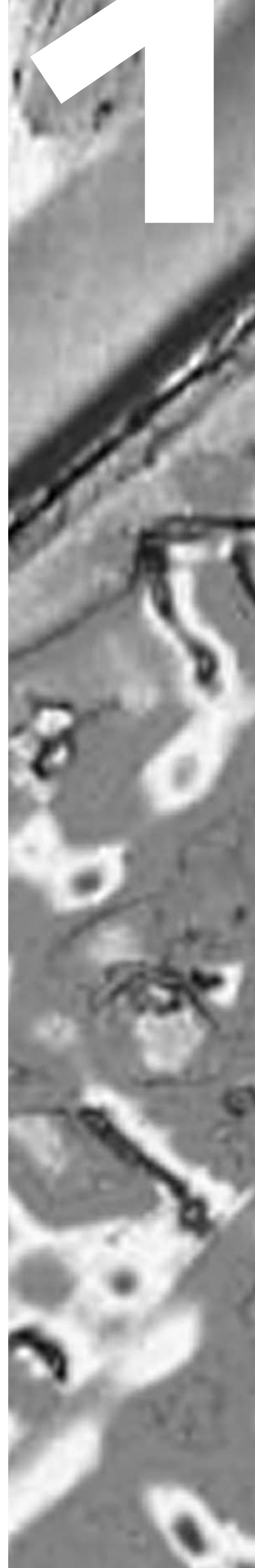

General introduction

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ESSENTIAL FATTY ACIDS

Linoleic acid (LA, C18:2n-6) and alpha-linolenic acid (ALA, C18:3n-3) are polyunsaturated fatty acids (PUFA) that cannot be synthesized *de novo* by human or animal cells. Nevertheless, these fatty acids are indispensable for normal development and function and therefore they must be provided by the diet. Their importance was first discovered in the 1920s by Mildred and George Burr, who found that rat pups fed a lipid-free diet developed growth retardation, infertility, steatosis, skin lesions and hair loss(1). Gradual reintroduction of lipid to the rats' diets failed to alleviate these symptoms, and only when linoleic acid and alpha-linolenic acid were supplied, symptoms disappeared. The importance of essential fatty acids for human nutrition became apparent in the 1970s, with the introduction of total parenteral nutrition (TPN) and infant formulas, that initially did not contain LA or ALA(2-5). In the first part of this chapter, the structure of EFA will be described, their conversion into LCPUFA and their functions and dietary requirements for the body. The second part will focus on the processes involved in dietary lipid absorption and metabolism, and the role of the liver herein, under physiological and cholestatic conditions.

EFA nomenclature and biosynthetic pathways

The structural formulas of LA and ALA are depicted below.

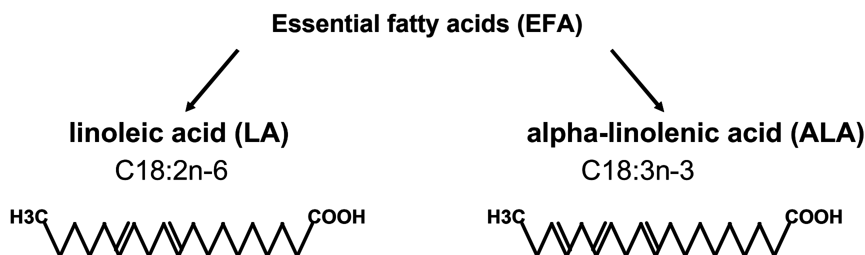


Figure 1

From the two "parent" essential fatty acids LA and ALA, two series of long-chain polyunsaturated fatty acid (LCPUFA) metabolites are formed; the omega-6 or n-6 series, which is synthesized from LA, and the omega-3 or n-3 series which has ALA as its precursor.

Formation of LCPUFA from EFA involves a series of alternating desaturation (insertion of a double bond) and elongation (addition of two carbon atoms) reactions, which occur predominantly in the endoplasmic reticulum of the liver(6;7).

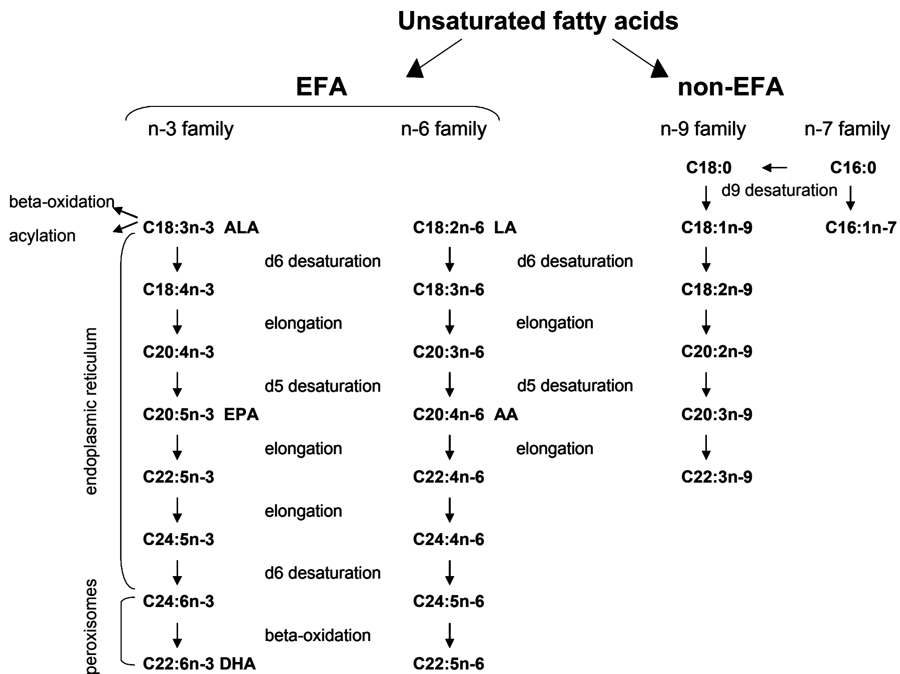


Figure 2

The omega- or n-number refers to the position of the terminal double bond, i.e., the number of carbon atoms between the methyl-end of the fatty acid and the last double bond. The number preceding the omega refers to the number of double bonds, and the C-number indicates the number of carbon atoms in the fatty acid chain.

The different PUFA series compete for the same desaturation and elongation enzymes, which have a preferential substrate affinity for n-3 fatty acids over n-6 fatty acids over n-9 fatty acids. During scarcity of n-3 and n-6 fatty acids, long-chain n-9 fatty acids are synthesized. The concentration of n-9 LCPUFA in body compartments is used to demarcate EFA deficiency[8]. Desaturase enzymes are not only competed for by the different fatty acid series, also feedback regulation occurs, mediated by the concentrations of desaturase and elongase substrates and end-products. In addition, hormonal and dietary factors regulate LCPUFA biosynthesis: desaturase activities are enhanced by insulin, thyroid hormones and high dietary protein intake and inhibited by glucagon, catecholamines, corticosteroids, and deficiencies of zinc, iron, calcium, selenium and vitamins B_6, C and E[9-11].

C16:0	hexadecanoic acid	palmitic acid
C16:1n-7	9-hexadecenoic acid	palmitoleic acid
C18:0	octadecanoic acid	stearic acid
C18:1n-7	11-octadecenoic acid	vaccenic acid
C18:1n-9	9-octadecenoic acid	oleic acid (OA)
C18:2n-6	9,12-octadecadienoic acid	linoleic acid (LA)
C18:3n-6	6,9,12-octadecatrienoic acid	gamma-linolenic acid (GLA)
C18:3n-3	9,12,15-octadecatrienoic acid	alpha-linolenic acid (ALA)
C20:0	eicosanoic acid	arachidic acid
C20:3n-9	5,8,11-eicosatrienoic acid	Mead acid
C20:3n-6	8,11,14-eicosatrienoic acid	dihomo-gamma-linolenic acid (DGLA)
C20:4n-6	5,8,11,14-eicosatetraenoic acid	arachidonic acid (AA)
C20:5n-3	5,8,11,14,17-eicosapentaenoic acid	timnodonic acid (EPA)
C22:0	docosanoic acid	behenic acid
C22:4n-6	7,10,13,16-docosatetraenoic acid	adrenic acid
C22:5n-6	4,7,10,13,16-docosapentaenoic acid	(DPA)
C22:5n-3	7,10,13,16,19-docosapentaenoic acid	clupanodonic acid (DPA)
C22:6n-3	4,7,10,13,16,19-docosahexaenoic acid	cervonic acid (DHA)
C24:0	tetracosanoic acid	lignoceric acid
C26:0	hexosanoic acid	cerotic acid

Figure 3: Nomenclature of polyunsaturated fatty acids

EFA functions

EFA and their long-chain metabolites fulfill a wide array of physiological functions in the body; they are structural membrane components, precursors of eisosanoids and ligands for nuclear receptors involved in lipid homeostasis. Similar to other fatty acids, EFA are also an important source of energy for the body; approximately 75% of EFA is not converted into LCPUFA but is oxidized[12]. Oxidation occurs in a preferential rank order of ALA > LA > oleic acid > palmitic acid > stearic acid. LCPUFA are oxidized at a very low rate[13].

Membrane PL

EFA and LCPUFA are structurally incorporated into cell membrane phospholipids (PL). The degree of PL acyl chain unsaturation modulates fluidity of the membrane lipid matrix, thus altering membrane permeability and function of membrane enzymes, receptors and transporters. Cellular membranes are typically constituted of bilayers of PL molecules (Figure 4), in which cholesterol and membrane proteins are inserted. Membrane PL are oriented with their hydrophobic acyl chains towards each other, and their hydrophilic headgroups towards the aqueous intra- or extracellular environment. Due to their amphiphilic nature, PL in membrane bilayers enable inter-actions between water-soluble and lipid-soluble substances, while simultaneously

allowing (sub)cellular compartmentalization. The PL bilayer provides an adaptable matrix for insertion of membrane proteins, that function as receptors, membrane-bound enzymes or transmembrane transporters. The nature of PL acyl chains can significantly affect membrane properties. Saturated fatty acids (SAFA) form a more rigid configuration, whereas double bonds make the membrane more flexible and reactive.

CNS membrane PL

Flexibility and reactivity is particularly important in the highly excitable membranes of the central nervous system (CNS), which are exceptionally rich in DHA (docosahexaenoic acid, C22:6n-3) and AA (arachidonic acid, C20:4n-6). In fact, lipids constitute 60% of brain dry weight, and 50% of PL acyl chains is DHA and AA[14]. The significant contribution of these fatty acids to CNS phospholipids has induced great scientific interest in EFA and LCPUFA contents of infant formulas as compared to breast milk. Human milk is a rich source of both n-3 and n-6 LCPUFA; it provides up to 0.5% of fatty acids as DHA and AA, which are incorporated preferentially into brain PL as compared to DHA and AA that originate from conversion of ingested precursor EFA[15-17]. Until recently, infant formulas only contained EFA and no LCPUFA. Both preterm and term infants can convert EFA into DHA and AA[18;19], but it is questionable whether this is sufficient to provide the high amounts of LCPUFA required by the rapidly developing brain during its growth spurt, from 3 months before to 18 months after birth. *In utero*, the placenta (which also has desaturase capacity) provides a selective LCPUFA transfer to the fetus by an as yet unidentified transport mechanism, resulting in up to 400-fold higher concentrations of DHA and AA in fetal compared to maternal blood[20]. A considerable deposition of LCPUFA in the brain occurs during late gestation. Breast milk supplies DHA and AA in amounts believed to equal intrauterine accretion rates, whereas feeding infant formulas devoid of preformed LCPUFA has been associated with decreased brain DHA and AA contents and with transiently impaired neurological maturation[21]. Whether LCPUFA supplementation of infant formulas, and in which amounts, has long-term beneficial effects on visual or cognitive development in term infants is still a matter of debate.

Enterocyte and biliary PL

Whereas CNS membranes contain high amounts of DHA and AA, enterocyte membranes and bile PL are particularly rich in LA and AA[22;23]. The intestinal mucosa is a dynamic structure that continuously undergoes biochemical and morphological modifications during enterocyte differentiation and maturation. Their short life span (~5 days in humans, ~2 days in rodents[24;25]) makes enterocytes highly sensitive to

changes in dietary lipids. A constant intestinal supply of EFA may be required for cell renewal and maintenance of membrane integrity and function[26;27]. Since bile phospholipids contain up to 40% of acyl chains as EFA or LCPUFA, bile is a quantitatively substantial supply of EFA for structural and functional needs of the intestine[28]. Enterocyte membranes are markedly EFA-depleted during EFA deficiency. This might play a role in the well-recognized phenomenon that EFA deficiency is not only a consequence of dietary lipid malabsorption, but can in itself also cause intestinal lipid malabsorption[29-32]. Intraluminal events involved in fat absorption (lipolysis by lipases, solubilization of lipolytic products by bile and uptake by enterocytes) seem undisturbed during EFA deficiency[32-35], yet qualitative changes in enterocyte membranes due to LA and AA depletion could impair intracellular processing of dietary lipid.

Eicosanoids

EFA derivatives are direct precursors for eicosanoids[36], which are involved in a wide variety of inflammatory and vascular processes. Eicosanoids are synthesized from C20 LCPUFA by cyclo-oxygenase and lipoxygenase enzymes, yielding prostaglandins, thromboxanes, leukotrienes and lipoxins. Arachidonic acid (AA) is the principal eicosanoid precursor in humans, next to dihomo-gamma-linolenic acid (DGLA, C20:3n-6) and eicosapentaenoic acid (EPA, C20:5n-3). As autocrine and paracrine hormones, eicosanoids mediate processes such as constriction or relaxation of endothelial cells, platelet aggregation, leucocyte activation and chemotaxis. Eicosanoids derived from n-6 fatty acids generally have pro-inflammatory effects whereas those with n-3 precursors are anti-inflammatory[37;38]. The latter has led to increased interest in EFA status, and particularly in n-3 to n-6 fatty acid balance, in patients with cystic fibrosis[39] and autoimmune diseases.

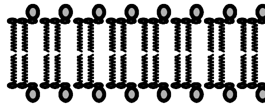
Nuclear receptors, lipid homeostasis

In recent years, EFA and LCPUFA have gained interest as regulators of genes involved in lipid homeostasis, by direct or indirect interactions with peroxisome proliferator-activated receptor alpha (PPARa), sterol regulatory element binding protein (SREBP), farnesoid X rexeptor (FXR) and liver X receptor (LXR). These nuclear receptors regulate expression of genes involved in lipogenesis (fatty acid synthase (FAS), acyl CoA carboxylase (ACC), stearyl CoA dehydrogenase (SCD)), lipid oxidation (carnitine palmitoyl transferase (CPT), acyl CoA oxidase) and lipoprotein metabolism (apoC2, C3, A1, E, scavenger receptor B1 (SR-B1), lipoprotein lipase (LPL), hepatic lipase (HL), phospholipid transfer protein (PLTP), cholesterol ester transfer protein (CETP), lecithin cholesterol acyl transferase (LCAT))[40-48]. Fatty acids differ in their

effects on plasma lipid and lipoprotein levels; generally, saturated fatty acids (SAFA) increase plasma TG and cholesterol concentrations, whereas PUFA lower plasma TG, yet species differences between animal models are considerable[49]. Enhancement of hepatic lipogenesis in EFA deficiency presumably results from removal of inhibitory PUFA, but how EFA and LCPUFA, as core (TG) and surface (PL) components of lipoproteins, affect physiological lipoprotein metabolism is not fully clarified. An overview of EFA and LCPUFA functions is depicted in Figure 4.

1 - energy

2 - membrane phospholipids



3 - eicosanoid precursors
-prostaglandins
-thromboxanes
-leukotrienes

4 - lipoprotein metabolism

5 - ligands for transcription factors

Figure 4: EFA functions

EFA sources and requirements

LA can be derived from the diet or, in adults, to a limited extent from mobilization from fat tissue. Healthy adults have approximately 1 kg of LA stored in adipose tissue, yet adipocytes contain hardly any ALA or LCPUFA. Infants, and particularly preterm neonates, have a limited adipose reserve, and are therefore especially dependent on continuous EFA intake from dietary sources. LA is found in plant seed oils such as corn oil and sunflower oil. ALA is present in green leafy vegetables, in nuts and in soybean-, linseed- canola- and blackcurrant seed-oils. The n-3 LCPUFA DHA and EPA are found in high concentrations in fatty fish such as mackerel, herring, salmon, tuna and trout. Both DHA and AA are present in egg yolk[50], and AA is also found in substantial concentrations in meat. As mentioned above, human milk is a rich source of both n-6 and n-3 EFA and LCPUFA.

Recommendations regarding adequate dietary intake of EFA and LCPUFA are highly variable between countries, and obviously vary with age, i.e., with adipose tissue stores and with growth rate. For adults, the minimal daily requirement for LA and ALA has been estimated at approximately 2 and 0.3 en%, respectively[51;52]. For healthy children, a daily intake of 1-5 en% of LA and 0.5 en% for ALA is

recommended[51;53]. For preterm babies, recommendations for infant formulas are currently 8-10% for LA, 1.5-1.75% for ALA, 0.5% for AA and 0.35% for DHA[54;55].
A deficiency of EFA can develop either when dietary intake is insufficient, when intestinal absorption is impaired or when body requirements or metabolism of absorbed EFA are (temporarily) increased. Thus, patients particularly prone for development of EFA deficiency include (preterm) babies, patients with impaired dietary fat absorption, such as cholestatic patients or patients with chronic intestinal disease, and patients with cystic fibrosis, in whom both absorption of EFA may be impaired and metabolism may be increased. An overview of the clinical symptoms associated with a deficiency of EFA is shown in Figure 5.

Skin scalinesss, hair loss
Increased skin permeability to water
Impaired visual development
Impaired cognitive development
Impaired psychomotor development
Growth retardation
Dietary lipid malabsorption
Steatosis
Infertility
Increased perinatal mortality
Increased bleeding tendency

Figure 5: Symptoms of EFA deficiency

LIPID ABSORPTION AND METABOLISM IN CHOLESTASIS

In the various aspects of lipid absorption and metabolism, the liver has a central role. Primarily, the liver produces bile, constituents of which are required for efficient intestinal fat absorption. Additionally, biliary secretion of cholesterol (as such, or after metabolism into bile salts) and phospholipids from the liver into the intestine is of major importance in body lipid homeostasis. The liver is the major source of plasma lipoproteins: it synthesizes apoproteins (i.e., apoA-I, apoB, apoE) that regulate metabolic interconversions between lipoprotein classes and lipoprotein lipid constituents as cholesterol, TG and PL. The liver is also the major site of clearance of circulating lipoproteins, which are subsequently catabolized in the hepatocytes. Additionally, the liver synthesizes enzymes such as LCAT, CETP, PLTP and LPL, which are involved in lipoprotein metabolism in the plasma compartment. Finally, the liver is the site of active synthesis, metabolism and/or oxidation of various lipid classes, including EFA and LCPUFA.

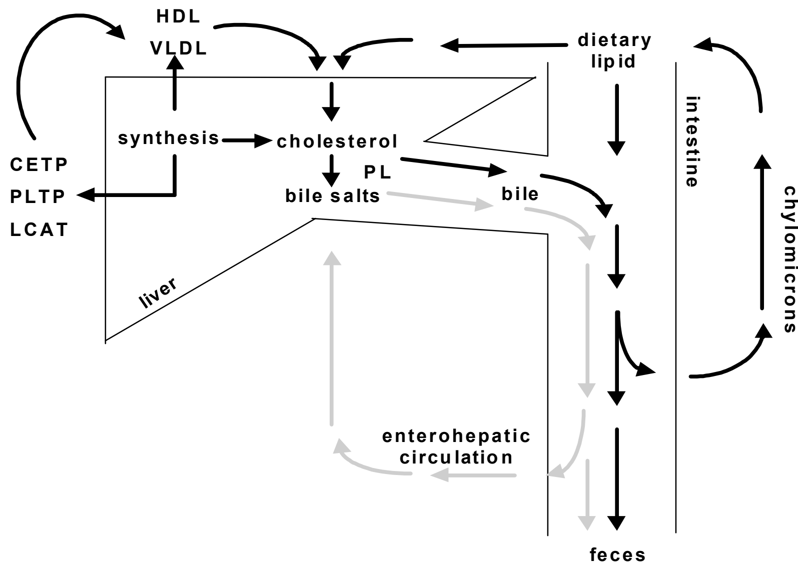


Figure 6

In view of this multitude of essential functions that are often strongly interrelated, it is evident that disturbances in bile formation in cholestatic liver disease will have a strong impact on various aspects of lipid metabolism in the body. Consequences of cholestasis, which is functionally defined as decreased or absent bile flow from the liver into the intestine, may be related to:

- absence of bile components at their sites of action, particularly in the intestine
- disruption of the continuous flux of lipids from the liver into bile and intestine, resulting in accumulation of toxic and non-toxic bile components in the body, most notably in hepatocytes, concomitantly altering hepatocyte or enterocyte function.
- characteristic alterations in plasma lipoproteins associated with cholestatic liver diseases, such as decreased HDL levels and the appearance of lipoprotein X.

Intestinal lipid absorption

Dietary lipid classification

Dietary fat comprises a wide array of lipid classes, which have been categorized according to the nature of their interactions with water into polar and non-polar lipids[30;56;57]. Polar lipids (cholesteryl esters, hydrocarbons and carotene), are insoluble in water and are divided into three subclasses. Firstly the insoluble non-swelling amphiphiles which form a thin stable monolayer in water; secondly the insoluble swelling amphiphiles, which form both stable monolayers in water and laminated

lipid-water structures called liquid crystals; and finally the soluble amphiphiles, which possess strong polar groups that render these molecules soluble in water at low concentrations, forming both unstable monolayers and micelles[29;33]. Examples of class 1 polar lipids are triglycerides (TG), diacylglycerols (DG), non-ionized long-chain fatty acids (LCFA), unesterified cholesterol and the fat soluble vitamins A, D, E and K. Class 2 insoluble swelling amphiphiles are monoacylglycerols (MG), ionized fatty acids (FA) and phospholipids (PL). Class 3 soluble amphiphiles are sodium salts of long-chain fatty acids and bile salts. The absence of bile during cholestasis will differentially affect solubilization and absorption of lipid classes due to their different interactions with water.

TG is the major fat in human diet, contributing ~90% of energy provided by dietary lipid. The majority of luminal phospholipid is phosphatidylcholine (PC), which is mostly of biliary origin (10-20 g daily in humans), with a dietary contribution of 1-2 g per day[29;30]. EFA are present in the diet mostly as acyl chains of TG (90%) and also as PL (10%). Biliary PL is an important source of intestinal EFA, since it is highly EFA- enriched.

Dietary EFA

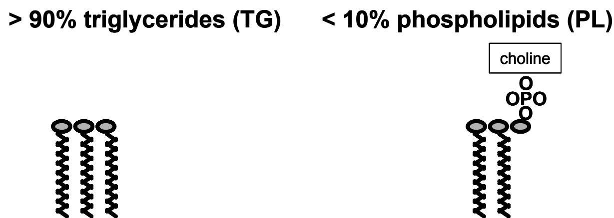


Figure 7

The predominant dietary sterol is cholesterol (0.5 g/day)[29;30], which is mostly of animal origin although small amounts are also present in vegetables. Beta-sitosterol is the most important plant sterol (which account for 25% of dietary sterols), but it is virtually not absorbed by humans under physiological conditions due to the intes-tinal half-transporters ABCG5/G8, which have been postulated to play a major role in efflux of absorbed sterols from enterocytes into the intestinal lumen, and from the liver into bile[58;59].

The fat-soluble vitamins A, D, E and K, required in small quantities for maintenance of normal cell and organ function[60-63], are class 1 polar lipids and depend on micel-lar solubilization for intestinal uptake. Absorption rates differ between vitamin

species, averaging from 50 to 80% for vitamins A, D and K but only ~25% for vitamin E[64]. Additionally, there may be competition between vitamin species for intestinal absorption- or transport-sites[65], although minimal information is available regarding the nature and function of these sites.

The processes involved in intestinal lipid absorption can be divided into intraluminal and intracellular events, which differ for TG and PL.

Intraluminal phase of lipid absorption

Before translocation from the intestinal lumen into enterocytes can occur, dietary lipids must undergo a number of physicochemical alterations. This is achieved in a sequence of events called the intraluminal phase of lipid digestion and absorption, including:

-emulsification of dietary lipid
-lipolysis
-solubilization (micelles, vesicles)
-translocation of lipolytic products across the enterocyte membrane

Emulsification and lipolysis

In humans, the first step in dietary fat digestion starts in the stomach with mechanical emulsification and partial TG hydrolysis by gastric lipase, resulting in the lipolytic products DG and free fatty acids (FFA). Gastric lipase does not hydrolyze PL or cholesterol ester, but its activity in the stomach accounts for 10-30% of TG lipolysis[30;56;66;67]. The remaining part of TG digestion is brought about in the duodenal lumen by pancreatic lipase, which acts mainly on the sn-1 and sn-3 position of TG molecules, releasing 2-MG and FFA[66;68]. Pancreatic (co-lipase-dependent) lipase is present in pancreatic secretions in large excess, in accordance with the clinical observation that only severe pancreatic insufficiency (as in CF) results in lipid malabsorption. In the presence of bile salts, pancreatic lipase requires the cofactor pancreatic co-lipase for adequate TG hydrolysis, since TG droplets covered with bile salts are not accessible to pancreatic lipase[56; 30]. Binding of pancreatic co-lipase to the TG/water interface facilitates binding of pancreatic lipase.

Digestion of PL occurs entirely in the duodenal lumen, predominantly by pancreatic phospholipase A_2. Phospholipase A_2 requires calcium and bile salts for activation, and hydrolyzes PL at the sn-2 position resulting in FFA and lyso-PL.
Dietary cholesterol is present for ~90% as free cholesterol, the remainder as cholesterol esters. Cholesterol esters are hydrolyzed in the small intestine by pancreatic

cholesterol esterase. Human cholesterol esterase (also known as carboxyl ester lipase, bile salt-stimulated lipase, monoglyceride lipase, pancreatic non-specific lipase or human milk lipase) can also hydrolyze TG (sn-1, -2, -3), PL (sn-1, -2) and lipidic vitamin esters[69] and its activity is enhanced by the presence of bile salts.

Solubilization of lipolytic products

For diffusion through the unstirred water layer, which separates the brush border membrane of enterocytes from the liquid luminal contents of the intestine, solubilization of lipolytic products is required. The most important function of biliary bile salts and PL in the intestinal lumen appears to be their ability to increase solubility of lipolytic products in the aqueous lumen by formation of mixed micelles. Mixed micelles were first described by Hoffman and Borgstrom[70] as disc-like aggregates of amphiphilic biliary and dietary components, oriented with their hydrophobic parts to the inside of the micelles and their hydrophilic polar headgroups towards the aqueous outside. This conformation increases solubility of FFA and MG 100-1000 fold[29]. Mixed micelles contain bile salts (class 3 polar lipids), hydrogenated fatty acids (class 1 polar lipids), fatty acid ions (class 2 polar lipids), MG (class 2 polar lipid), PL (class 2 polar lipids) and cholesterol (class 1 polar lipid), and are ~4 nm in diameter[29;30;33;56;57]. Carey[65] described the co-existence of mixed micelles in the intestinal lumen with unilamellar liquid crystalline vesicles or liposomes. He demonstrated that only when intraluminal bile salt concentrations exceed the so-called critical micellar concentration, mixed bile salt / lipid micelles are formed. However, when bile salt concentrations are low or decreased by dilution, large (20-60 nm) unilamellar liquid crystalline vesicles or liposomes predominate[71;72]. All classes of lipolytic products can be incorporated into disc-shaped micelles as well as liquid crystalline vesicles. Since both phases co-exist, quantifying the relative contribution of the two phases remains difficult, especially due to continuous exchange of 2-MG and FA between both structures. Dissociation rates of lipolytic products from vesicles and their subsequent translocation across the enterocyte membrane are slower than dissociation rates from mixed micelles[73].

The existence of liquid crystalline vesicles is thought to have specific pathophysiological consequences for lipid absorption in conditions where intraluminal bile salt concentrations are diminished, as in cholestasis. It has been demonstrated that fat uptake can still occur rather efficiently, based on balance studies, but that fat absorption rates are profoundly slower in bile salt-deficient states. Porter *et al*. reported on a bile fistula patient who continued to absorb up to 80% of dietary lipid, despite the obvious bile salt deficiency and the 100-fold decreased FFA concentration in the

aqueous phase of the small intestinal lumen[74]. Mansbach *et al*. found similar results in patients with bile salt malabsorption, where the strong decrease in solubilized fatty acid concentration led only to a mild degree of lipid malabsorption[75]. Solubilization of lipolytic products into liquid crystalline vesicles during intestinal bile-salt deficiency, in combination with the reserve capacity of the length of the small intestine to absorb fat, could explain the slower but preserved rate of lipid absorption in cholestasis.

Nishioka *et al*.[76] studied the importance of PL-cholesterol vesicles for lipid absorption during bile deficiency. Intraduodenal administration of ^{13}C-labeled linoleic acid (LA) or palmitic acid (PA) to bile-diverted rats was associated with strongly decreased plasma concentrations of ^{13}C-LA and ^{13}C-PA. Subsequent intraduodenal supplementation with PL-cholesterol vesicles reconstituted plasma concentrations of labeled PA. Yet, there appeared to be a delay in plasma appearance of both lipids, since at 5h after lipid administration plasma concentrations were still increasing. These observations are in concordance with the slower dissociation and translocation rates of lipolytic products from vesicles compared to mixed micelles, as proposed by Narayan and Storch[73].

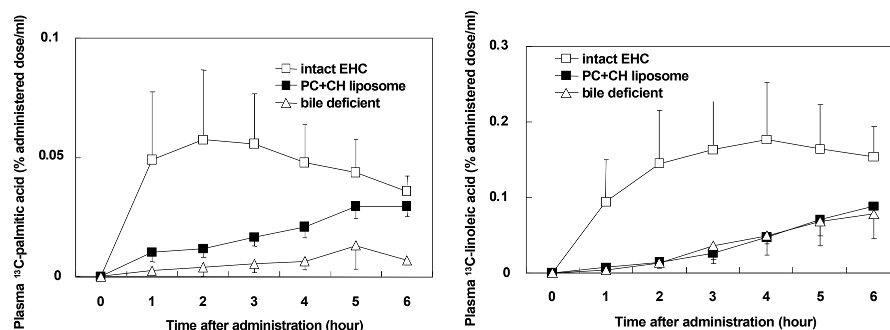


Figure 8

It is important to note the lipid-class difference in the dependence on bile for solubilization and consecutive uptake of fats. PUFA are less dependent on bile solubilization due to their lower hydrophobicity, compared to SAFA. Even in complete absence of intestinal bile salts, absorption of PUFA has been demonstrated to be relatively well preserved (up to 80%) compared to that of saturated long-chain fatty acids (<30%), although absorption remained significantly lower than in the presence of bile (~97%, Figure 9)[25].

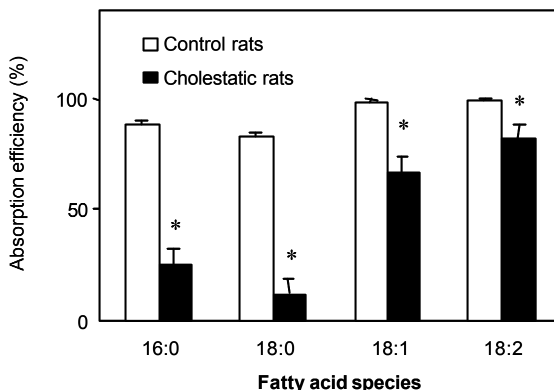


Figure 9

In contrast to long-chain lipids, short- and medium-chain lipids do not depend on luminal presence of bile salts for adequate uptake, and can directly be transferred from the intestinal lumen through the enterocytes. Medium-chain triglyceride (MCT)-based formulas are therefore widely used as energy providers in conditions where intestinal solubilization is impaired. For absorption of cholesterol and fat-soluble vitamins, however, micellar solubilization by bile salts is crucial(77).

Translocation

For many years, translocation of lipolytic products across the unstirred water layer and the enterocyte membrane has been assumed to occur through passive diffusion. Yet reports from Stremmel and Schulthess *et al*.(78;79) suggested that an active carrier-mediated process by fatty acid binding proteins (Fabp) is involved in transport of FA across the intestinal brush border membrane. Members of the Fabp family appeared to have both fatty acid transport and esterification capacities(80;81), yet studies in mice in which intestinal Fabp was genetically inactivated (Fabpi $^{-/-}$ mice) revealed that this protein is not essential for dietary fat absorption(82). Stahl *et al.* addressed FATP4, abundantly present in apical membranes of enterocytes, as the principal intestinal transporter of long-chain fatty acids(83). A fatty acid transporter not specific for the enterocyte was identified by Harmon *et al.*(84), who isolated a 88-kDa membrane protein termed FAT (fatty acid transporter) which appeared to be the rat homologue of human CD36. CD36 is expressed in platelets, macrophages and endothelial cells as well as in intestine, adipose tissue, heart and muscle, where it mediates long-chain fatty acid uptake. Impaired CD36 function is associated with a large (~70%) deficit in FA uptake in these tissues(85). Some authors have suggested interactions between different fatty acid transporters to regulate intestinal fatty acid uptake(86), yet the exact molecular mechanism by which translocation of lipolytic products occurs is still a matter of debate.

The net uptake of cholesterol is highly specific, since the plant sterol ß-sitosterol is poorly absorbed under physiological conditions despite its structural similarity to cholesterol. In healthy individuals, ~60% of dietary cholesterol is taken up, whereas absorption of plant sterols is less than 1%[29;87]. The half-transporters ABCG5/G8, implied in the autosomal recessive disorder sitosterolemia, are held responsible for efficient efflux of absorbed dietary sterols from enterocytes into the intestinal lumen, and from liver into bile[58;59], possibly with different affinities for sterol species. Sitosterolemia patients, in whom ABCG5/G8 function is genetically impaired, have 30-fold increased plasma plant sterol levels, increased cholesterol absorption and decreased biliary sterol secretion, resulting in sterol accumulation and atherosclerosis[88;89].

Intracellular phase of lipid absorption

After translocation across the apical enterocyte membrane, dietary lipids migrate to the endoplasmic reticulum. At the cytosolic membrane of the smooth endoplasmic reticulum (SER), re-esterification of absorbed fatty acids into TG takes place. Two different biochemical pathways are involved in TG resynthesis, of which the monoacyl glycerol (MG) pathway is the most important under physiological conditions. In the MG pathway, 2-MG is re-acylated to DG and subsequently to TG by monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase enzymes (DGAT1 and 2)[90;91], respectively. The alternative route of re-esterification is the alpha-glycerophosphate pathway, which involves conversion of glycerol-3-phosphate via phosphatidic acid to DG and then to TG, also mediated by DGAT. Under physiological conditions there is an abundant supply of 2-MG and FFA during lipid absorption, and the 2-MG route will predominate over the alpha-glycerophosphate route.
Newly synthesized TG from both pathways are thought to be metabolically distinct: TG from the 2-MG route is secreted more rapidly across the basolateral membrane than TG originated from the alpha-glycerophosphate route. It has been suggested that the DG from each pathway enter into separate intracellular pools. DG from the alpha-glycerophosphate route is preferentially used for *de novo* PL synthesis.

Absorbed cholesterol, either from biliary or dietary origin, enters the free cholesterol pool inside the enterocyte, which also contains cholesterol originating from absorption of shed intestinal mucosal membranes and from *de novo* synthesis. Cholesterol is transported into the lymphatic system mainly as cholesterol ester (CE) in the neutral lipid core of chylomicrons. It is unclear whether a fraction of cholesterol escapes the lymphatic route and crosses the basolateral membrane of the enterocyte via Abca1. The enzymes involved in cholesterol esterification are the acyl-CoA

cholesterol acyltransferases ACAT-1 and -2. Inhibition of ACAT activity decreases absorption of dietary cholesterol, associated with lymphatic release of aberrant apoB containing lipoproteins devoid of CE, containing mostly TG in their cores[90;92-94].

Newly synthesized TG and CE form lipid droplets in the SER, where packaging occurs, mainly into lipoprotein particles called chylomicrons (CM). In the SER, nascent chylomicrons associate with PL, cholesterol and apoA-I, apoA-IV and apoB48. Under physiological conditions, surface coat PL of lymph chylomicrons are of biliary origin rather than of dietary sources whereas chylomicron TG fatty acids closely correspond with those of dietary TG[95]. As fat absorption and TG resynthesis proceeds, lipoprotein particles increase in size and number and eventually end up in vesicles filled with pre-chylomicrons, which are transported to the Golgi apparatus. Here, modification of pre-CM into mature CM occurs, followed by translocation to the lateral surface of the enterocyte where CM are exocytosed into the interstitium, ending up in mesenteric lymph. Nascent CM have diameters between 100-1000 nm. Mesenteric lymph ducts drain into the thoracic duct, which enters the systemic circulation at the level of the jugular vein.

In recent years, it has become appreciated that biliary PL secretion is necessary for proper intestinal CM assembly and thus for secretion of dietary lipid into lymph. Studies in rats with interrupted enterohepatic circulations, by cholestyramine feeding or manipulation of bile composition by dietary means[96, 97], revealed a dietary lipid accumulation in enterocytes. This phenomenon was also seen by Tso in bile-diverted rats, where subsequent administration of bile acids only partially reinstated lipid transport into lymph. Only after administrating biliary PL, lymphatic lipid transport was fully restored[98].
Voshol *et al*. demonstrated a delayed plasma TG appearance after an oral lipid bolus in *Mdr2* $^{-/-}$ mice lacking biliary PL secretion. This aberrant postprandial plasma TG response was accompanied by normal fecal fat excretion and accumulation of lipid droplets in the intestinal wall, suggesting a relatively well-preserved intestinal lipid uptake into enterocytes in the absence of biliary PL, but a delay in subsequent CM secretion[99]. Intestinal PL requirements for CM production might be comparable to that of the liver for VLDL secretion. In choline deficiency, decreased hepatic PC synthesis results in impaired VLDL production[100]. Enterocytes might similarly require biliary PL for appropriate intestinal CM production. A schematic overview of the various steps involved in absorption of TG and PL is depicted in Figure 10.

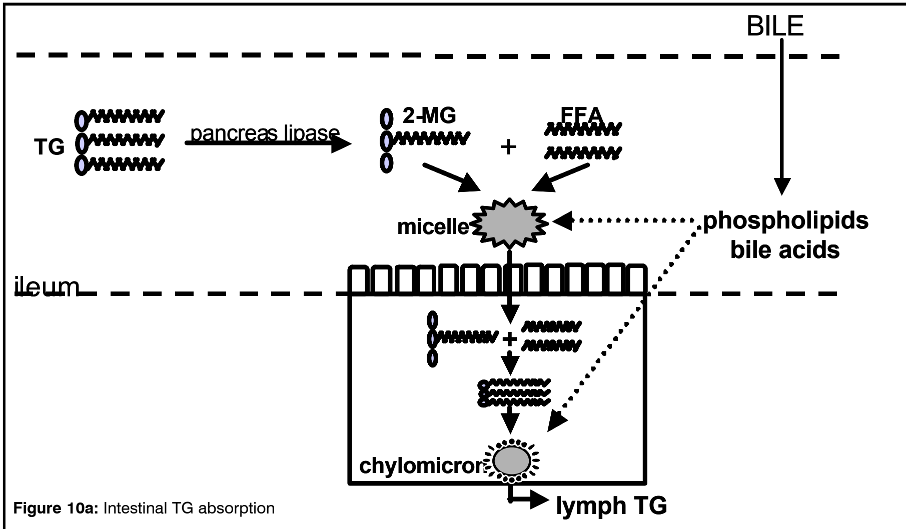


Figure 10a: Intestinal TG absorption

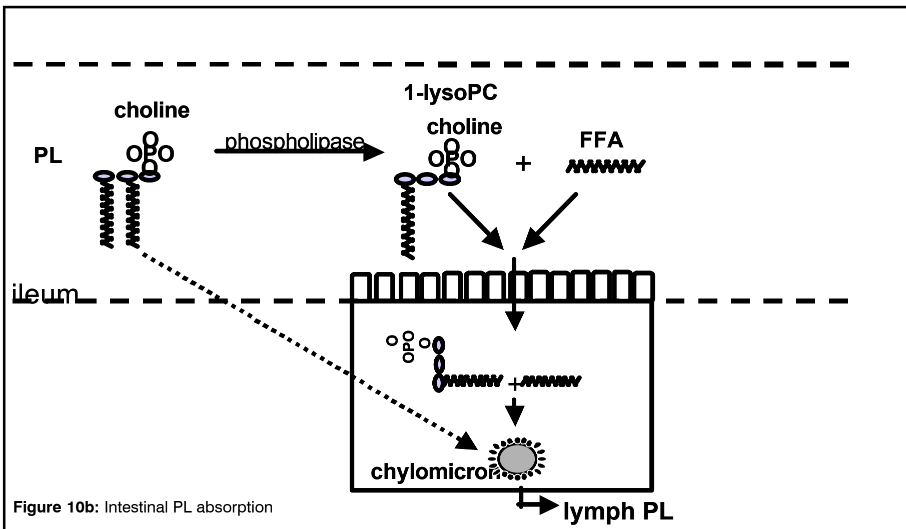


Figure 10b: Intestinal PL absorption

Absorption of enteral PL exceeds 90%, 20% of which is absorbed as intact PL, 40% as lyso-PL, and 40%[101;102] is degraded to glycerophosphocholine and phosphoryl choline, which is taken up via the portal vein. After absorption, PL acyl chains are biologically highly available for the organism[103;104].

Alterations in lipid homeoastasis during cholestasis

Lipid malabsorption

Fat malabsorption as a consequence of disturbed bile secretion is associated with weight loss due to energy deficiency (in children complicated by impaired growth and development), with fat-soluble vitamin deficiencies and with EFA deficiency.
Several compensatory mechanisms for fat malabsorption during bile deficiency have been described in animal models for cholestasis. Minich *et al*. reported on lipid malabsorption in rats with chronic bile diversion. In this model, bile is absent from the intestinal lumen, but in contrast to the cholestatic condition, (toxic) biliary components do not accumulate in the body. Bile-diverted rats appeared to compensate for their markedly decreased dietary lipid absorption by strongly increasing their food ingestion[25]. Subsequent morphological examination of the small intestine revealed that both villus and crypt height were significantly increased in bile-diverted rats compared to controls.
Porter and Knoebel *et al*. described another compensatory mechanism designated as 'the absorptive reserve of the small intestine'[74;105]. Under physiological conditions, only the proximal part of the intestine is involved in fat absorption. In situations where proximal fat absorption appears impaired, more distal parts can also contribute.
Minich *et al*. also demonstrated that the amount of dietary lipid strongly affects the efficacy of lipid absorption in bile deficient states. In rats with chronic bile diversion, absorption of dietary lipid remained highly efficient (84% of ingested lipid) when regular low-fat chow was fed. However, when rats were fed a high-fat diet, lipid absorption coefficients decreased to around 50%, indicating that compensatory mechanisms for lipid absorption in bile deficiency have limited capacity[33]. Detailed knowledge of different compensation mechanisms and alternative routes of lipid absorption during bile deficiency are important for developing dietary treatment strategies for nutritional deficiencies in cholestatic patients.

Fat-soluble vitamin deficiency

Although great variability exists between studies in defining biochemical vitamin deficiency, many reports have indicated the existence of significantly decreased fat-soluble vitamin levels under cholestatic conditions[106-108]. Fat-soluble vitamins (class I polar lipids) are highly dependent on intraluminal solubilization by bile acids, and lack of bile flow usually results in malabsorption and depletion of fat-soluble vitamin stores. The extent of deficiency appears to be highly vitamin-species specific; Phillips *et al*.[106] reported biochemical deficiencies of vitamin A, D, E and K in 34%, 13%, 2% and 8% of PBC patients, respectively. Similar values were found by Kaplan and Kowdley *et al*.[64;107;108]. Vitamin A deficiency in chronic cholestasis can not only result

from malabsorption, but hepatic secretion of retinol binding protein (RBP) may also be diminished, leading to low plasma levels of retinol and impaired delivery to target tissues such as retina and epithelial cells[60]. Deficiency of vitamin K can lead to life-threatening haemorrhages due to the vitamin K dependence of clotting factors II, VII, IX, X and proteins S and C for haemostatic activity. Biliary atresia can present itself with intracranial haemorrhage as first consequence of cholestasis-induced lipid malabsorption[109]. Although vitamin D deficiency during lipid malabsorption can partially be circumvented by endogenous photosynthesis of vitamin D_3 in the skin, many chronically ill patients are not adequately exposed to sunlight, resulting in low vitamin D and calcium levels and impaired bone mineralization[60;61]. Prolonged vitamin E deficiency in cholestatic children leads to degenerative neuromyopathy eventually resulting in peripheral neuropathy, muscular weakness, ophthalmoplegia and retinal dysfunction which appears partly irreversible. The irreversibility and severity of many of the symptoms associated with fat-soluble vitamin deficiencies mandate strict monitoring and correction of vitamin status in cholestatic patients.

EFA deficiency

EFA deficiency as a consequence of overall lipid malabsorption in cholestasis is well recognized. Additionally, it has become apparent that EFA deficiency in itself can impair lipid absorption. Levy *et al*. observed decreased bile salt secretion rates in EFA-deficient rats[110], implying impaired bile formation as a possible cause for EFA deficiency-induced fat malabsorption. EFA deficiency may also affect intracellular events of dietary fat absorption occurring in the enterocyte. In both situations, EFA deficiency during cholestasis may further compromise dietary fat absorption.

Lipid metabolism during cholestasis

During lipid absorption, both intestine and liver release large amounts of TG-rich lipoproteins into the circulation. During fasting, the liver is the major source of TG-rich lipoproteins by secreting VLDL. Both liver and intestine are capable of synthesizing HDL, which are secreted as nascent particles containing predominantly PL and unesterified cholesterol. Another major lipoprotein, LDL, is predominantly formed in the plasma compartment as a product of VLDL catabolism. Additionally, the liver synthesizes apoproteins that are essential structural and enzymatic components of lipoproteins. Apoproteins act as cofactors for enzymes crucial for cholesterol esterification or TG lipolysis. ApoA-I activates the cholesterol esterifying enzyme LCAT, which is also synthesized in the liver. ApoC-II is required for lipoprotein lipase (LPL) activation, which hydrolyzes lipoprotein TG, thus converting CM into CM-remnants and VLDL into IDL and ultimately LDL. ApoE and apoB are crucial for receptor-mediated

uptake of lipoproteins by peripheral cells, and for hepatic uptake of end products of lipoprotein catabolism[111;112]. Lipoprotein remnant uptake by the liver, mediated by SR-B1 and hepatic lipase (HL), and dependent on lipoprotein type also by LDL-receptor and LDL-receptor-related protein (LRP), provides a feedback inhibition mechanism for cholesterol homeostasis by regulating activity of HMG-CoA reductase, the key enzyme in hepatic cholesterol synthesis. Hepatocyte damage due to toxic accumulation of bile acids in cholestasis may disrupt synthesis of apoproteins and other enzymes involved in lipoprotein formation and metabolism such as LCAT, CETP and PLTP, with concomitant derangements in plasma and hepatic lipid homeostasis which will be discussed below.

Lipoprotein X in cholestasis

Biliary excretion is the principal route for cholesterol disposal from the body (either direct or after conversion into bile acids), and cholestasis thoroughly deranges body sterol balance. The well-recognized increase in plasma free cholesterol observed in some forms of cholestasis can be accompanied by an equimolar elevation of plasma PL[113;114]. Hypercholesterolemia in (extrahepatic) cholestasis is accompanied by plasma appearance of the aberrant lipoprotein X (LpX)[115;116]. LpX is a 40-100 nm bilamellar vesicle with an aqueous lumen, predominantly composed of PL and free cholesterol in equimolar amounts and containing only 3% of TG and 2% of cholesteryl ester[117;118]. Gradient ultracentrifugation revealed that LpX is isolated in the LDL-fraction[119] and contains apoC and albumin. Manzato *et al*. hypothesized that LpX particles represent biliary vesicles regurgitated from liver into plasma of cholestatic subjects[114], since both LpX and bile vesicles are composed of PL and free cholesterol. The presence of apoC and albumin, and the observation that the cholesterol-PL ratio in LpX differs from that in bile, can be explained by plasma interactions of LpX with other lipoproteins. LpX is not readily taken up by the liver, thus LpX-cholesterol does not participate in feedback inhibition of hepatic cholesterol synthesis. This could contribute to the paradox of increased hepatic cholesterol neosynthesis in hypercholesterolemia during cholestasis. Felker observed LpX-like vesicles in bile canaliculi of bile duct-ligated rats, indicating a biliary origin of the particle[119-121]. Oude Elferink *et al*. demonstrated the biliary origin of LpX in mice, since in *Mdr2* $^{-/-}$ mice, which secrete PL-free bile, bile duct ligation was not associated with appearance of LpX[122].

HDL in cholestasis

Apart from increased lipid contents in the LDL fraction in the form of LpX, appearance of TG-rich LDL and decreased plasma VLDL concentrations, chronic cholestasis is

associated with strongly decreased plasma HDL concentrations (<10%)[123]. The underlying mechanism may involve either increased HDL clearance during cholestasis, or decreased HDL synthesis. Recent work indicates that bile salts, accumulating in hepatocytes during cholestasis, can suppress apoA-I gene transcription via a negative farnesoid X receptor (FXR) response element mapped to the C-site of the apoA-I promoter[124]. As HDL is partly derived from CM surface remnants, low plasma HDL levels could also result from decreased CM formation during intestinal bile deficiency, or from defective HDL formation from CM surface remnants by PLTP[125]. Upon ultracentrifugation, HDL isolated in cholestasis is in the density range of bilamellar discoidal particles, enriched in free cholesterol and PL with decreased apoA-I and apoA-II contents and increased apoE, resembling so-called 'nascent' HDL particles. Such particles are normally not found in plasma in considerable amounts because of rapid transformation by concerted actions of LCAT, CETP and PLTP.

Lipoprotein-metabolizing enzymes in cholestasis

LCAT: Lecithin cholesterol acyl transferase (LCAT) and hepatic lipase (HL) are key enzymes in lipoprotein metabolism. Both proteins are produced in the liver, but LCAT is active in the circulation at the HDL surface whereas HL resides at the hepatic endothelial cell lining. LCAT is a 60 kDa glycoprotein that converts cholesterol and PL into cholesteryl esters and lyso-PL, and is activated by apoA-I. Its cholesterol esterifying activity not only moves cholesterol from the HDL surface into the core, thereby promoting the flux of cholesterol from cell membranes into HDL, but it also leads to morphological changes of HDL particles. Nascent disc-shaped HDL becomes spherical as cholesteryl esters accumulate in the HDL core. HL and LCAT hydrolytic activities together account for over 80% of PL disappearance from plasma[126]. Impaired hepatic synthesis of these enzymes in cholestasis could thus contribute to increased plasma PL concentrations. Both plasma cholesteryl ester and LCAT concentrations are decreased in cholestatic subjects, and plasma appearance of 'nascent' discoidal HDL particles is considered a direct result of defective LCAT functioning. Furthermore, discoidal HDL has been associated with primary familial LCAT deficiency[113].

CETP: Cholesteryl ester transfer protein (CETP) transfers excess cholesteryl esters from HDL to VLDL and LDL in exchange for TG[127;128], thus participating in the so-called reverse cholesterol transport. CETP activity results in homogenous fatty acid species distribution between lipoprotein fractions. Activity of CETP is decreased 25% in cholestasis, associated with a decreased LA content of VLDL-TG and CE

compared to HDL[127]. Faust *et al*. demonstrated that fatty acid absorption regulates CETP secretion in CaCo-2 cells[129;130], and several animal and human studies[131-134] revealed that high-fat diets can increase CETP activity. Freeman *et al*. suggested that serum TG levels >1.4 mmol/l are required for significant CETP-mediated lipid exchange between LDL and VLDL[135]. The mechanism for decreased CETP activity in cholestatic subjects is not obvious because of the multiple origins of CETP synthesis (liver, intestine, adipose tissue, macrophages)[136;137]. However, since hepatocytes are the predominant source of CETP, impaired hepatic CETP synthesis remains a likely contributor to the decreased CETP activity in cholestasis[127].

PLTP: Plasma phospholipid transfer protein (PLTP) circulates bound to HDL and mediates transfer of PL from apoB-containing lipoproteins into HDL, thus modulating HDL size and lipid composition. PLTP activity generates pre-ß-HDL, the major acceptor of cholesterol in the reverse-cholesterol transport route. PLTP knockout mice have markedly reduced HDL levels due to defective transfer of PL from TG-rich lipoproteins into HDL[138]. Liver, adipocytes and lung are presumably the major sources of circulating PLTP. Impaired hepatic synthesis of PLTP in cholestatic conditions can markedly reduce circulating HDL levels, and due to the stimulatory effect of PLTP on CETP activity[139], it can further deteriorate the already impaired CETP function. Recently, PLTP was identified as an FXR target gene[140], providing a molecular basis for reduced PLTP gene expression under cholestatic conditions.

EFA metabolism in cholestasis

Socha *et al*. reported on decreased plasma arachidonic acid (AA) levels in pediatric cholestatic patients, which was attributed to impaired hepatic microsomal desaturase and/or elongase activity[141;142]. However, Minich *et al*. demonstrated that conversion of [^{13}C]-LA to [^{13}C]-AA was not significantly different in short-term bile duct-ligated rats compared to controls. Accordingly, delta-6-desaturase activity determined in hepatic microsomes was not altered[143]. These results are in agreement with observations of de Vriese *et al*., who found equal delta-9-, delta-6- and delta-3-desaturase activities in liver microsomes of cholestatic and non-cholestatic rats[144]. Decreased LA uptake by cholestatic subjects appears the predominant cause of low plasma AA levels, rather than impaired post-absorptive EFA metabolism. Cholestasis may also impair hepatic ß-oxidative capacity, which on one hand may preserve EFA from oxidation but on the other hand may inhibit the final step in forming C22:6n-3 and C22:5n-6. In rats with long-term bile duct ligation impaired hepatic ß-oxidative capacity has been reported[145].

Nutritional therapy in cholestasis

Chronic cholestasis is often accompanied by nutritional deficiencies due to inadequate dietary intake, maldigestion, malabsorption and/or defective metabolism of nutrients. Additionally, requirements of energy or specific nutrients may increase during cholestasis. The recommended caloric intake for chronic cholestatic patients is ~130% of RDI, usually accomplished by dietary supplementation with glucose polymers and/or MCT oil. For prevention or treatment of EFA deficiency, EFA-rich oils are frequently recommended, but the effects hereof have remained unsatisfactory[146;147]. The enteral route is preferred for dietary supplementations but in pediatric patients with severe chronic malabsorption, nasogastric and nocturnal feedings are often required[127]. For fat-soluble vitamin deficiency in cholestatic children, adequate and rapid correction is required. To treat vitamin D deficiency, a regimen of oral 25-OHD is recommended, at a dose of 2-4 μg/kg/d in children and 50-100 μg in adults, with regular measurements of cholecalciferol levels in plasma to prevent toxicity. Vitamin K supplements of 2.5-5 mg 2-7 times a week are currently recommended as prophylaxis for children with chronic cholestasis[60;61]. Most cholestatic children absorb the phylloquinone form of vitamin K to reach functionally adequate levels, if supplied in high dosages. In adults, vitamin K supplements are only recommended when blood tests suggest deficiency. For correction of vitamin E deficiency, oral forms of vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, alpha-tocopheryl succinate) are recommended at doses from 10-25 IU/kg/d up to 100-200 IU/kg/d. When normalization of plasma vitamin E levels is not reached, a water-soluble form of vitamin E called d-alpha-tocopheryl polyethylene glycol-1000 succinate (TPGS) can improve vitamin E status in cholestatic patients[148]. Argao *et al*. demonstrated that absorption of other fat-soluble vitamins is greatly enhanced by simultaneous administration with TPGS[149]. Recommended dosage of vitamin A in chronic cholestasis is 10000 IU if given with TPGS[148]. Irrespective of the form of vitamin supplementation that is chosen, plasma vitamin levels should be carefully monitored to avoid excessive serum levels and toxicity.

Conclusion

Cholestatic liver disease can disturb many aspects of lipid absorption and metabolism. Accumulation of potentially toxic bile components in hepatocytes due to disruption of the flux of bile from the liver to the intestine can damage hepatocytes, resulting in impaired synthetic function and decreased production of enzymes involved in lipoprotein metabolism. Also, lipoprotein secretion is disturbed during cholestasis, reflected by decreased HDL levels and appearance of the aberrant lipoprotein X in plasma. The absence of biliary components from the intestinal lumen

during cholestasis can strongly impair uptake of dietary fat and fat-soluble vitamins, resulting in nutritional deficiencies such as EFA deficiency and vitamin A, D, E and K deficiency. Current prolonged survival of patients with chronic cholestasis will require critical evaluation of nutrient deficiencies and adequate treatment strategies in order to prevent sequelae and to improve quality of life.

SCOPE OF THIS THESIS

Essential fatty acids and their long-chain polyunsaturated metabolites are crucial for normal function and development of human and animal cells. Low levels of essential fatty acids in the body are associated with dietary fat malabsorption, steatosis and impaired neurological development in infants. Pediatric conditions with lipid malabsorption (cholestasis, cystic fibrosis, prematurity) can be complicated by EFA deficiency. A deficiency of essential fatty acids is common in children with cholestatic liver disease, despite high intakes of dietary EFA. Dietary EFA are predominantly present in the form of triglycerides (TG), which are malabsorbed during cholestasis, whereas phospholipids (PL) are relatively well absorbed during bile deficiency, and have been postulated to have a high post-absorptive bioavailability.

This thesis focuses on the role of oral phospholipids (PL) as compared to triglycerides (TG) as vehicles for EFA supplementation under cholestatic conditions in experimental animals (mice) and pediatric patients with end-stage liver disease. We aim to clarify the role of EFA-rich phospholipids in intestinal and hepatic lipoprotein formation, and in the development of fat malabsorption and steatosis during EFA deficiency. Also, we aim to evaluate the role of fat malabsorption and EFA metabolism on the frequently observed alterations of EFA status in cystic fibrosis, a condition also often associated with EFA deficiency, by analyzing EFA concentrations and metabolism in different body compartments of mouse models for CF.

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