyme replacement therapy frequently fails to correct intestinal lipid malabsorption completely in cystic fibrosis (CF) patients. The reason behind therapy failure in these patients is unknown. We investigated whether lipid malabsorption in CF patients treated with pancreatic enzymes is caused by insufficient lipolysis of triacylglycerols or by defective intestinal uptake of long-chain fatty acids. In 10 CF patients receiving their habitual pancreatic enzymes, lipolysis was determined by analysis of breath $^{13}CO_2$ recovery after oral ingestion of 1,3-distearoyl, 2[1-^{13}C]octanoyl glycerol ([^{13}C]-MTG). Intestinal uptake of long-chain fatty acids was determined by analysis of plasma [^{13}C]-linoleic acid ([^{13}C]-LA) concentrations after oral ingestion of [^{13}C]-LA. For 3 d, dietary intake was recorded and feces was collected. Fecal lipid excretion ranged from 5.1 to 27.8 g/d (mean ± SD: 11.1 ± 7.0 g/d) and lipid absorption ranged from 79 to 93% (89 ± 5%). After ingestion of [^{13}C]-MTG no relationship was observed between breath $^{13}CO_2$ recovery and dietary lipid absorption (r=0.04). In contrast, a strong relationship was observed between 8 h plasma [^{13}C]-LA concentrations and dietary lipid absorption (r=0.88, P<0.001). Our results suggest that continuing lipid malabsorption in CF patients on enzyme replacement therapy is not likely due to insufficient lipolytic enzyme activity, but rather due to either incomplete intraluminal solubilization and/or reduced mucosal uptake of long-chain fatty acids.

Introduction

In humans, triacylglycerols composed of long-chain fatty acids constitute 92 to 96% of dietary lipids [1]. Absorption of these lipids comprises two main processes. Firstly, lipolysis, by lipolytic enzymes originating predominantly from the pancreas, leads to hydrolysis of triacylglycerols into fatty acids and 2-monoacylglycerols. And secondly, intestinal uptake involves the formation of mixed micelles composed of bile components and lipolytic products, followed by the desintegration of the mixed micelles in the unstirred water layer, and the translocation of the lipolytic products across the intestinal epithelium [1-4].

Most CF patients have a considerable malabsorption of dietary lipids due to pancreatic insufficiency leading to impaired lipolysis [5,6]. The symptoms of pancreatic insufficiency, such as steatorrhea and poor growth, can be alleviated by oral supplementation of pancreatic enzymes. However, despite recent improvements in the pharmacokinetics of the supplements, many patients continue to experience a certain degree of steatorrhea [7-9], with lipid absorption reaching 80 to 90% of their dietary lipid intake. It has not been elucidated if the remaining lipid malabsorption is due to an insufficient dosage of pancreatic enzyme replacement therapy. This possibility is not unlikely because a decreased pancreatic bicarbonate secretion may negatively affect enzyme activity by sustaining a low pH in the duodenum [10,11]. At a low duodenal pH, the release of the enzymes from the (micro)capsules is inhibited and the denaturation of the enzymes is stimulated [11,12]. However, it has been demonstrated that increasing the pancreatic enzyme dosages does not completely correct lipid malabsorption [13]. In addition, attempts to increase lipolysis by high-strength pancreatic enzyme supplements has led to the reported association with fibrosing colonopathy [14-16].

An alternative explanation for the continuing lipid malabsorption in CF patients on pancreatic enzyme replacement therapy may involve inefficient intestinal uptake of fatty acids [7,17]. Impaired uptake in CF patients can be due to an altered bile composition, decreased bile salt secretion by the liver, bile salt precipitation, a decreased bile salt pool size, and/or bile salt inactivation at low intestinal pH [9,17-20]. Furthermore, small bowel mucosal dysfunction or alterations in the mucus layer contribute to inefficient intestinal uptake of long-chain fatty acids in CF patients [5,21].

The gold standard for monitoring enzyme replacement therapy is the lipid balance. A drawback of the lipid balance is that it does not provide insight into the pathophysiology of lipid malabsorption. Insight into the adequacy of these separate processes (lipolysis, intestinal uptake) would enable treatment in individual patients by modulating diet therapy, pancreatic enzyme replacement therapy and supplementation of antacids and bile salts. So far, it has not been possible to determine whether continued lipid malabsorption in enzyme-treated CF patients is due to impaired lipolysis or due to impaired uptake of long-chain fatty acids. Therapeutic improvements of lipid absorption may be of benefit for CF patients, as a positive correlation has been observed between a good nutritional status and long-term survival or well-being of CF patients [22].

The aim of the present study was to determine whether continued lipid malabsorption encountered in pediatric CF patients on their habitual pancreatic enzyme replacement therapy results from either insufficient lipolysis or from defective intestinal uptake of long-chain fatty acids in the lumen. We choose to measure lipolysis and uptake by two indepedent tests in CF patients *in vivo*. Previously, a test to determine lipase activity was described and validated in CF patients, based on oral ingestion of a [^{13}C]-labeled mixed triglyceride ([^{13}C]-MTG, 1,3-distearoyl, 2[1-^{13}C]octanoyl glycerol) and excretion of [^{13}C] in breath [23-26]. Inadequate intestinal uptake can be measured by oral ingestion of long-chain fatty acids, e.g. [^{13}C]-LA [27,28]. The concentration of [^{13}C]-LA in plasma and the expiration of $^{13}CO_2$ could then serve as parameters to quantify uptake of [^{13}C]-LA.

Subjects and Methods

Patient characteristics

The study protocol was approved by the Medical Ethics Committee of the University Hospital Groningen, and included informed consent obtained from the parents and the children.

Patients. The study group included 10 pediatric CF patients, three male and seven female, ranging in age from 7 to 18 y. The diagnosis of CF had been established by the sweat test and a DNA genotype analysis [29]. The ΔF508/ΔF508 genotype was present in six patients, and the ΔF508/other in four (subjects 1, 4, 8, 9; Table 1). All patients were pancreatic insufficient and therefore received enteric coated pancreatic enzymes. None of the patients received antacids.

Table 1. *Comparison of energy intake, ingested lipase enzymes, and fasting plasma concentrations of bile salts in individual CF patients and mean ± S.D.*

Patient	Age (y)	Wt (kg)	Energy (%RDA)	CHO (% en)	Lipid (%en)	Protein (%en)	Lipase (IU/g lipid)	Plasma bile (μmol/L)
1 (F)	18	55	66	52	33	15	560	13.8
2 (F)	18	58	104	52	31	17	710	13.5
3 (M)	16	53	115	48	39	13	1820	20.6
4 (M)	15	56	113	48	38	15	680	12.8
5 (F)	9	34	91	52	35	13	440	23.4
6 (F)	9	26	102	57	30	13	1520	13.5
7 (M)	8	23	110	50	37	13	830	11.8
8 (F)	7	27	111	52	35	13	590	18.2
9 (F)	7	24	92	56	32	12	860	11.6
10 (F)	7	23	121	54	35	11	460	30.3
Mean ±SD			103 ± 16	52 ± 3	35 ± 3	14 ± 2	850 ± 460	16.6 ± 5.9

F, female; M, male; CHO, carbohydrates. Normal range fasting plasma bile salts, 1-10 μmol/ L.

Anthropometry

Anthropometric evaluation consisted of weight, height, midarm circumference, and skinfold thickness measurements at 4 sites (biceps, triceps, subscapula, and suprailiac), done by one

pediatrician. The Z-scores of all these anthropometric parameters were calculated based on the reference data for Dutch children described by Gerver and De Bruin [30]. The Z-score is defined as $X-\bar{x}/S$ where X is the patient's measurement, $\bar{x}$ is the median value for age and sex, and S is the standard deviation of $\bar{x}$. A negative value indicates a value under the median reference value.

Pulmonary function

Pulmonary function was assessed by standard spirometric techniques and was characterized by the parameters forced vital capacity, and forced expiratory volume in one second. For each patient, results were expressed as percentage of predicted (control) values for sex and height [31].

Liver function tests

Liver function had been screened during a standard routine control at the time of the study using serum enzyme activities: g-glutamyl transpeptidase, aspartate transaminase, and alanine transaminase.

Diet evaluation

Intake of nutrients was calculated from 3 d consecutive food diaries by a clinical dietitian using The Netherlands Nutrients Table "NEVO" 1993. Intakes were expressed as the recommended dietary allowance (RDA) for weight, age and sex (Table 1).

[^{13}C]-labeled substrates

The mixed triglyceride (1,3-distearoyl, 2[1-^{13}C]octanoyl glycerol; S*OS) was purchased from Euriso-Top (Saint Aubin Cedex, France) and was 99% [^{13}C]-enriched. In the original literature, the breath test performed with the use of this molecule has been named the mixed-triglyceride breath test or the [^{13}C]-MTG breath test [23,25]. For reasons of consistency, we adhered to this nomenclature. Uniformly labeled [^{13}C]-LA, obtained from Campro Scientific B.V. (Veenendaal, The Netherlands), had an enrichment exceeding 97%. [^{13}C]-LA was included into a gelatin capsule coated with an acid-resistant layer consisting of 4.8% cellulose acetate hydrogen phthalate in acetone.

Study protocol

The subjects were instructed to avoid consumption of naturally [^{13}C]-enriched foods (e.g. corn or corn products, pineapple, cane sugar) for at least two days prior to the study. The [^{13}C]-LA test and the [^{13}C]-MTG test were performed on two subsequent days. On day 1, after an overnight fast, the patients received a capsule with [^{13}C]-LA (1 mg/kg BW), together with their habitual breakfast (bread, butter, ham, cheese, etc.) and pancreatic enzymes. A baseline blood sample (EDTA) was collected before consumption of breakfast, every 2 h for 8 h, and at 24 h. Immediately after sampling, plasma was isolated and stored frozen (-20ºC) until further analysis. Breath samples were collected in duplicate at baseline and every 30 min for 6 h. On day 2, the patients received [^{13}C]-MTG (4 mg/kg BW) mixed with their habitual breakfast and pancreatic enzymes. Breath samples were collected in duplicate at baseline and every 30 min

for 6 h. The fecal lipid balance and both breath tests were performed in the same 3 d period. On the day before the [^{13}C]-LA test, a feces sample was collected for baseline [^{13}C]-measurements. After consumption of the breakfast on the first day, all feces passed was collected for 3 d (72 h) to determine the presence of lipid malabsorption and the amount of [^{13}C]-LA excretion into the feces. Collected feces was stored at -20°C. During this period, intake of nutrients was determined from food diaries also. During the first 6 h of both tests, no additional food or liquids were permitted except for non-caloric drinks such as water and tea (without milk and sugar). After 6 h, patients were allowed to have their habitual lunch, including pancreatic enzymes.

Analytical techniques

Breath sample analysis

End expiratory breath was collected via a straw into a 10 ml tube (Exetainers; Labco Limited, High Wycombe, United Kingdom), from which aliquots were taken to determine [^{13}C]-enrichment by means of continuous flow isotope ratio mass spectrometry (Finnigan Breath MAT, Finnigan MAT GmbH, Bremen, Germany), conform previous experiments [24]. The [^{13}C]-abundance of breath CO_2 was expressed as the difference per mil from the reference standard Pee Dee Belemnite limestone ($\delta^{13}C_{PDB}$, ‰).

Mean values of whole body CO_2 excretion were measured by indirect calorimetry (Oxycon, model ox-4, Dräger, Breda, The Netherlands) at two separate periods of 5 min during both test days. This sampling method was compared to sampling every 30 min (results not shown). The results indicated that, under the test conditions chosen, the mean values of the CO_2 production obtained from two randomly chosen periods were within the 95% confidence interval of the mean values obtained when sampling occurred every 30 min.

Plasma fatty acids

Plasma lipids were extracted and fatty acids were hydrolyzed and methylated according to Lepage and Roy [32]. Resulting fatty acid methyl esters were analyzed both by gas chromatography and by gas chromatography combustion isotope ratio mass spectrometry. Quantification of the resulting fatty acid methyl esters was performed with the use of heptadecanoic acid (17:0) as an internal standard.

Fecal lipids

After thawing, feces was weighed and homogenized. Fecal lipid was determined according to the method of Van de Kamer et al. [33] and expressed as g lipid/d. The percentage of total lipid absorption was calculated using the calculations described in Chapter 2. Aliquots of freeze-dried feces were extracted according to the method of Bligh and Dyer [34], and subsequently hydrolyzed and methylated [32]. Resulting fatty acid methyl esters were analyzed by both gas chromatography and gas chromatography combustion isotope ratio mass spectrometry (see Chapter 2).

Plasma and fecal bile salts. Fasting and postprandial plasma bile salts up to 8 h were determined by an enzymatic fluorimetric assay [35]. Results were expressed as µmol/L plasma. Fecal bile salts were extracted from an aliquot of dried homogenate of a 24 h feces fraction [36] and fluorimetrically measured [35].

Gas liquid chromatography
Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard gas chromatograph Model 5880 equipped with a CP-SIL 88 capillary column (Chrompack; 50 m x 0.32 mm) and an FID detector [37,38] using the method described in Chapter 2.

Gas chromatography combustion isotope ratio mass spectrometry
[^{13}C]-enrichment of the palmitic acid methyl esters was determined on a gas chromatography combustion isotope ratio mass spectrometer (Delta S/GC Finnigan MAT, Bremen, Germany) [39] as described in Chapter 2.

Statistics

The experimental data are reported as means ± S.D. Corresponding to the literature [40-42], relationships between the percentage of total lipid absorption and either plasma [^{13}C]-LA concentrations or breath $^{13}CO_2$ expiration were considered exponential. All other correlations were assumed to be linear. Correlations between variables were calculated with the least square method and are expressed as Pearson's coefficient of variation *r*. Differences between means were considered statistically significant at the level of $P<0.05$.

Results

Patient characteristics

Z-scores for all anthropometric parameters in CF patients were low to normal. For all parameters, the 95% confidence interval does include the reference 50th centile line (Z-score 0), indicating that there is no significant difference between our study group and the healthy reference population. Most patients had some degree of lung disease. Subjects 1 and 6-10 had normal liver biochemistry. Previously, subject 3 was diagnosed as having liver cirrhosis with portal hypertension. This patient receives ursodeoxycholic acid (750 mg/d) and the condition of this patient has been stable for the past few years. The bile salt concentration in plasma of this subject is in the same range as that of the other patients (Table 1). Analysis of 3 d dietary food records is shown in Table 1. Energy intake of 7 patients exceeded the recommended dietary allowance. In all patients approximately 50% of the energy was derived from carbohydrates, 35% from lipid, and 15% from protein. Patients took pancreatic enzyme supplements in a dosage of approximately 440 - 1820 IU lipase per gram lipid ingested (Table 1).

Table 3. *Results of the ^{13}C-LA test, and ^{13}C-MTG test in 10 individual CF patients*

Patient	^{13}C-LA test			[^{13}C]-MTG test
	Breath	**Plasma**	**Feces**	**Breath**
	6 h cum $^{13}CO_2$ (% dose)	8 h [^{13}C]-LA (%dose/L)	[^{13}C]-LA (%dose)	6 h cum $^{13}CO_2$ (% dose)
1	11.0	1.5	0.6	2.4
2	1.6	1.9	0.2	9.7
3	2.2	0.9	1.8	15.1
4	1.7	0.6	0.6	5.8
5	0.2	1.3	0.0	30.8
6	3.6	2.0	0.7	11.3
7	1.1	1.3	0.2	40.2
8	3.1	0.5	1.3	28.9
9	2.4	0.5	0.3	11.5
10	0.5	1.2	0.2	8.7
Mean ± SD	2.7 ± 3.1	1.2 ± 0.5	0.6 ± 0.6	16.4 ± 12.5

LA, linoleic acid; MTG, mixed triglyceride. Normal range fecal bile salt: 0.1-1 mmol/kg fecal wet weight.

Lipid balance

In the studied CF patients, dietary intake of lipid over the 3 d period ranged from 54 to 130 g/d, and the excretion of lipid in feces ranged from 4.9 to 27.8 g/d (Table 2). The percentage of total lipid absorption ranged from 79 to 93% (Table 2). Under physiological conditions, healthy individuals excrete approximately 4-6 g/d of lipid via the feces [43], which generally means that over 96% of dietary lipids entering the intestinal lumen is absorbed [43]. These observations were confirmed by experiments performed in our own laboratory with dietary records and feces of healthy human adults (n=13, fecal lipid excretion: 3.0 ± 0.9 g/d, total lipid absorption: 97 ± 2%, data not shown). Despite standard pancreatic enzyme replacement therapy, fecal lipid excretion in 8 out of 10 patients was higher than 6 g lipid per day, and the percentage of total lipid absorption was below 96% in all patients studied. According to the prevailing reference values [43], all patients but 2 have lipid malabsorption.

Table 2. *Results of the fecal bile salt concentrations and fecal lipid balance in 10 individual CF patients*

Patient	Fecal bile salts (mmol/kg wet wt)	Lipid intake (g/d)	Fecal lipid (g/d)	Total lipid absorption (%)
1	30.2	54	4.9	91
2	10.6	85	7.0	92
3	0.7	124	14.8	88
4	8.7	133	27.8	79
5	22.1	92	9.6	90
6	20.5	66	5.1	92
7	5.3	85	6.1	93

8	6.8	93	16.7	82
9	16.1	64	9.2	86
10	16.7	88	7.1	92
Mean ± SD	13.8 ± 9.0	84 ± 22	11.1 ± 7.0	89 ± 5

Normal range fecal bile salt: 0.1-1 mmol/kg fecal wet weight.

In studies in infants between 0 and 6 months, Fomon et al. [44] found that fecal lipid excretion per kg body weight correlated with lipid intake per kg body weight. In our study we observed a similar curvilinear correlation (r=0.71, P<0.05) despite a considerably lower intake of lipid/kg BW compared to infants [44]. However, when we compared lipid intake per kg body weight with percentage of total lipid absorption, no correlation was observed (r=-0.06), indicating that lipid malabsorption in our study was not due to high lipid intake. In addition, no correlation was observed between the percentage of total lipid absorption and the amount of pancreatic enzymes ingested (r=0.12).

^{13}C-MTG test

The baseline ^{13}C-abundance in breath prior to consumption of the [^{13}C]-MTG label was -23.2 ± 2.6‰ (range -25.5 to -17.1‰). After ingestion of the [^{13}C]-MTG label, different time-course patterns were observed for the excretion of [^{13}C] in breath over the 6 h study period (Fig.1a). When expressed as a proportion of administered [^{13}C], the excretion rate reached a mean maximum value of 4.9 ± 3.1% per h between 3 and 6 h after administration of the label (range 0.7 to 10.7%). Over the 6 h study period the cumulative excretion of [^{13}C] in breath was 16.4 ± 12.5% of that administered, ranging between 2.4 and 40.2% (Fig. 1b, Table 3). If defective lipolysis would be responsible for the continuing lipid malabsorption in CF patients, then a low percentage of lipid absorption would be expected to correlate with low expiration of $^{13}CO_2$ after [^{13}C]-MTG ingestion. However, no significant relationship was observed between 6 h cumulative $^{13}CO_2$ expiration and either daily fecal lipid excretion (r=-0.02) or the percentage of total lipid absorption (r=0.04).

[^{13}C]-LA test

The baseline [^{13}C]-LA abundance in plasma prior to consumption of [^{13}C]-LA was -29.1 ± 2.2‰ (range -32.6 to -25.5‰). [^{13}C]-LA concentration in plasma samples, expressed as percentage of the dose per liter plasma, increased steeply after approximately 6 h (Fig. 2). Peak values of [^{13}C]-LA concentrations in plasma after administration occurred between 8 and 24 h. At 24 h after ingestion of the label, the enrichment of [^{13}C]-LA in plasma had not yet returned to the level of baseline [^{13}C]-abundance. Plasma 8 h [^{13}C]-LA concentrations varied from 0.5 to 2.0% dose/L plasma (Table 3).

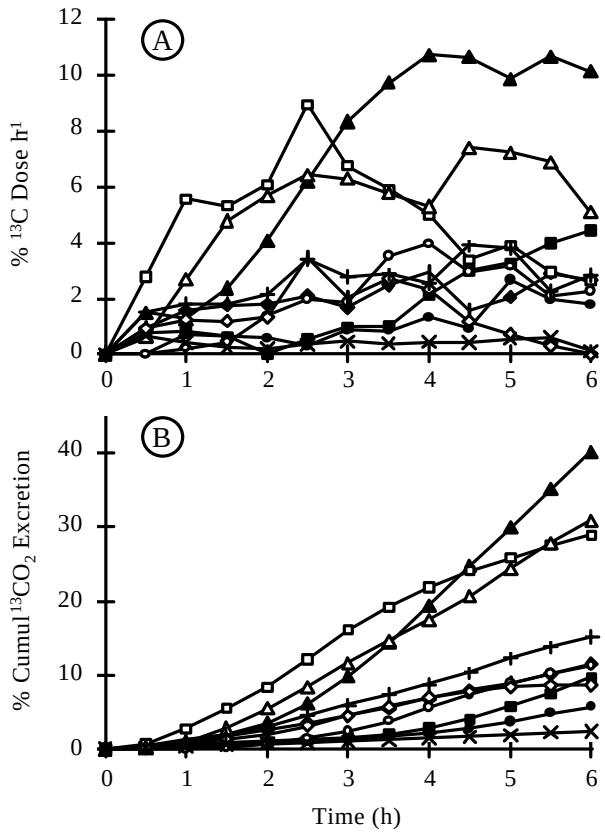


Figure 1. *Time courses for the excretion of [^{13}C] in breath over the 6-h study period following oral ingestion of [^{13}C]-MTG (4 mg/kg BW) at time 0 in 10 CF patients. Each symbol represents a patient. Figure (A) represents the excretion rate, whereas figure (B) represents the cumulative $^{13}CO_2$ excretion.*

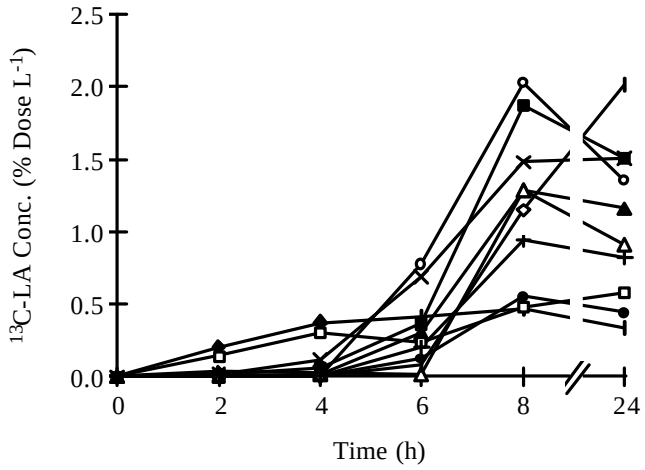
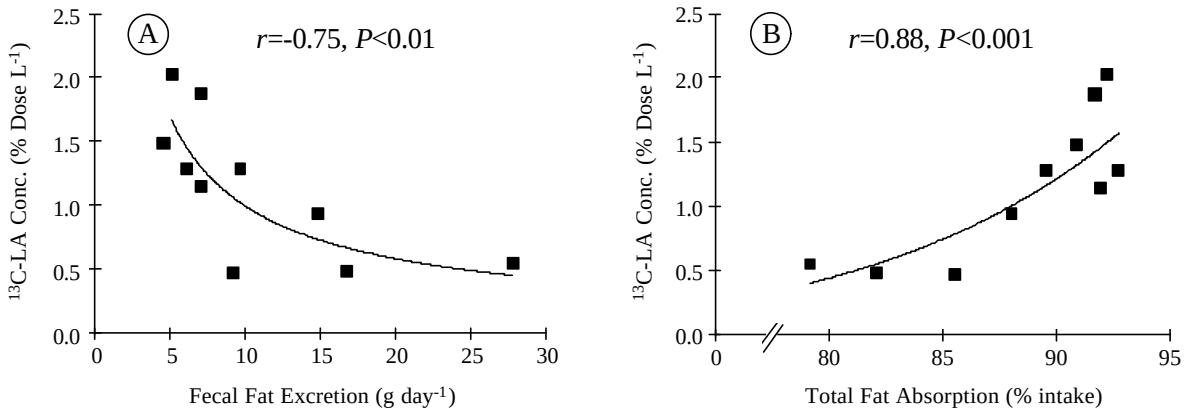


Figure 2. *Time courses of [^{13}C]-LA appearance in plasma of 10 CF patients after a single oral dose of [^{13}C]-LA (2 mg/kg body weight) at time 0. Each symbol represents a patient.*

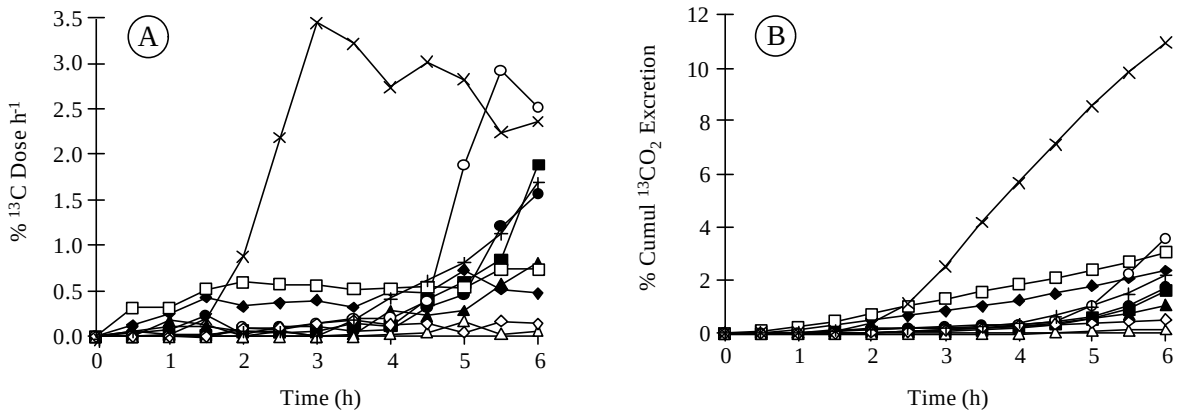
If defective intestinal uptake of long-chain fatty acids would be responsible for the continuing lipid malabsorption in CF patients, then a low percentage of lipid absorption would be expected to correlate with low concentrations of [^{13}C]-LA in plasma after [^{13}C]-LA ingestion. Figure 3 shows the relationship between the 8 h plasma [^{13}C]-LA concentrations and either fecal lipid excretion or the percentage of total lipid absorption. A strong, negative relationship was observed between fecal lipid excretion and 8 h plasma [^{13}C]-LA concentrations (Fig. 3a; r=-0.75, P<0.01) and, correspondingly, a strong, positive relationship was observed between the percentage of total lipid absorption and 8 h plasma [^{13}C]-LA concentrations (Fig. 3b; r=0.88, P<0.001).



Figures 3a and 3b. *Relationship between the results of the 72 h fecal lipid balance and the 8 h plasma [^{13}C]-LA concentration after a single oral dose of [^{13}C]-LA (1 mg/kg body weight) at time 0 in 10 CF patients. Fig. (A) represents the relationship between daily fecal lipid excretion and 8 h plasma [^{13}C]-LA concentration and fig. (B) the relationship between the percentage of total lipid absorption and 8 h plasma [^{13}C]-LA concentration.*

Since a breath test would be more convenient to the patient than a test requiring blood sampling, we investigated whether similar information on intestinal uptake of long-chain fatty acids could become available using breath $^{13}CO_2$ analysis after [^{13}C]-LA ingestion. The baseline [^{13}C]-abundance in breath prior to consuming the [^{13}C]-LA label was -24.3 ± 2.3‰, (range -27.2 to -20.9‰). In most subjects, the [^{13}C] excretion rate in breath was low during the first hours, then increased rapidly and reached a possible maximum value at 6 h after administration of the label (Fig. 4a). In most subjects no decay was observed during the time course of the study. This time course pattern was very similar to the pattern obtained for [^{13}C]-LA concentrations in plasma, except for subject 1, whose [^{13}C] excretion rate in breath already increased after 90 min. The 6 h cumulative $^{13}CO_2$ expiration (Table 3) amounted to 2.7 ± 3.1% dose. In Fig. 4b the 6 h cumulative $^{13}CO_2$ expiration for all patients is plotted. In contrast to plasma values, no significant relationship between 6 h cumulative $^{13}CO_2$ expiration and either fecal lipid excretion (r=0.00) or the percentage of total lipid absorption (r=-0.13) was observed. In addition, there was no correlation between plasma [^{13}C]-LA concentrations and cumulative breath $^{13}CO_2$ expiration (r=0.32), indicating that the multitude of metabolic processes limits the utility of breath samples to measure uptake of long-chain fatty acids [45]. The results indicate that for the measuring intestinal uptake of long-chain fatty acids, plasma sampling cannot be easily replaced by breath sampling.

Finally, we investigated the excretion of [^{13}C]-LA in feces. The apparent absorption of [^{13}C]-label was determined from the difference between the amount of [^{13}C]-LA administered and that excreted in feces. [^{13}C]-LA excretion in feces over the 3 d period was very low and varied between 0.0 and 1.8% of the dose administered (Table 3). No metabolites of [^{13}C]-LA were observed in the feces. There was no significant correlation observed between the excretion of [^{13}C]-LA and of total lipid in feces (r=0.22, P=0.54).



Figures 4a and 4b. *Time courses for the excretion of [^{13}C] in breath over the 6h study period following oral ingestion of [^{13}C]-LA (1 mg/kg body weight) at time 0 in 10 CF patients. Fig. (A) represents the excretion rate, whereas fig. (B) represents the cumulative $^{13}CO_2$ excretion.*

Total bile salt concentrations in plasma and feces

Total bile salt concentrations were determined in plasma and feces. Fasting plasma total bile salt concentrations in CF patients were high when compared with normal healthy control values and ranged from 11.6 to 30.3 µmol/L (mean 17.2 µmol/L) (Table 1). Following a meal there was no significant change in total plasma bile salts (data not shown). Fecal total bile salt concentrations in most CF patients were elevated (range 0.7-30.2; mean 13.8 mmol/kg fecal wet weight) when compared with healthy control values, indicating that they had bile salt malabsorption (Table 2). Bile salt malabsorption could result in a decreased amount of bile salts available for the formation of mixed micelles, leading to lipid malabsorption. However, no significant correlation was found between percentage of dietary lipid absorption and fecal bile salt concentrations (r=0.26).

Discussion

In CF patients, pancreatic enzyme replacement therapy frequently does not correct disordered lipid absorption to values obtained in controls. Our results of the 3 d lipid balance confirm the presence of mild to moderate lipid malabsorption (percentage of total lipid absorption: 79-93%) in a group of pediatric CF patients on enzyme replacement therapy despite good clinical conditions. The aim of the present study was to elucidate whether lipid malabsorption in CF patients receiving habitual pancreatic enzyme replacement therapy is due to deficient lipolysis of triacylglycerols or due to impaired intestinal uptake of fatty acids.

We applied two lipid substrates with different physical and chemical properties, i.e., [^{13}C]-MTG and [^{13}C]-LA. The principle of the [^{13}C]-MTG breath test is based on lipolysis-dependent $^{13}CO_2$ excretion via the breath. Efficient absorption of [^{13}C] from the MTG is limited primarily by lipolysis [23], and the [^{13}C]-MTG test therefore distinguishes pancreatic insufficiency from deficient intestinal uptake of long-chain fatty acids. After [^{13}C]-MTG ingestion, no relationship was observed between recovery of $^{13}CO_2$ in breath and percentage of

total lipid absorption, indicating that lipid malabsorption in CF patients on their habitual enzyme replacement therapy is probably not related to defective lipolysis. The recovery of expired $^{13}CO_2$ obtained in the present study was similar to those obtained in other studies, indicating sufficient supplementation of pancreatic enzymes to the CF patients in this study. In healthy adults the 6 h cumulative percentage of [^{13}C] expired via the breath after ingestion of [^{13}C]-MTG varied between 23 and 52% of the dose in one study [23] and between 3 and 48% in another study [24]. The recovery of expired $^{13}CO_2$ in CF patients receiving regular amounts of pancreatic enzymes varied between 0 and 45% [23,25]. In neither of these studies total lipid absorption was related to the percentage of [^{13}C] recovered in the breath.

Efficient absorption of [^{13}C]-LA, a long-chain unesterified fatty acid, differs predominantly from [^{13}C]-MTG in its dependence on adequate intestinal uptake [27]. Minich et al. [28] showed in a rat model for lipid malabsorption (permanently interrupted enterohepatic circulation) that measuring plasma [^{13}C]-LA concentrations is a valuable method to assess the intestinal uptake of long-chain fatty acids and correlates with lipid absorption. The [^{13}C]-LA test therefore distinguishes deficient intestinal uptake of long-chain fatty acids from pancreatic insufficiency [28]. After ingestion of [^{13}C]-LA, a strong relationship was observed between 8 h plasma [^{13}C]-LA concentrations and total lipid absorption, indicating that the observed lipid malabsorption in CF patients on their habitual enzyme replacement therapy is due to defective intestinal uptake of long-chain fatty acids.

Impaired intestinal uptake of long-chain fatty acids may result from several processes. In the absence of adequate bicarbonate secretion, gastric acid entering the duodenum may lower intestinal pH until well into the jejunum [11]. Bile salts are readily precipitated in an acid milieu [17], and duodenal bile salt concentration may fall below the critical micellar concentration, thereby exacerbating lipid malabsorption. Precipitated bile salts also appear to be lost from the enterohepatic circulation in greater quantities, thus reducing the total bile salt pool and decreasing the fraction of bile salts conjugated with glycine [20]. Intracellullar events may also contribute to impaired uptake of long-chain fatty acids in CF patients, e.g. due to absent fatty acid binding proteins or impaired chylomicron assembly and secretion [46]. Viscous, thick intestinal mucus, with altered physical properties, may have a deleterious effect on the thickness of the intestinal unstirred water layer, limiting translocation of long-chain fatty acids over the intestinal epithelium [5,21]. Our data on increased fecal bile salt losses are in agreement with several other studies [47-49] and could be in agreement with a diminished bile salt pool in CF patients. Watkins et al. [18] showed that bile acid pool size was nearly doubled upon treatment with pancreatic enzymes in a group of CF patients with normal fecal bile salt losses. Although the present data suggest that the problem is related to insufficient long-chain fatty acid uptake, they do not allow a clear identification of the individual process responsible for impaired uptake.

The [^{13}C]-LA bolus was administered in an acid-resistant coated capsule, preventing the capsule from being opened at a low pH environment (gastric or intestinal). In patients with a low intestinal pH, e.g. due to inadequate bicarbonate secretion [10,11], the bioavailability of

[^{13}C]-LA was hypothesized to be impaired, resulting in a decreased amount of [^{13}C]-LA incorporated into plasma LA. Since low intestinal pH affects uptake of long-chain fatty acids, we reasoned that the acid-resistant capsule probably enhances the effect of the [^{13}C]-LA test in correctly diagnosing solubilization disorders. The release of the substrate may be delayed in some patients, which can explain the differences in timing for the onset of the individual [^{13}C]-LA curves. In addition, delayed time courses for the onset of $^{13}CO_2$ in breath have been observed before and may be explained by, e.g., delayed gastric emptying [24,50,51].

The study was designed such that the patients served as their own controls. Thus, in each individual patient we calculated the percentage of total lipid absorption and related these results to the measurements of the [^{13}C]-MTG breath test and the [^{13}C]-LA test. We reasoned that these controls would be the most appropriate, given the fact that neither the optimal positive control group (pancreatic sufficient CF patients with known impaired intestinal uptake) nor the optimal negative control group (pancreatic sufficient CF patients without intestinal uptake disorder) exists or is available. The present approach allowed us to relate the results of total lipid absorption to the results of lipolysis and intestinal uptake in the individual patient.

In conclusion, lipid balance data indicate that, despite enzyme replacement therapy, pediatric CF patients have increased fecal lipid excretion and, correspondingly, decreased percentage of lipid absorption. The results of the [^{13}C]-MTG test and [^{13}C]-LA test indicate that continuing lipid malabsorption is not likely due to insufficient enzyme replacement therapy, but rather due to either incomplete intraluminal solubilization and/or reduced mucosal uptake of long-chain fatty acids. Indirect indications exist that an increased bile salt loss leading to a diminished bile salt pool may contribute to this problem. Therapeutic attempts to normalize lipid absorption in pediatric CF patients need to include a strategy to improve intestinal uptake of long-chain fatty acids.

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CHAPTER 8

General Summary

Experiments performed in this thesis aimed to quantitate the absorption and status of the major dietary essential fatty acid (EFA), linoleic acid (LA; 18:2n-6), under conditions in which the quantity or qualitative composition of bile entering the intestinal lumen was altered by surgical or dietary means, and by transgenic techniques. The overall conclusion is that the efficacy of LA absorption is rather independent of intestinal bile secretion. Furthermore, the status of LA in plasma does not seem to be influenced by the effects of bile on absorption. However, it is to be argued whether the LA concentration in plasma is a valid reflection of net absorption as determined by fecal balance techniques. Compared with saturated fatty acids, LA absorption and status remain relatively well-preserved despite quantitative or qualitative changes in intestinal bile salt secretion. The reason for the preservation of LA absorption is not known, but may be due to its physiochemical properties. In addition, a sparing effect, for example, decreased beta-oxidation of LA after its absorption may contribute to the observed maintenance of LA status under varying bile deficient conditions. Although its quantitative absorption was rather unaffected in conditions of absent or little bile availability in the intestinal lumen, the qualitative absorption of LA strongly depended on changes in the quantity or composition of intestinal bile.

The bile-diverted rat (**Chapter 2**) experienced impaired LA status in plasma. After only one week of bile diversion, LA status was decreased in bile-diverted rats compared with controls (16.4 ± 0.8% vs. 21.2 ± 0.4%; $P<0.001$). Malabsorption of dietary LA was tested as a mechanism for this decrease. Although fecal excretion of dietary LA was increased approximately 20-fold in bile-diverted rats (0.72 ± 0.11 mmol/day vs. 0.03 ± 0.00 mmol/day), net intestinal absorption of LA was similar between bile-diverted and control rats due to a ~40% increase in ingestion of chow. Mechanisms underlying this adaptation to food intake are unclear, but it can be speculated that decreased intestinal release of apo A-IV may play a role in regulation of satiety. In addition to their increased food intake, the intestinal mucosa of bile-diverted rats had longer villi compared with control rats, which may be associated with an increased surface area for absorption. Despite these adaptations, the plasma concentration of [^{13}C]-LA for 6 h after its intraduodenal administration was considerably lower in bile-diverted rats, as in bile duct-ligated rats, compared with controls. Thus, the absence of bile in the intestinal lumen may not be essential for quantitative uptake of LA, but may influence its efficiency of delivery to the blood from the enterocyte. Finally, metabolism of LA to arachidonic acid (AA: 20:4n-6) seemed to be enhanced in the bile-diverted rat, as evidenced by higher molar percentages of AA in plasma and by increased [^{13}C]-AA in plasma after [^{13}C]-LA administration. Accelerated metabolism to AA may, therefore, be another contributor to decreased LA concentrations in plasma. Reasons for this enhanced conversion are not known. It remains to be established whether this apparently increased conversion is related to an upregulation of the responsible metabolic pathways (i.e., delta-6 desaturase and/or elongase enzymes).

The effects of intestinal bile deficiency with concomitant accumulation of non-secreted compounds in the body on LA absorption and status were studied in the bile duct-ligated rat model (**Chapter 3**). As no bile is available in the intestine for the formation of mixed micelles, lipid absorption is expected to decrease. Indeed, overall dietary lipid absorption was significantly decreased in rats with bile duct ligation after one week compared with control rats (53.7 ± 5.0% vs. 94.2 ± 0.6%; $P<0.001$); however, of the four fatty acids measured:

palmitic acid (16:0, PA), stearic acid (18:0, SA), oleic acid (18:1n-9, OA) and LA, LA kept the highest percentage absorption (~80%). In analogy to these data on dietary lipids, after intraduodenal administration of [^{13}C]-LA and [^{13}C]-PA, [^{13}C]-LA was absorbed to a higher extent than [^{13}C]-PA, based on fecal excretion. However, when expressed as net absorption from the diet (moles), LA (along with other fatty acids) absorption was decreased in the bile duct-ligated rats compared to control rats. This phenomenon of increased food intake upon bile-diversion was not observed by bile-duct ligated rats. Despite the decreased net absorption of LA, its status in plasma and in liver was not changed in the bile duct-ligated rats compared with control rats, in contrast to the bile-diverted rat. We hypothesize that this is due to a relatively preserved absorption of LA and/or to a retention of biliary phospholipid-LA in plasma. Although quantitative absorption of LA appeared to be relatively spared, bile duct-ligated rats experienced delays in the appearance of administered [^{13}C]-LA and -PA in plasma. Finally, the metabolism of [^{13}C]-LA to [^{13}C]-AA was not different between bile duct-ligated and control rats. This result was confirmed by measurement of similar delta-6 desaturase activities in both groups of rats.

Whereas the bile duct-ligated and bile-diverted rat lack bile secretion into the intestinal lumen, the *mdr*2 knockout mouse (**Chapters 4-6**) has increased bile flow, normal secretion of biliary bile salts and absent biliary phospholipid secretion. Biliary cholesterol excretion is strongly reduced (~97%); however, cholesterol is not significantly implied in the absorption of dietary lipid. This model allowed for the study of the importance of biliary phospholipids for LA status and absorption. Three different types of diets were used to study LA status and absorption: low-fat (6 wt%, ~50% LA), high-fat (16 wt%, ~30% LA) and EFA-deficient (16 wt% lipid, 3% LA). Relative concentrations of LA in plasma were similar in knockout and control mice after feeding either a low-fat or high-fat diet. The classical measure of EFA deficiency (EFAD), the triene:tetraene ratio, was not significantly different between knockout and control mice on a low-fat diet. Values were slightly increased in knockout mice on a high-fat diet (0.035 ± 0.003 vs. 0.018 ± 0.001), however, they were well below the cutoff value for EFAD (>0.2). After two months on an EFA-deficient diet, both knockout and control mice were EFA-deficient according to triene:tetraene ratios in plasma (0.46 ± 0.03 vs. 0.66 ± 0.05; $P<0.01$. On low- and high-fat diets (**Chapters 4-5**), dietary lipid absorption in knockout mice was found to be comparable with that of controls, and within the range of normal dietary lipid absorption (>95%). Similar to bile-diverted rats, net absorption of dietary and [^{13}C]-LA was unaffected in knockout mice compared with control mice. Knockout mice exhibited a delay in appearance of [^{13}C]-LA at 2 h after its intragastric administration (**Chapter 4**) and an abolished plasma triglyceride response after an intragastric fat load (**Chapter 5**). Thus, the overall conclusion from studies with the *mdr*2 mouse is that biliary phospholipid secretion is not necessary for quantitative absorption and for plasma status of LA; however, the lack of biliary phospholipids results in a significant delay in plasma appearance of administered lipids.

Levy et al. [1] has demonstrated that EFAD results in lipid malabsorption and decreased bile flow and biliary bile salt and phospholipid secretion in rats. Indeed, EFA-deficient mice (**Chapter 6**) exhibited decreased overall absorption ($70.1 \pm 1.6\%$ vs. $99.1 \pm 0.1\%$) with the absorption of LA (~98%) remaining rather well-preserved, when compared with PA (~68%), SA (~67%) or OA (94%). In contrast to the findings of Levy et al. [1], EFA-deficient mice had

increased bile flow and secretion of biliary bile salts and phospholipid, indicating that alterations in bile formation do not seem to be directly responsible for the lipid malabsorption encountered in EFAD in mice. The role of biliary phospholipids in lipid absorption was confirmed by a more pronounced decrease in lipid absorption in EFA-deficient *mdr*2 knockout mice compared with EFA-deficient control mice (60.4 ± 2.1% vs. 70.1 ± 1.6%). In summary, studies with *mdr*2 knockout mice demonstrated that biliary phospholipids are not necessary for the absorption and status of LA in mice when a diet rich or deficient in LA is fed.

Finally, dietary lipid and [^{13}C]-LA absorption was studied in patients with cystic fibrosis (CF) (**Chapter 7**). CF is an autosomal recessive inherited disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The majority of CF patients have pancreatic exocrine insufficiency, even during early infancy, and if untreated it leads to lipid malabsorption, and eventually malnutrition [2]. Pediatric patients with CF often have impaired plasma levels of EFAs and long-chain polyunsaturated fatty acids (LCPUFAs) [2-8] in addition to bile disturbances, such as altered bile composition, decreased bile salt secretion by the liver, bile salt precipitation, a decreased bile salt pool size, and/or bile salt inactivation at low intestinal pH [9-13]. In this experiment, we focused on the pathophysiology of lipid malabsorption in human CF patients. Specifically, we determined whether it involved pancreatic insufficiency and/or impaired uptake of long-chain fatty acids. The substrates, [^{13}C]-mixed triglyceride (MTG) and uniformly labeled [^{13}C]-LA, were both applied to determine whether the rate-limiting step behind lipid malabsorption was either impaired lipolysis or impaired intestinal uptake of long-chain fatty acids, respectively. Dietary lipid absorption ranged from 79 to 93%. After ingestion of [^{13}C]-MTG no relationship was observed between breath $^{13}CO_2$ recovery and dietary lipid absorption (r=0.04). In contrast, a strong relationship was observed between 8 h plasma [^{13}C]-LA concentrations and dietary lipid absorption after ingestion of [^{13}C]-LA (r=0.88, P<0.001). Our results suggest that lipid malabsorption in CF patients on enzyme replacement therapy is not likely due to insufficient lipolytic enzyme activity, but rather due to defective intestinal uptake of long-chain fatty acids. Therefore, therapeutic attempts to normalize lipid absorption in CF patients need to include a strategy to improve intestinal uptake of long-chain fatty acids. It is not known whether the impaired uptake is related to bile alterations. Based on the animal experiments, this certainty seems to be an attractive explanation. However, it cannot be excluded that other events involved in long-chain fatty acid uptake, such as the unstirred water layer, apical translocation, and/or chylomicron production, may be altered in CF.

Summary

In summary, this thesis has provided information on the role of bile in the absorption and status of the EFA, LA. For this purpose, various animal models of hepatobiliary disease were described and characterized: bile duct-ligated rats to study the effects of acute cholestasis, bile-diverted rats to study bile deficiency in the intestinal lumen without the accumulation of nonsecreted compounds in the blood, *mdr*2 knockout mice to study the absence of biliary phospholipid secretion, and EFA-deficient control and *mdr*2 knockout mice to study the effects of EFAD on bile flow and biliary lipid secretion. These animal models are potentially

valuable in elucidating the mechanism(s) of decreased EFA status in hepatobiliary disease, with the ultimate goal to develop novel therapeutic strategies. In addition, the potency of diagnostic tests such as the [^{13}C]-MTG breath test and the [^{13}C]-LA test was investigated to characterize the etiology behind lipid malabsorption in patients with CF. Although present results on LA absorption and status in animal models need confirmation in patients with hepatobiliary disease, the findings in this thesis suggest that decreased bile output into the intestine is not be the primary factor involved in the depletion of EFAs and LCPUFAs in plasma. Rather, other factors related to their condition, such as decreased synthesis of LCPUFAs from their precursors in the liver or increased metabolic demands for energy, may need to be addressed by increasing oral administration of EFAs and LCPUFAs. Finally, future investigations are necessary to examine the implications of impaired chylomicron formation and secretion in the status of EFAs and LCPUFAs in patients with hepatobiliary disease.

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SAMENVATTING

Linolzuur (18:2n-6) en linoleenzuur (18:3n-3) worden ook wel *essentiële vetzuren* (EFA's: essential fatty acids) genoemd omdat ze wel nodig zijn voor specifieke functies in het lichaam maar niet door het lichaam zelf gemaakt kunnen worden. Afhankelijk van de behoefte van het organisme kunnen ze, na inname en de daarop volgende opname door het lichaam, worden omgezet in langeketen meervoudig onverzadigde vetzuren (LCPUFA's: long-chain polyunsaturated fatty acids). Van LCPUFA's, zoals arachidonzuur (20:4n-6) en docosahexaeenzuur (22:6n-3) is aangetoond dat ze belangrijk zijn voor respectievelijk de vorming van prostaglandines en voor het functioneren van het zenuwstelsel. Verlaagde aanwezigheid van EFA's of LCPUFA's in het lichaam kan pathologische gevolgen hebben, zoals een verminderde groei, huidaandoeningen, degeneratieve veranderingen in de nieren, longen, testes en in de lever, en onvruchtbaarheid. Theoretisch kunnen verlaagde EFA concentraties in het bloed een gevolg zijn van een aantal factoren, zoals een lage inname van EFA's via het dieet, een gestoorde metabolisme in het lichaam, en door een verstoorde vetopname in de darmen.

De aanwezigheid van gal in de darm is belangrijk voor de opname van vetten (zoals EFA's) door het lichaam. Gal bevat galzouten, fosfolipiden, cholesterol, afvalstoffen (zoals bilirubine) en diverse eiwitten. Eén van de belangrijkste eigenschappen van gal is dat het de oplosbaarheid van verteerde vetten in de waterige darminhoud 100 tot 1000 maal verhoogt door de vorming van galzout-vet bolletjes, de zogeheten micellen. Daarnaast kan het belangrijkste fosfolipide in gal, fosfatidylcholine (PC), nog een extra rol hebben in de opname van vetten. PC komt voor in de buitenkant van vetbolletjes (chylomicronen) die in de darmcel gemaakt worden. Er zijn aanwijzingen dat PC uit gal hiervoor sneller wordt gebruikt dan PC uit het dieet. Door de specifieke vetzurencompositie van PC afkomstig uit gal, is dit PC verantwoordelijk voor 50% van de dagelijkse opname van EFA's in de darm van de mens. Het belang van deze specifieke vetzuren van gal PC is niet bekend, maar het is waarschijnlijk erg belangrijk voor de opname van vetten uit het dieet door het afstaan van vetzuren aan de membraan van de mucosa in de darmen.

De logische consequentie hiervan blijkt bij kinderen met een galvormingstoornis: een verstoorde galproductie leidt tot een suboptimale EFA status, in het bijzonder van n-6 vetzuren. Het is mogelijk dat een sub-optimale EFA status in deze patiënten, vooral met betrekking tot LCPUFA's, gerelateerd is aan een minder efficiënte vet opname uit de darm, aan een verstoorde synthese van LCPUFA's vanuit de precursors in de lever, en/of aan een verhoogd gebruik van LCPUFA's voor prostaglandine synthese. De mechanismen achter de afgenomen EFA en LCPUFA concentraties gedurende een verstoorde galuitscheiding zijn echter niet goed bekend. Deze informatie is nodig om een optimale behandeling van EFA-deficiëntie in te stellen.

Het doel van dit proefschrift was om meer inzicht te krijgen in de opname van linolzuur, de belangrijkste EFA in plasma bepalen, onder invloed van verandering in samenstelling van gal door middel van diëten of door chirurgische technieken, of met behulp van transgene technieken. Hiervoor zijn meerdere ratten- en muizenmodellen gebruikt. Uiteindelijk is ook een klinische studie verricht met kinderen die lijden aan cystische fibrose die een enzymvervangende therapie ondergingen.

In **hoofdstuk 1 en 2** is het belang van de aanwezigheid van gal in de darm voor de linolzuurconcentratie in het bloed en opname onderzocht. Dit is gedaan in twee modellen: de rat waar de galstroom buiten het lichaam wordt geleid (*bile-diverted rat*) en de rat met galgang onderbinding (*cholestatic rat*). In beide modellen is er geen gal in de darm aanwezig. Het verschil is dat in de bile-diverted rat er een ophoping plaatsvindt van stoffen die normaliter wordt uitgescheiden via de gal. Opname van dieetvet (berekend door de inname van vetten en de uitscheiding in de feces te meten) was afgenomen tot respectievelijk 53.7 ± 5.0% en 58.6 ± 4.1% in de bile-diverted en cholestatic ratten. Linolzuur opname uit het dieet was relatief ongehinderd vergeleken met verzadigde en enkelvoudig onverzadigde vetzuren (16:0, 18:0, 18:1n-9) (respectievelijk 81.3 ± 3.3% en 73.3 ± 4.1 % voor bile-diverted en cholestatic ratten). Deze resultaten werden bevestigd in een experiment waarin vetzuren gelabeld waren met stabiele isotopen. In de bile-diverted rat werd in de feces aanmerkelijk meer [^{13}C]-palmitinezuur gevonden dan [^{13}C]-linolzuur (4.67 ± 0.50 mmol/48 uur respectievelijk 0.54 ± 0.19mmol/48 uur; $P<0.05$). In de cholestatic rat was de netto hoeveelheid linolzuur die werd opgenomen significant afgenomen. Echter, aangezien galgang-afgeleide ratten ongeveer 40% meer voedsel tot zich nemen, hadden ze toch een vergelijkbare opname van linolzuur als controle ratten.

Desondanks bleek de linolzuur status afgenomen, na slechts één week van het afleiden van gal, vergeleken met controle ratten (16.4 ± 0.8% respectievelijk 21.2 ± 0.4%; $P<0.001$). Een verminderde netto opname kon dus worden uitgesloten als mogelijke oorzaak. Een toegenomen omzetting van linolzuur in arachidonzuur kan verantwoordelijk zijn voor de relatieve afname van linolzuur status in plasma. De mechanisme voor toegenomen omzetting is nog niet bekend. Zowel cholestatic als bile-diverted ratten vertoonden een vertraagde opname van [^{13}C]-linolzuur dat in de darm was ingebracht vergeleken met controle ratten. De afwezigheid van gal in de darm lijkt dus niet zo zeer essentieel te zijn voor de kwantitatieve opname van linolzuur, maar wel voor de snelheid van het transport van de darmcel naar het bloed.

In de **hoofdstukken 4-6** werd het belang van gal-fosfolipiden in de opname van dieetvetten en linolzuur en EFA status onderzocht in *mdr2* knockout muizen. Deze muizen produceren fosfolipiden-vrije gal en hebben een normale galzoutproductie. In dit model werd de linolzuur status en opname bestudeerd met drie verschillende diëten: laag-vet, hoog-vet en EFA-deficiënt. Zowel op laag-vet als op hoog-vet dieet was de linolzuur status in plasma van *mdr2* knockout muizen gelijk aan die van controle muizen. Na twee maanden op een EFA-deficiënt dieet waren zowel *mdr2* knockout muizen als controle muizen EFA-deficiënt op grond van de triene:tetraene ratio's in plasma (0.46 ± 0.03 respectievelijk 0.66 ± 0.05; $P<0.01$). Op een

laag- en hoog-vet dieet was de opname van dieet vetten en linolzuur vergelijkbaar in *mdr2* knockout muizen en controle muizen, binnen de grenzen van normale vetopname (>95%). EFA-deficiënte muizen vertoonden een verslechterde opname van dieetvetten (respectievelijk 70.1 ± 1.6% en 99.1 ±0.1% in EFA-deficiënte en controle muizen; P<0.0001), maar de opname van linolzuur was onveranderd zeer efficient (~98%). *Mdr2* knockout muizen hadden een meer verlaagde vetopname (60.4 ± 2.1%), wat laat zien dat de uitscheiding van fosfolipiden in gal de effecten van EFA-deficiëntie gekoppelde lipiden malabsorptie gedeeltelijk tegengaat. In tegenstelling tot EFA-deficiënte ratten hadden EFA-deficiënte muizen een *toegenomen* galvorming en uitscheiding van galzouten en fosfolipiden, wat aangeeft dat veranderingen in galvorming niet verantwoordelijk zijn voor de EFA-deficiëntie-geïnduceerde verstoring van de vetopname in muizen.

Vergelijkbaar met de uitkomsten van bile-diverted en cholestatic ratten, vertonen *mdr2* knockout muizen een vertraagde verschijning van [^{13}C]-linolzuur in plasma nadat het in de maag is ingebracht. Ook vertonen ze geen plasma triglyceride respons na toediening van een vetdosis in de maag. De algemene conclusie die uit de *mdr2* studies kan worden getrokken is dat gal fosfolipiden niet noodzakelijk zijn voor kwantitatieve vet opname en voor de plasma status van linolzuur; de afwezigheid van gal fosfolipiden resulteert echter wel in een significante vertraging van de verschijning van de toegediende vetten in plasma.

Tot slot werd de dieetvet- en [^{13}C]-linolzuur opname bestudeerd in patiënten met cystische fibrose (taaislijm ziekte). Cystische fibrose is een genetische afwijking waarbij er onvoldoende pancreatisch lipase (een vetafbrekend enzym) aanwezig is in de darm om de dieetvetten af te breken. Deze patiënten worden dan ook behandeld met lipaseenzymen bij elke maaltijd. Kinderen met cystische fibrose hebben vaak lage plasma concentraties van EFA's en LCPUFA's, en vertonen vaak stoornissen in galvorming. In experimenten beschreven in **hoofdstuk 7** gaven we een [^{13}C] gemengvet (MTG) en [^{13}C]-linolzuur aan deze patiënten, om te bepalen of de snelheidsbepalende stap in hun verstoorde vetopname een afgenomen triglyceride afbraak was, danwel dat het oplossen van de vetzuren zelf verstoord was. De [^{13}C]-MTG ademtest kan worden gebruikt om de mate van vetafbraak te bepalen. Na het innemen van dit product worden delen van dit molekuul afgesplitst, en de rest wordt opgenomen door de darmcellen, onafhankelijk van het oplosbaar maken door de gal. Dit molekuul wordt snel geoxideerd tot $^{13}CO_2$. Het principe van de [^{13}C]-MTG ademtest is dus gebaseerd op het feit dat, na inname van het substraat, de hoeveelheid $^{13}CO_2$ die uitgeademd wordt, afhankelijk is van de activiteit van het (niet alleen) geproduceerde vetafbrekende enzym. Een verstoorde 'oplosbaarheid' van dieetvetten kan worden aangetoond met behulp van langeketen vetzuren (bijvoorbeeld [^{13}C]-palmitaat of [^{13}C]-linolzuur). Vrije vetzuren hebben de vetafbrekende enzymatische activiteit niet nodig. Hun opname is echter wel afhankelijk van de aanwezigheid van gal en de daaropvolgende vorming van miceldeeltjes. De opname van langeketen vetzuren kan worden gemeten door de concentratie van deze vetzuren of afbraakproducten ervan in het plasma of in de adem te bepalen. Zo kan dus met twee verschillende substraten onafhankelijke informatie worden verkregen over twee belangrijke stappen in de vetopname. Dieetvet opname varieerde van 79 tot 93%. Na inname van [^{13}C]-

MTG was er geen relatie te vinden tussen de in de adem gevonden $^{13}CO_2$ en dieetvet opname. (r=0.04). Er was echter wel een sterke relatie tussen de concentratie van [^{13}C]-linolzuur die acht uur na inname in het plasma was aangetroffen en dieetvet opname (r=0.88, P<0.001). Onze resultaten suggereren dat de verminderde vetopname in patiënten met cystische fibrose die een enzym vervangingstherapie ondergaan, waarschijnlijk niet wordt veroorzaakt door een verminderde lipolytische activiteit in de darmen, maar eerder door een verminderde opname van langeketen vetzuren. De resultaten suggereren dat optimalisatie van langeketen vetzuren opname bij patiënten met cystische fibrose verbeterd moet worden, naast enzym vervanging. Het is niet bekend of de verminderde opname gerelateerd is aan veranderingen in de gal of dat andere mechanismen die betrokken zijn bij langeketen vetzuur opname, zoals de opname in de darmcellen, en/of chylomicronen productie, veranderd zijn in cystische fibrose.

De conclusie van dit proefschrift is dat de opname van linolzuur in grote mate onafhankelijk is van galsecretie in de darm, althans in kwantitatieve zin. Bovendien wordt de status van linolzuur in plasma niet bepaald door de effecten van gal op de vetopname. Vergeleken met verzadigde vetzuren is de opname en status van linolzuur betrekkelijk goed beschermd in situaties met veranderende galsecretie. De mechanisme hiervoor is niet bekend, maar zou gerelateerd kunnen zijn aan de fysiochemische eigenschappen van het vetzuur of aan een specifiek “sparing effect” in weefsels. Alhoewel de kwantitatieve opname in afwezigheid of kleine aanwezigheid van gal in de darm nauwelijks veranderd was, was de kwalititatieve (=tijdsafhankelijkheid) opname van linolzuur sterk afhankelijk van veranderingen in galvorming.

De resultaten suggereren in dit proefschrift dat de afgenomen galuitscheiding in de darm niet de belangrijkste factor is in de afname van de EFA en LCPUFA concentraties in plasma. Bovendien moeten andere factoren die verband houden met hun toestand, zoals afgenomen vorming van LCPUFA’s vanuit hun uitgangsproducten in de lever of een toegenomen behoefte in de cel (bijvoorbeeld verbranding om energie te verkrijgen), worden bepaald. Ten slotte moet worden opgemerkt dat er meer onderzoeken nodig zijn om de gevolgen van een verlaagde vorming en uitscheiding van chylomicronen op de EFA en LCPUFA status te bepalen in patiënten met cholestatische leverziekten.

SUMMARY

(*for non-biologists)*

What are essential fatty acids (EFAs)?

Our body can make most of the fats it needs. However, there are certain fats that are required for specific functions, but cannot be made in the body. Therefore, they must be obtained from dietary sources. These fats are called *essential fatty acids* (EFAs). Specifically, these fats are linoleic acid (chemical structure:18 carbon atoms with 2 double bonds) and alpha-linolenic acid (chemical structure: 18 carbon atoms with 3 double bonds) and can be found in plant oils (linoleic acid: sunflower and corn oils; alpha-linolenic acid: linseed and soybean oils). Linoleic and alpha-linolenic acids are the 'parent' EFAs and can be converted further in the body to other long-chain fats (usually containing 18-22 carbon atoms) which are highly unsaturated (more than 3 double bonds). These EFA metabolites are often referred to as long-chain polyunsaturated fatty acids (LCPUFAs). Directly and indirectly, LCPUFAs perform specific immune and nervous system functions within the body.

What can happen if the body does not have enough EFAs and LCPUFAs?

In general, EFA and LCPUFA deficiency in the body can result in several consequences such as skin lesions, hair loss, infertility, growth retardation, and nervous system dysfunction such as numbness, leg pain, impaired vision and reduced learning capacity. There are also many degenerative changes in major organs like the liver and the kidneys. Some researchers believe that low levels of EFAs and LCPUFAs can lead to heart disease and cancer because of the role of these fatty acids in providing a properly functioning immune system.

Patients with liver disease have low levels of EFAs in the body.

Pediatric patients with liver disease requiring liver transplantation usually have low levels of EFAs in the body, which can be disadvantageous to their condition. The cause(s) of low EFA levels in these patients is (are) not known, but may be related to one or more of the following factors:

1. Increased dietary EFAs in feces and constant dietary intake (*malabsorption*)
2. Conversion of EFAs into other fats or used for energy (*changes in metabolism)*
3. A shift in the amount of EFAs and LCPUFAs between body tissues *(redistribution)*

Malabsorption of dietary EFAs is thought to be largely responsible for low EFA levels in the body since patients with liver disease usually have strongly reduced amounts of bile in their

intestine. Bile is a mixture of fats and organic salts that is made in the liver and released in the intestine during a meal. Bile assists in the absorption of dietary fat due to its emulsifying property and to its ability to contribute specific components for the packaging of fat from the intestinal cell to the blood.

In addition to malabsorption of EFAs, patients with liver disease may have changes in their fat metabolism. Since the liver is the organ where a majority of fat metabolism takes place, patients with damaged liver cells may not be able to efficiently convert EFAs to LCPUFAs. Additionally, these patients may use EFAs and LCPUFAs for energy due to increased energy needs or for making potent hormone-like substances called prostaglandins, which are produced in a number of tissues in response to a variety of stimuli.

So, changes in absorption and/or metabolism in liver disease patients can largely contribute to low levels of EFAs and LCPUFAs in their body.

The primary aim of this thesis:

Eventually, we want to develop a (dietary) treatment for patients with liver disease to correct their low levels of EFAs and LCPUFAs. In order to do this, we attempted to understand why they have low levels of EFAs and LCPUFAs in their blood. Due to the fact that they have disturbed bile secretion into the intestine and that their liver cells are damaged, it would seem likely that changes in absorption and metabolism of EFAs and LCPUFAs could be affected in patients with liver disease. In our experiments, we focused primarily on how the lack of bile in the intestine affects the absorption and metabolism of the major dietary EFA, linoleic acid. We did this by using animals and patients known to have changes in either the amount of bile they have in the intestine or in the composition of bile. If we would find that EFAs are poorly absorbed in the absence of bile, then it would be helpful to design fats that are easily absorbed in bile-deficient conditions. If we would find that EFAs are poorly metabolized in the absence of bile, then a mere increase in the oral intake of EFAs and LCPUFAs seems helpful.

Major findings of experiments performed in this thesis:

1. In our rat model in which all bile was continuously collected outside of the body (called **bile diversion**), we found that linoleic acid levels in the blood were decreased. We checked if this decrease was due to malabsorption of linoleic acid (because there is no bile in the intestine), but it was not. These rats actually *ate more* food, which completely compensated for the amount of dietary linoleic acid lost in the feces. So, when you subtract how much linoleic acid was in their feces compared to how much they ate, they were actually ‘absorbing’ the same (‘net’) amount of linoleic acid as control rats. We also measured the metabolism of linoleic acid to one of its LCPUFAs in the blood. Our findings show that bile-diverted rats seem to have a more rapid conversion of linoleic acid to its LCPUFA. This change could be responsible for the decreased levels of linoleic acid in blood.

2. We used another rat model in which no bile was allowed to enter the intestine. However, in this model, the passage where bile enters the intestine was tied, blocking the release of bile into the intestine. Under these circumstances, the bile components normally secreted into bile will accumulate in the liver and in the blood, giving the skin and eyes a yellow ('jaundiced') look. This condition (called **bile-duct ligation**) is similar to what happens in pediatric patients with end-stage liver disease. Again, we studied linoleic acid absorption and metabolism in these rats. Our findings were rather different from what we found in the bile-diverted rats. Indeed, these rats had a high amount of linoleic acid in their feces, or increased malabsorption of dietary linoleic acid. When we calculated the amount of linoleic acid in the feces compared to what they were eating, we found that they were 'absorbing' significantly less linoleic acid than normal rats. However, of all the different dietary fats, linoleic acid was absorbed to the greatest extent, suggesting that linoleic acid absorption is relatively preserved under these conditions. We also noticed that the appearance of linoleic acid in the blood was delayed in bile duct-ligated rats compared to control rats. So, in bile duct-ligated rats, the net absorption of linoleic acid is decreased; however, relative to other fatty acids, its absorption is preserved.

3. In the bile-diverted and bile duct-ligated rat, there is an absence of bile in the intestine. Using these models, we could study if the *quantity* of bile is important for linoleic acid absorption. In order to study the effect of the *quality* of bile, or its composition, on linoleic acid absorption, we used a different animal model. Specifically, we wanted to determine how important certain lipids in bile (called phospholipids) were for linoleic acid absorption. We proposed that phospholipids were important for EFA levels in the body because 1) they assist in the packaging of fat into large fat droplets (called chylomicrons) in the intestinal cell so that it can be transported to the blood and 2) their structure contains high amounts of EFAs and LCPUFAs. An attractive way to study the effect of biliary phospholipids on EFA levels in the body and on EFA absorption is to use an animal which has been genetically altered so that it produces bile without phospholipids. Presently, there are mice that produce phospholipid-free bile called *mdr2 knockout mice*. We did three separate studies in which we used different types of diets in order to study linoleic acid and dietary fat absorption. The types of diets used were as follows: low-fat (14 en%, ~50% linoleic acid), high-fat (35 en%, ~30% linoleic acid) and high-fat, EFA-deficient (35 en%, 3% linoleic acid). The levels of linoleic acid in the mdr2 knockout mice were similar to that in control mice, regardless of diet. On low- and high-fat diets, dietary fat absorption in mdr2 knockout mice was comparable with that of controls, and within the range of normal dietary fat absorption (>95%). Similar to bile-diverted and bile duct-ligated rats, the mdr2 knockout mice had a delayed, and even absent, appearance of fat in the blood after administration of a fat load. These findings suggest that phospholipids in bile are not necessary for linoleic acid levels in the body, or for linoleic acid absorption; however, their absence results in a delay in the plasma appearance of administered fats.

In the experiment in which we used the EFA-deficient diet, we found that EFA-deficient mice have decreased fat absorption (~70%). In contrast to what has been found in studies with EFA-deficient rats, EFA-deficient mice had an *increased* flow and secretion of bile components. This finding suggests that changes in bile in EFA deficiency are not responsible for fat absorption in this condition in mice. Mdr2 knockout mice on an EFA-deficient diet had an even lower fat absorption (~60%), which suggests that phospholipids in bile may at least partially protect against further fat malabsorption in EFA deficiency. In summary, studies with mdr2 knockout mice on different diets have revealed that phospholipids in bile are not necessary for the absorption of linoleic acid or for the levels of linoleic acid in the body.

4. Finally, we studied fat absorption in children with cystic fibrosis. Cystic fibrosis is a genetic disease which leads to malfunctioning of several major organs, such as the pancreas, lungs and even the liver. Many patients with cystic fibrosis malabsorb their dietary fat. To increase the amount the fat absorbed, most patients are given oral pancreatic enzymes since they do not produce their own. However, oral enzyme therapy does not completely correct malabsorption. They continue to absorb only 80-90% of their dietary fats. Efficient absorption of dietary EFAs and LCPUFAs is especially important in these patients because they already have low levels of these fats in their blood. In our study, we tried to investigate why they had continued malabsorption of dietary fat despite the pancreatic enzyme therapy. By giving them certain fats, we could distinguish which step in their absorption process was disturbed. We found that they had malabsorption of dietary fats because of problems with the solubilization step (in which bile plays a key role) in absorption. Fat malabsorption in cystic fibrosis patients can be eliminated by correcting the solubilization step in absorption, in addition to pancreatic enzyme therapy.

"Take home messages.."

The absorption of dietary fats requires many steps, including the transport of fat from the diet into the intestine cell, and the transport of fat from the intestine cell to the blood. Of all the dietary fats, the major dietary EFA, linoleic acid, seems to be transported relatively efficiently from the diet into the intestine cell in the absence of bile in the intestine, as determined by measuring diet intake and fecal fat excretion. However, the appearance of linoleic acid in the blood (so, the exit of fat from the intestine into the blood) is delayed significantly in bile-deficient animals. Specifically, this effect appears to be related to the absence of biliary phospholipids.

This conclusion strongly suggests that impaired EFA and LCPUFA status in hepatobiliary disorders can be alleviated by increasing their oral/enteral administration.

About the author

Deanna Marie Minich was born in Chicago, Illinois, USA on December 28, 1970. After high school, she attended Augustana College in Rock Island, Illinois where she studied biology and English literature. Upon completion of her Bachelor of Arts Degree in 1992, she continued her study in Human Nutrition and Metabolism, in which she received a Master of Science Degree from the University of Illinois at Chicago. Since September 1995, she has pursued a doctoral study in Groningen, The Netherlands, under the supervision of Henkjan Verkade and Roel Vonk.

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