CHAPTER 1

General introduction
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1.1 Dietary lipids

On average, adult Western diets contain approximately 100 g of lipids per day, of which 92% to 96% are long-chain triacylglycerols [1,2]. Triacylglycerols (also referred to as triglycerides) are fatty acid triesters of glycerol (Figure 1.1). Triacylglycerols differ according to the identity and position of their three fatty acid residues. Most triacylglycerols in nature contain long-chain free fatty acids [1], although for example, triacylglycerols in human milk are mixtures containing both medium- and long-chain fatty acids [3]. Fatty acids in biological systems usually contain an even number of carbon atoms, typically between 14 and 24. The alkyl chain may either be saturated or it may contain one or more double bonds. The predominant fatty acid residues in nature are those of the C16 and C18 species palmitic, oleic, linoleic, and stearic acids (Figure 1.2) [4]. Fatty acids can be divided into three main classes according to their chain length: 1. short-chain fatty acids, less than 6 carbon atoms; 2. medium-chain fatty acids, from 6 to 12 carbon atoms; 3. long-chain fatty acids, 14 or more carbon atoms [5]. The properties of fatty acids are markedly dependent on their chain length and degree of saturation. Unsaturated fatty acids are more fluid than saturated fatty acids of the same length. By virtue of their smaller molecular size, medium-chain fatty acids are relatively soluble in water [6].
An adequate intake of dietary lipid is essential for life and well-being. Lipids serve several important functions in the human body. Firstly, they represent the major source of energy (9 kcal g\(^{-1}\)), double that of sugars and protein [4]. In the average Western diet, lipids provide approximately 40% of the caloric energy [1], which can be stored in the human body for more than several days, in contrast to carbohydrates and proteins. The lipid content of normal humans (21% for men, 26% for women) enables them to survive energy starvation for 2 to 3 months. Secondly, lipids are the major constituent of cell membranes in the form of phospholipids, sphingolipids and cholesterol. Furthermore, lipids are the only source of essential fatty acids, the precursors of eicosanoids such as prostaglandins, thromboxanes, and leucotrienes [7,8]. Finally, lipids are necessary for the solubilization and uptake of the fat-soluble vitamins A, D, E, and K.

Lipids are particularly needed during periods of growth and development. In light of the important physiological roles of lipids, an efficient high-capacity absorption mechanism is
required. Impaired lipid absorption has been associated with physical complications, such as diarrhea, retarded growth, and essential fatty acid deficiency. The aim of this thesis is to obtain mechanistic information on the various pathophysiological processes involved in fat malabsorption, with the purpose to increase diagnostic and eventually therapeutic possibilities in patients with fat malabsorption. The mechanisms by which lipids are taken up will be discussed in paragraph 1.2, and subsequently lipid malabsorption with special emphasis on the disease cystic fibrosis will be discussed in paragraph 1.3. The distinct methods to diagnose and quantify lipid malabsorption will be discussed in paragraph 1.4.

1.2 Intestinal absorption and digestion of dietary lipids

In order for intestinal lipid absorption to take place, lipids must undergo a number of physico-chemical changes to enable transport from the intestinal lumen to the plasma compartment. This is achieved by both mechanical and chemical means [5]. The overall process of intestinal lipid absorption and digestion can be classified as a chain of events, including:

1. Emulsification; the dispersion of bulk fat globules into finely divided emulsion particle.
2. Lipolysis; the enzymatic hydrolysis of fatty acid esters at the emulsion-water interface.
3. Micellar solubilization; the desorption and dispersion of insoluble lipid products into an absorbable form.
4. Membrane translocation; the transport of a lipid from the intestinal lumen across the membrane of the intestinal mucosa cell.
5. Intracellular events; this intracellular phase of lipid absorption involves re-esterification of fatty acids and monoacylglycerols into triacylglycerols, the packaging of the lipids into chylomicrons and secretion of these chylomicrons at the basolateral side of the enterocyte [1,2,5,9-13]. The overall process of lipid absorption is shown in Figure 1.3.

![Figure 1.3 Schematic of intestinal lipid absorption.](image-url)
**Emulsification**

Processing of lipids starts in the mouth with emulsification. The purpose of emulsification is to increase the surface area of the lipid droplets, thereby increasing the area on which the digestive enzymes can act effectively. Chewing breaks down large pieces of fat into smaller sizes. Following ingestion, food enters the stomach, the major site for emulsification of dietary lipids. Muscle contraction of the stomach - particularly peristalsis against a closed pylorus and the squirting of lipid through a partially opened pyloric canal - produces the shear forces sufficient for emulsification [1]. These peristaltic movements further grind the smaller pieces of lipid into a fine emulsion [14], which together with other emulsified foodstuffs is referred to as chyme. In addition to emulsifying food, the grinding action of the antrum mixes food with various digestive enzymes derived from the mouth and the stomach. Similarly, intestinal peristalsis continuously mixes luminal contents with digestive enzymes to ensure complete digestion [5].

**Lipolysis**

The main objective of lipolysis is to convert triacylglycerols, which are virtually insoluble in the aqueous phase of the gastrointestinal tract, into other forms of lipid with an increased ability to interact with water. Enzymatic hydrolysis of dietary triacylglycerols in humans beyond the breast feeding period is mainly catalyzed by preduodenal and pancreatic lipases, and therefore in this thesis only these enzymes will be discussed. Preduodenal lipase is secreted from different tissues depending on species [15-17] and has therefore been assigned different names, e.g. gastric lipase [18], pharyngeal lipase [19], and lingual lipase [20]. In humans the lipase is entirely a product of the chief cells of the gastric mucosa, and is therefore called gastric lipase [2]. Regardless of species or tissue origin, the preduodenal lipases share molecular and kinetic properties and it is assumed that they all have a common physiological function, i.e. to initiate triacylglycerol digestion in the stomach. Gastric lipase preferentially acts on the sn-3 position of the triacylglycerol molecule to release diacylglycerols and free fatty acids [21,22]. The level of hydrolysis in the stomach by gastric lipase in humans under physiological conditions accounts for approximately 10 to 30% of total lipid ingested [23].

The lipid emulsion enters the small intestine as fine lipid droplets less than 0.5 µm in diameter [1]. Pancreatic colipase-dependent lipase, secreted by pancreatic acinar cells, completes dietary lipid digestion in the proximal small intestine [24]. Pancreatic lipase acts mainly on the sn-1 and sn-3 positions of the triacylglycerol molecule to release 2-monooacylglycerol and free fatty acids [21,22]. Pancreatic lipase is one of the most studied and best characterized lipases, and is considered to be responsible for quantitative digestion of all triacylglycerols in the adult. In healthy human adults, the level of enzyme secreted into the intestinal lumen was calculated to be in 1000-fold excess of what would be required for hydrolysis of daily lipid intake [2]. Pancreatic lipase is clearly essential for efficient dietary lipid digestion as evidenced by the steatorrhoea present in patients with for example cystic fibrosis [25] or congenital pancreatic lipase deficiency [26,27].

The recent description of the primary and tertiary structures of pancreatic lipase has provided insight into the molecular detail of pancreatic lipase-catalyzed lipolysis [28,29]. Pancreatic lipase is an enzyme with a marked substrate preference for triacylglycerols.
Enzymatic hydrolysis of lipids can occur only on the surface of a lipid droplet, that is, at the interface between the lipid droplet and the surrounding aqueous solution. When an oil-water interface is encountered, pancreatic lipase activity increases markedly, a property termed interfacial activation [29]. Although pancreatic lipase is secreted into the duodenum along with bile salts, the enzyme is inhibited by physiological concentrations of bile salts and is dependent on another pancreatic protein, colipase, for activity in the presence of bile salts [30].

The action of both gastric and pancreatic lipase is facilitated for medium-chain triacylglycerols when compared with long-chain triacylglycerols due to their more expanded surface films in water [1]. Consequently, medium-chain triacylglycerols are hydrolyzed both faster and more completely than long-chain triacylglycerols [6]. In the case of mixed triacylglycerols the medium-chain fatty acids are liberated preferentially [6].

**Micellar solubilization**

Bile is secreted by the liver and enters the intestine through the biliary tract. One of the important properties of bile is its ability to increase the solubility of lipolytic products (i.e. 2-monoacylglycerols and free fatty acids) in the aqueous intestinal lumen by the formation of mixed micelles. Micelles are structures in which the polar group projects into the aqueous phase while the nonpolar hydrocarbon chain forms the center. This macromolecular structure has a high water solubility. Micellar solubilization increases the aqueous concentration of fatty acids and monoacylglycerols 100 to 1000 times [9].

Much of our current understanding on the uptake of dietary lipids was derived from the work of Hofmann and Borgström [31,32] and subsequent studies [33-35], who describe the importance of micellar solubilization of lipids in the uptake of lipid digestion products by enterocytes. To understand the importance of micellar solubilization, it is important to discuss the unstirred water layer, a concept introduced by Westergaard and Dietschy [36] (Figure 1.4). According to this concept, the brush border membrane of the enterocytes is separated from the bulk fluid phase in the intestinal lumen by an unstirred water layer, which is relatively impermeable for the lipolytic products, especially the long-chain fatty acids. The rate of long-chain fatty acid monomer diffusion in water is greater than that of aggregates of mixed micelles [12]. The increased concentration of fatty acids by micellar solubilization overcomes the slower diffusion rate, so that the net effect of micelle formation is an increase in the transfer of lipolytic products across the unstirred water layer [36]. Thus, mixed micelles would act as lipid shuttles to overcome the unstirred water layer [36].

The validity of this concept was later challenged by Carey and his associates, who discovered the coexistence of unilamellar liposomes with bile salt-lipid mixed micelles in the small intestine [37]. They proposed that when the bile salt concentration in the lumen exceeds the critical micellar concentration, the lipids in the intestinal lumen will be incorporated into mixed micelles [1]. When the amount of lipids in the aqueous phase increases further and the amount of bile salts does not increase, this eventually results in the formation of liquid crystalline vesicles (liposomes) [1]. However, so far the relative roles of the micelle and the liquid crystalline vesicle in the uptake of fatty acids and monoacylglycerols have not been resolved [9].
The lipolytic products of medium-chain triacylglycerols are absorbed faster than those of long-chain triacylglycerols. As lipolysis of medium-chain triacylglycerols is more complete than that of long-chain triacylglycerols, the medium-chain triacylglycerols (unlike long-chain triacylglycerols) are absorbed mainly as free fatty acids, and only rarely as mono- and diacylglycerols [6]. Because of the increased water solubility of medium-chain fatty acids, absorption of medium-chain fatty acids is not dependent on micellar solubilization [38-40]. Thus, for long-chain fatty acids passage across the unstirred water layer is rate limiting, whereas passage of medium-chain fatty acids is only limited by the brush border membrane [41].

Figure 1.4 Diagrammatic representation of the effect of bile salt micelles (or vesicles) in overcoming the diffusion barrier resistance offered by the unstirred water layer. In the absence of bile acids, individual lipid molecules must diffuse across the barriers overlying the microvillus border of the intestinal epithelial cells (arrow 1). Hence, uptake of these molecules is largely diffusion limited. In the presence of bile acids (arrow 2), large amounts of these lipid molecules are delivered directly to the aqueous-membrane interface so that the rate of uptake is greatly enhanced [9].

Translocation
The mechanism by which lipids are taken up by the enterocyte across its apical membrane remains unresolved. Previously, it has been accepted that the uptake of free fatty acids and monoacylglycerols by the enterocytes is a passive diffusion process [10,42]. Recently, the possibility has been raised that some lipids may be taken up by enterocytes by carrier-mediated processes [43-47]. It was shown that fatty acid binding proteins and/or fatty acid translocase, associated with the brush border membrane, seem to play a role in the uptake of fatty acids by enterocytes [43,47]. However, the exact role of the protein has not been resolved yet, and the issue of whether fatty acids are taken up by passive diffusion or by a carrier-mediated process needs further investigation.
Intracellular events
In the intestinal cell the various absorbed lipids migrate from the site of absorption to the endoplasmic reticulum. It has been suggested that the migration of the lipids is mediated via fatty-acid-binding proteins (FABP) located in the intestine (intestinal FABP and liver FABP) [48]. Re-esterification of free fatty acids and monoacylglycerols into triacylglycerols takes place at the cytoplasmic surface of the endoplasmic reticulum [49] mainly via the monoacylglycerol pathway [50,51]. This involves reacylation to diacylglycerols and triacylglycerols by monoacylglycerol-acyltransferase and diacylglycerol-acyltransferase, respectively [9]. The other route of triacylglycerol synthesis, the alpha-glycerophosphate pathway, involves conversion of glycerol-3-phosphate via phosphatidic acid to diacylglycerols and, subsequently, to triacylglycerols by various enzymes [9]. Under physiological circumstances, the monoacylglycerol pathway predominates relative to the alpha-glycerophosphate pathway [9].

Triacylglycerols are then transferred by a transfer protein to the inside of the endoplasmic reticulum [52] and packaged into lipoprotein particles called chylomicrons. Chylomicrons are made exclusively by the small intestine, and consist mainly of phospholipids, dietary triacylglycerols and apolipoproteins apo A-I, apo A-IV, and apo B-48 [9]. Data from both animals and humans indicate that the fatty acid composition of the triacylglycerol of chylomicrons closely resembles the dietary lipid fed [53,54]. The chylomicrons are released into the bloodstream via the lymph system for delivery of triacylglycerols to the tissues.

1.3 Fate of lipids in the colon
The digested nutrients that enter the colon encounter a large population of bacteria capable of a wide range of metabolic activities. For example, the colonic flora play a major part in the fermentation of carbohydrates to produce short-chain fatty acids. Although these short-chain fatty acids may play a role in the prevention of colonic inflammation, further discussion is beyond the scope of this thesis and the interested reader is referred to [55,56].

The small amounts of long-chain fatty acids escaping absorption and entering the large bowel have been regarded as of trivial biological significance. However, there is evidence that colonic bacteria can metabolize dietary fats: colonic bacteria secrete lipase enzymes [57], they have active transport mechanisms for medium- and long-chain fatty acids, and are capable of oxidation, desaturation and hydroxylation of fatty acids [58]. In the past several studies showed that the unabsorbed fraction of lipid may have important effects on bacterial metabolism of the colon [59,60], and may even play a role in the etiology of colonic cancer [61].

Obviously, the daily input of lipids into the colon increases considerably in the case of various lipid malabsorption syndromes. However, the role of large amounts of lipids in the colon has only been partially resolved and further research is necessary [58,62].
1.4 Lipid malabsorption

Fat malabsorption is characterized by increased fecal excretion of mostly dietary lipids. Increased fat content of the feces is also known as steatorrhoea, which may be a first symptom of underlying diseases affecting fat absorption. It has been convenient to divide fat malabsorption into those disorders with an impaired digestion of triacylglycerols from those disorders with impaired intestinal uptake, which includes impaired mixed micelle formation and translocation of fatty acids over the intestinal mucosa.

**Impaired lipolysis**

Under physiological conditions, pancreatic lipase is present in pancreatic juice in abundance. Its high concentration in pancreatic secretions and its high catalytic efficiency ensure the efficient digestion of dietary lipid. However, impaired lipolysis of dietary triacylglycerols, caused by a lack of sufficient pancreatic lipases, is a well-recognized cause of steatorrhoea. Pancreatic lipase deficiency can either be due to the (relative) absence of the enzymes involved or due to inactivity of these enzymes [63]. Steatorrhoea in lipase deficiency is usually not severe, unless lipase concentration in the upper intestinal tract is less than 10% of normal. Such impaired lipolysis may be secondary to cystic fibrosis, chronic pancreatitis, pancreatic resection, or pancreatic carcinoma [38,63].

The most effective treatment of lipase deficiency is to restore lipase activity. This is accomplished either by eliminating causes of lipase inactivation, such as correcting gastric acid hypersecretion, or to supply exogenous lipase [38,64].

**Impaired uptake of long-chain fatty acids**

Patients with a biliary fistula, biliary obstruction, chronic liver disease, or an interruption of the bile salt enterohepatic circulation by ileal resection or disease have a decreased bile salt secretion rate. With less bile salt present in the intestinal lumen, fewer mixed micelles form, impairing solubilization of ingested lipids. However, total absence of bile in the intestine does not completely inhibit fat absorption. Even up to 80% of dietary lipids were found to be absorbed in a study in adults with biliary fistula [33]. An explanation could be the observation of liquid crystalline vesicles by Carey et al. [1]. They suggested that when the amount of fat in the aqueous intestinal phase is high compared with the amount of bile, liquid crystalline vesicles are formed. These vesicles may play an important role in the uptake of fats by enterocytes in disease states [33]. The finding of liquid crystalline vesicles may have important pathophysiological implications. Because patients with low intraluminal bile salt concentrations or with bile fistulae can have reasonably good lipid absorption, it was proposed that the liquid crystalline vesicles may play an important role in the uptake of fatty acids and monoacylglycerols by enterocytes in these disease states [1]. In addition, in the absence or at low concentrations of bile salts, the absorption of fatty acids occurs to a relatively lower and slower extent [65]. Brand and Morgan [66] showed that fat absorption occurs largely from the proximal small intestine in control rats, whereas, in the absence of bile distal small intestine is also involved. Presumably, the absorptive reserve of the distal small intestine is called upon in
the case of bile diversion and much of the fat which failed to enter the proximal intestinal mucosa is absorbed more distally [67].

Therapy is directed toward either restoring the enterohepatic circulation of bile salts or by substituting medium-chain triacylglycerols in the diet [38]. Because of the increased water solubility of medium-chain fatty acids, bile salts are not as necessary for efficient absorption of medium-chain fatty acids.

**Cystic fibrosis**

A frequently encountered genetic disorder associated with fat malabsorption is cystic fibrosis [68,69]. The pathophysiology of fat malabsorption in cystic fibrosis patients involves both pancreatic insufficiency and deficient intestinal uptake of long chain fatty acids. Cystic fibrosis is an autosomal recessive disorder in which defective transepithelial chloride transport results in the production of mucus with increased viscosity in various organs. Among the organs commonly affected, the lungs and the pancreas frequently are involved in serious symptoms at young age [70]. The basic defect is the cystic fibrosis transmembrane regulator (CFTR), a protein responsible for chloride ion transport. Both pancreatic insufficiency and high energy expenditure due to increased respiratory work are thought to contribute to the frequently observed poor nutritional status of these patients [68,71]. The positive correlation between a good nutritional status and long-term survival or well-being of cystic fibrosis patients is well documented [72]. This observation has led to increased attention for optimization of nutrient intake and absorption in cystic fibrosis patients [68]. Recommendations for treatment of cystic fibrosis patients include consumption of 120-150% of the recommended daily allowance of energy for healthy individuals [73], with a normal to high lipid (40 energy %) intake to offset increased energy requirements [74].

Despite recent improvements in the pharmacokinetics of the supplementary pancreatic enzymes, many patients continue to experience a certain degree of steatorrhoea [75-77], 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 [64]. At a low duodenal pH, the release of the enzymes from the (micro)capsules is inhibited and the denaturation of the enzymes is stimulated [64,78]. However, it has been demonstrated that increasing the pancreatic enzyme dosages does not completely correct lipid malabsorption [79]. In addition, attempts to increase lipolysis by high-strength pancreatic enzyme supplements has led to the reported association with fibrosing colonopathy [80-82].

An alternative explanation for the continuing fat malabsorption in CF patients on pancreatic enzyme replacement therapy may involve inefficient intestinal uptake of fatty acids [75,83]. 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 [77,83-86]. Furthermore, small bowel mucosal dysfunction or alterations in the mucus layer may contribute to inefficient intestinal uptake of long chain fatty acids in CF patients [68,87].
Although it is known that the pathophysiology of fat malabsorption in cystic fibrosis patients involves both pancreatic insufficiency and deficient intestinal uptake of long chain fatty acids, the relative contribution of these two processes frequently remains unclear. Insight into the contribution of either of these processes would benefit cystic fibrosis patients, however, it is difficult to obtain mechanistic information in patients. In an attempt to further elucidate the pathophysiology of cystic fibrosis, several mouse models of cystic fibrosis were developed [88,89].

1.5 Methods to measure lipid malabsorption

The efficiency of intestinal lipid absorption in patients is routinely determined by means of a lipid balance, requiring detailed analysis of daily lipid intake and the complete recovery of feces for 72 h. However, in the case of lipid malabsorption, this method does not discriminate between the potential causes, such as impaired intestinal lipolysis or disturbed micellar solubilization of long-chain fatty acids. Since different therapies are selected for the different causes, it is important to know the etiology behind the fat malabsorption. In the development of novel diagnostic strategies, stable isotope techniques have been introduced. In this chapter the several aspects regarding the fecal fat balance will be discussed first. Thereafter, attention will be paid to stable isotope tests measuring impaired lipolysis and/or disturbed uptake of long-chain fatty acids.

Fecal fat balance

The conventional method by which lipid absorption is evaluated is the 3-day fecal fat balance. Estimation of a fat balance is carried out as follows: the patient is kept on a diet containing a known amount of lipid and dietary intake is recorded for a period of 3 days. The feces excreted during the same period is collected accurately, and lipid is determined quantitatively. Since its first description in 1949, the titrimetric procedure of Van de Kamer [90] has been used as a reference method for the measurement of lipid in the feces. The percentage of total dietary lipid absorption is calculated from the amount of lipid ingested and the amount of lipid excreted via the feces by the following equation:

\[
\text{Percentage of total fat absorption} = \frac{\text{Fat intake (g day}^{-1}) - \text{Fecal fat output (g day}^{-1})}{\text{Fat intake (g day}^{-1})} \times 100\%
\]

The 3-day fecal fat balance in Western adult humans shows that intake of major dietary lipids, principally triacylglycerols, constitutes approximately 100 g day\(^{-1}\). In addition, substantial amounts of endogenous lipids are delivered to the intestinal lumen from bile [91], desquamated cells [92], and dead bacteria [93,94]. Intestinal epithelial cells are being sloughed off into the lumen continuously [92] and it can be estimated to amount to about 450 g of cells per day of which 2 to 6 g are membrane lipids that are mostly digested and absorbed [1,91].

Although measurement of fecal lipid excretion during a standard lipid intake is generally considered to be the most accurate screening test for detecting lipid malabsorption,
the test is not widely used because of its poor acceptability by patient, physician, and clinical chemist. For the patient, the test involves the inconvenience of eating a defined diet and the mechanical and esthetic problems of collecting, storing, and transporting stools. For the physician, the test involves scientific uncertainty as to the completeness of the fecal collection and also may involve storage and transport problems. For the clinical chemist, the test involves the storage of bulky specimens and the unpleasant task of sample homogenization and sampling [95]. Finally, in the case of lipid malabsorption, the lipid balance method does not discriminate between the underlying mechanisms, such as impaired intestinal lipolysis or disturbed intestinal solubilization of long chain fatty acids. In order to investigate the underlying mechanisms, stable isotope techniques have been introduced, which will be discussed in the next paragraph.

Stable isotopes
The renaissance of interest in stable isotopes in the last ten years is based upon the development of new instrumentation, such as the availability of the quadruple mass spectrometers interfaced with the gas chromatograph (GC/MS) and the development of isotope ratio mass spectrometers (IRMS), which made possible the convenient use of selective ion monitoring for the quantification of isotope enrichment. An increased awareness of the health hazards of radioactivity, as well as greater availability of stable isotopes, also stimulated the use of stable isotopes.

The most obvious advantage of stable isotopes is that they are nonradioactive and present little or no risk to human subjects and they are even suitable for the study of infants, children, and pregnant women [96]. Carbon 13 is a naturally occurring isotope present to the extent of approximately 1.1% of the major isotopic species, carbon 12 [97]. Since carbon 13 naturally contributes 1.1% of the carbon pool, and since it has not been possible to demonstrate more than trivial in vitro isotopic effects on chemical reactions with carbon 13-labeled substrates [98,99], significant side effects in vivo are not expected from administration of tracer doses of carbon 13.

Among the numerous applications of stable isotopes in physiology and medicine, the investigation of lipid absorption and metabolism poses considerable challenges because of the complexity of the subject, the multitude of influencing factors and the demanding analytical requirements [100]. Various labeled fatty acids and labeled triacylglycerols are available and can be given orally. When a $^{13}$C-labeled fat is ingested, the substrate may be digested, absorbed and enters metabolic pathways leading to enrichment of bicarbonate, protein, lipid and carbohydrate within the body. Unabsorbed amounts of the $^{13}$C-labeled fat are excreted via the feces. After absorption, the $^{13}$C-labeled fat enters the oxidative pathways and is excreted as $^{13}$CO$_2$ via the breath.

When stable isotopes are used to measure fat digestion and absorption, between ingestion of the labeled fat and appearance of $^{13}$C in plasma and excretion of $^{13}$CO$_2$ in the breath, many factors can influence the outcome of the test and expression of the results, such as gastric emptying rate, absorption rate, hepatic clearance etc. [101]. Choice of substrate and choice of sampling compartment are the first factors in determining the sensitivity and
specificity of the test. The rate-limiting step of interest in the handling of substrates by the body determines the selection of a substrate.

\textbf{\textsuperscript{13}}C-\textit{TRIOLEIN}
Since triolein is a long-chain triacylglycerol, its efficient absorption depends upon the overall process of fat absorption, thus, adequacy of lipolysis, bile salt solubilization and intact mucosal surface. Hence, this substrate is a sensitive indicator of steatorrhoea, but will not distinguish between the underlying mechanisms [102-104]. It has been proposed that the triolein test is preferred when compared to the trioctanoin or the tripalmitin test for the screening of total lipid malabsorption arising from a broad spectrum of gastrointestinal disorders because of its higher sensitivity and specificity [95,105,106]. However, it has been shown that the test has not the ability to predict the severity of malabsorption [107]. So far \textsuperscript{13}C-triolein has only been used with collection of breath and analysis of \textsuperscript{13}CO\textsubscript{2}, but its radioactive form, \textsuperscript{14}C-triolein, has also been used with measurements of postprandial serum [108,109].

\textbf{\textsuperscript{13}}C-\textit{HIOLEIN}
Naturally occurring hiolein provides a new tracer for lipid absorption studies. Hiolein is a long chain triacylglycerols mixture obtained from algae which is uniformly labeled with \textsuperscript{13}C and enriched by 98%. The major fatty acid composition of hiolein is oleic acid (51%), palmitic acid (17%) and linoleic acid (20%) [110]. Since hiolein consists mainly of triacylglycerols, efficient absorption depends on the same processes as triolein, i.e. lipolysis, bile salts solubilization and intact mucosa. Patients with significantly impaired lipolysis, bile salt deficiency, or mucosal disorders, excrete the substance in their stool, and have decreased amounts of \textsuperscript{13}CO\textsubscript{2} in their breath [110-113].

\textbf{\textsuperscript{13}}C-\textit{TRIOCTANOIN}
Trioctanoin is a medium-chain triacylglycerol. Although both medium and long-chain triacylglycerols require lipolysis by gastric and pancreatic lipase, medium-chain triacylglycerols, being water soluble, do not depend critically on the presence of bile salts for their digestion and absorption. The \textsuperscript{13}C-trioctanoin test thus focuses on lipase activity and a reduction in trioctanoin absorption reflects the level of lipolytic activity present in the patients digestive tract. Digestion and absorption of trioctanoin have been assessed by \textsuperscript{13}CO\textsubscript{2} excretion via the breath [105,114-119]. The choice of a medium-chain triglyceride has an additional advantage in that the lipolytic products are rapidly absorbed and oxidized [120], thus shortening the overall study period [114]. The \textsuperscript{13}C-trioctanoin test distinguishes pancreatic from non-pancreatic causes of steatorrhoea, and it has been applied for measurements of lipid maldigestion in adults [119] and in children with cystic fibrosis [115,116], and for measurements of lipid utilization in preterm and full-term neonates [116,121]. A disadvantage of the \textsuperscript{13}C-trioctanoin breath test is that the rate of lipolysis is facilitated for medium-chain triacylglycerols when compared with long-chain triacylglycerols [1,6]. Hence, the test does not exactly reflect lipolytic rate of dietary fats, because they mainly consist of long-chain triacylglycerols.
**13C-MIXED TRIGLYCERIDE**

A substrate that has the advantages of the 13C-trioctanoin breath test (short study period) and avoids the disadvantages (facilitated lipolysis for medium-chain triacylglycerols) is the so-called 13C-mixed triglyceride breath test [122,123]. The mixed triglyceride used is 1,3-distearoyl, 2[carboxyl-13C]octanoyl glycerol. This molecule contains a 13C-labeled medium chain fatty acid (octanoic acid) at the sn-2 position, and long-chain fatty acids (stearic acid) at the sn-1 and sn-3 positions of the glycerol backbone of the triacylglycerol [123]. The two stearoyl chains have to be hydrolyzed by lipolytic enzymes in the intestine before 13C-octanoate can be absorbed, thereby avoiding the disadvantage of the 13C-octanoin substrate which contains only medium-chain fatty acids. Thus, the principle of the mixed triglyceride breath test is based on lipolysis-dependent 13CO2 excretion via the breath. The applicability of the mixed triglyceride breath test has been demonstrated in healthy adults [124,125], pancreatic insufficiency patients [123,126], and preliminary data on the potential applicability in children are available [127]. The mixed triglyceride breath test has a sensitivity of 89% and a specificity of 81% compared with direct measures of lipase in patients with pancreatic and non-pancreatic causes of steatorrhoea [123].

**CHOLESTERYL-[1-13C]OCTANOATE**

The utilization of cholesteryl-[1-13C]octanoate is another attractive substrate for measuring pancreatic exocrine insufficiency. This substrate differs little from the cholesteryl esters naturally present in food. It undergoes hydrolysis by pancreatic cholesteryl esterase, and the labeled octanoate molecule is rapidly absorbed and oxidized [114,120]. Because cholesteryl octanoate is not hydrolyzed by gastric lipase it may be used to assess pancreatic exocrine function alone [128]. However, pancreatic carboxyl ester lipase activity requires the presence of bile salts and therefore, this test will not only measure pancreatic function but also solubilization by bile [128]. The test has been successfully used to diagnose exocrine pancreatic insufficiency [129] and to monitor pancreatic enzyme replacement therapy in patients with pancreatic insufficiency [128,130,131].

**[1-13C]PALMITIC ACID**

Efficient absorption of long-chain fatty acids, e.g. palmitic acid, is not dependent on lipolysis since free fatty acids are already hydrolyzed substrates. Thus, application of this test to patients with gastrointestinal complaints would identify individuals with inadequate solubilization of long chain fatty acids or intestinal mucosa disease [105,132,133]. It is difficult to discriminate between the two processes, which may be due to the fact that impaired solubilization is rarely an isolated event [105]. The test has been performed in healthy controls [134,135] and in patients with gastrointestinal diseases [105,136]. The advantage of using palmitic acid as a substrate instead of other fatty acids is that palmitic acid is a saturated fatty acid and both solubilization and translocation across the intestinal mucosa of saturated fatty acids are more difficult when compared to unsaturated fatty acids. In addition, palmitic acid is the most predominant fatty acid in the Western diet, and therefore experiments mimic dietary fat absorption as much as possible.
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[U-\textsuperscript{13}C]LINOLEIC ACID

[U-\textsuperscript{13}C]linoleic acid is a long-chain fatty acid and, thus depends on the same absorptive processes as [1-\textsuperscript{13}C]palmitic acid does, i.e. adequate solubilization by bile components and intact mucosa of the intestine. In addition, linoleic acid is an essential fatty acid and therefore may be used for studies with respect to the essential fatty acid status.

1.6 Scope of this thesis

As discussed before, the process of lipid absorption can be viewed as a chain of events occurring after lipid ingestion, including emulgation, lipolysis, solubilization, uptake in the enterocyte, and chylomicron assembly. Under physiological conditions, the efficacy of lipid absorption ranges from 96 to 98\% [1,2]. Until now, most attention has been paid towards the efficacy of the overall process of lipid absorption, yet, insight into the individual mechanisms causing fat malabsorption has remained rather incomplete. A detailed insight into the underlying mechanisms would enable not only improvements in diagnostic methodologies, but also treatment in individual patients by modulating diet therapy, pancreatic enzyme replacement therapy and supplementation of antacids and/or bile salts. Thereby, it is a reasonable expectation that the prognosis of (pediatric) patients with impaired lipid absorption can be improved, given the positive correlation between a good nutritional status and long-term survival or well-being [72-74].

The aim of the thesis is to obtain mechanistic information on the various processes involved in fat malabsorption, with the purpose to increase diagnostic and eventually therapeutic possibilities in patients with fat malabsorption. The approach to achieve this aim involves studies in experimental animals, in human volunteers and in patients. The studies were chosen to investigate in detail the two most frequently occurring pathophysiological processes involved in human fat malabsorption, namely, impaired lipolysis and disordered bile formation, as well as the most frequently encountered disease in children associated with fat malabsorption, cystic fibrosis. Since our purpose was to increase diagnostic and eventually therapeutic possibilities in patients, we applied stable isotopes in our experiments, allowing physiological studies in humans in a non-harmful way. The applicability of stable isotope labeled lipids for quantitative studies on lipid absorption has only been investigated to a very limited extent.

A non-invasive test that has been described to characterize pancreatic insufficiency in a functional way is the \textsuperscript{13}C-MTG breath test [123]. However, widely variable results have been obtained in children, healthy adults, and in cystic fibrosis patients with and without pancreatic enzyme replacement therapy [125,126]. The origin of this variability has not been elucidated. In fact, a quantitative relationship between the extent of fat malabsorption due to impaired lipolysis and the corresponding result of the \textsuperscript{13}C-MTG breath test has never been demonstrated in humans or in defined animal models. Therefore, in this thesis the efficiency and repeatability of the \textsuperscript{13}C-MTG breath test were investigated in rats treated with the lipase inhibitor orlistat (chapter 2) and in healthy adults (chapter 3), respectively.
Chapter 1

Few attempts to develop a specific test for the detection of impaired intestinal uptake of long chain fatty acids have been reported [105]. Intestinal uptake involves solubilization of lipolytic products by the formation of mixed micelles composed of bile components and lipolytic products, followed by the translocation of the lipolytic products across the intestinal epithelium [2,9,34,35]. Potential substrates for the detection of impaired intestinal uptake are $^{13}$C-labeled long chain fatty acids. In this thesis, the potency of $^{13}$C-labeled palmitic acid to detect impaired intestinal uptake was determined in rats with long-term diversion of the biliary tract (chapter 4). In addition, the sensitivity of the $^{13}$C-labeled palmitic acid test was investigated in healthy adults supplemented with calcium in order to achieve mild fat malabsorption due to decreased amounts of bile in the intestine (chapter 5).

A relatively frequently encountered disorder in Caucasian populations associated with fat malabsorption is cystic fibrosis. Although it is known that the pathophysiology of fat malabsorption in cystic fibrosis patients involves both pancreatic insufficiency [68,69] and deficient intestinal uptake of long chain fatty acids [75,83], the relative contribution of these two processes frequently remains unclear. In order to obtain more insight into the impaired processes of fat malabsorption in cystic fibrosis we performed a study in pediatric cystic fibrosis patients treated with their usual pancreatic enzyme replacement therapy (chapter 6). The substrates $^{13}$C-MTG and uniformly labeled $^{13}$C-linoleic acid were both applied to determine whether the rate-limiting step behind their remaining fat malabsorption was either impaired lipolysis or impaired intestinal uptake of long chain fatty acids, respectively. Based on the results of this study, we further explored the mechanisms involved in deficient intestinal uptake of long chain fatty acids in further detail in two recently generated cystic fibrosis mouse models (chapter 7). 1. Mice with the ΔF508 mutation in the $cfr$ gene, ΔF508/ΔF508 mice. 2. Mice with complete inactivation of the $cfr$ gene, $cfr$ -/- mice [137,138].

References


General introduction


