UNIVERSITY OF SOUTHAMPTON

DIGESTION, ABSORPTION AND METABOLIC DISPOSAL OF DIETARY LIPID IN CYSTIC FIBROSIS PATIENTS AND CONTROL SUBJECTS

BY

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ABSTRACT

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An impaired functional capacity of the gastrointestinal tract to digest and absorb dietary lipid in cystic fibrosis may contribute to energy and nutrient deficiencies sufficient to limit growth and cause weight loss. Patients commonly have excessive stool lipid losses despite current management regimens including pancreatic enzyme replacement therapy. The factors that determine lipid digestion and absorption in cystic fibrosis are poorly understood and present methods of assessing lipid handling by balance studies fail to differentiate between maldigestion and malabsorption. Little is known about metabolism of absorbed lipid in cystic fibrosis and in the absence of direct measurements is generally assumed not to differ from that of healthy individuals. The aims of this thesis were 1) to develop and evaluate a method which may be used to differentiate between maldigestion and malabsorption of dietary lipid, 2) to determine amount and type of stool lipid losses and 3) to investigate metabolic disposal of absorbed lipid in patients compared to controls.

A combination of novel stable isotope technology and traditional techniques was used to study gastrointestinal handling and metabolic disposal of dietary lipid. Cystic fibrosis patients with gastrostomies (9 females, 3 males, aged 7.0 to 30.9 years) and controls subjects (5 females, 3 males, aged 7.8 to 16.5 years) received [1,1,1-^{13}C]tripalmitin (10 mg/kg body weight) as an emulsion drink with a standardised breakfast. Maldigestion and malabsorption of dietary lipid were determined as label losses in stools and stool lipid fractions whilst metabolic disposal was determined as label excretion on breath over the 10 hour postprandial period. Patients omitted enzyme preparations with the labelled meal to investigate the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid.

Total stool losses of ^{13}C-label expressed as a percentage of administered dose were elevated in patients (median 51.2%, range 6.8 to 77.9%) compared to controls (median 1.9%, range 0 to 10.9%, $P<0.001$). The ^{13}C-label excretion in triacylglycerol fraction (index of maldigestion) of 5 patients and the ^{13}C-label excretion in fatty acid fraction (index of malabsorption) in 11 patients exceeded the upper limit of the 95% confidence interval for total label excretion in stools in controls. No difference was found in postprandial net lipid or carbohydrate oxidation, assessed by indirect calorimetry, between the groups but the excretion of ^{13}C-label on breath corrected for stool losses of the label was lower in patients (median 5.6%, range 0 to 15.9%) compared to controls (median 22.6%, range 17.5 to 33.7%, $P<0.001$).

The results highlight differences between patients in the extent to which they may digest and absorb dietary lipid and the need to target the management according to causes of stool lipid losses. Whilst malabsorption was indicated in most patients, fewer patients appeared to have evidence of maldigestion. Thus, adjusting enzyme therapy has no advantages in patients who malabsorb dietary lipid and management should be focused on resolving malabsorption. The lower excretion of label on breath suggested that metabolism of absorbed lipid was altered in these patients and was due to a reduced oxidation of dietary lipid over the postprandial period. This altered metabolism may impact upon subsequent growth and body composition of patients and has implications for the use of breath tests in defining the capacity of gastrointestinal tract to digest and absorb dietary lipid. A model for the assessment and management of patients with weight loss or disturbed growth was proposed.

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Publications

Unless otherwise indicated by acknowledgements or references to published literature, the work contained in this thesis is the work of the author. The findings presented in this thesis have been reported, in part, in the following publications:

Laiho K., Gavin J., Murphy J., Connett G. & Wootton S. (1999). Stool lipid in CF: maldigestion or malabsorption? *Ped. Pulm.* (Suppl 19): 302.(Abs).

Laiho K.M., Gavin J., Murphy J.L., Connett G.J., Wootton S.A. Altered metabolism of ^{13}C-labelled tripalmitin in cystic fibrosis patients compared to controls. *Proc. Nutr. Soc.* (Abs, in the press).

Laiho K., Gavin J., Murphy J., Connett G. & Wootton S. (1999). Metabolism of absorbed fat is altered in cystic fibrosis. *Neth. J. Med.* **54**(Suppl): 63. (Abs).

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Abbreviations and terminology

BMR	Basal metabolic rate
C14:0	Tetradecanoic acid (common name: myristic acid)
C16:0	Hexadecanoic acid (common name: palmitic acid)
C18:0	Octadecanoic acid (common name: stearic acid)
C18:1	Octadecenoic acid (common name: oleic acid)
C18:2	Octadecadienoic acid (common name: linoleic acid)
CF-IRMS	Continuous flow isotope ratio mass spectrometry
CFTR	Cystic fibrosis transmembrane conductance regulator
CO_2	Carbon dioxide
DAG	Diacylglycerol
Tripalmitin	1,2,3-trihexadecanoylglycerol
FA	Fatty acid
FAME	Fatty acid methyl ester
FEV_1	Forced expiratory volume in 1 s
GC	Gas chromatography
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
GSL	Gas solid liquid interface
HCl	Hydrochloric acid
HDL	High-density lipoprotein
H_2SO_4	Sulphuric acid
HSL	Hormone sensitive lipase
Internal standard	Tricosanoic acid methyl ester added to samples prior to GC-C-IRMS analysis
IRMS	Isotope ratio mass spectrometry
LDL	Low-density lipoprotein
LBM	Lean body mass
LPL	Lipoprotein lipase
MAG	Monoacylglycerol

NEFA	Non-esterified fatty acid
PDB	Pee Dee Belemnite
PERT	Pancreatic enzyme replacement therapy
RER	Respiratory exchange ratio
Surrogate FA standard	Fatty acid standard, heneicosanoic acid, added in stools prior to lipid extraction
Surrogate MAG standard	Monoacylglycerol standard, 1-mono[cis-13-docosenoyl]-rac-glycerol (common name: monoerucin (C22:1, [cis]-13), added in stools prior to lipid extraction
Surrogate TAG standard	Triacylglycerol standard, 1,2,3-triheptadecanoylglycerol (common name: triheptadecanoin), added in stools prior to lipid extraction
TAG	Triacylglycerol
TLC	Thin layer chromatography
VLDL	Very low-density lipoprotein

Chapter 1: Background to the research

The metabolic demand for energy to support basic body functions, physical activity and growth requires a dietary source of lipid and other nutrients. Dietary lipid, which consists mainly of triacylglycerols (TAG), is assimilated from the gastrointestinal tract through processes of digestion and absorption [Senior 1964]. The absorbed lipids are partitioned to body tissues and organs where they are retained or oxidised to provide energy. The capacity of the gastrointestinal tract to digest and absorb dietary lipid in healthy individuals is generally good, on average more than 95% of the lipid intake being absorbed, as shown previously in balance studies and stable isotope tracer studies [Wollaeger *et al.* 1947; Murphy *et al.* 1998]. The almost complete digestion and absorption of dietary lipid in healthy individuals is achieved by a sufficiently functioning gastrointestinal tract, which provides the necessary digestive enzymes and bile salts, appropriate pH and mucosal properties for the digestion and absorption of dietary lipid. The function of the gastrointestinal tract is co-ordinated in such a way that energy and nutrients are provided to satisfy the metabolic demand generated by the vital body functions, physical activity and growth in children. Digestion and absorption of dietary lipid requires a sequence of effectively functioning physiological processes, which are relatively well described in the literature. However, the metabolic regulation and the factors that control or limit lipid digestion and absorption are poorly understood. Patients with gastrointestinal disorders may present with an altered functional capacity of the gastrointestinal tract to digest and absorb dietary lipid or with disorders of the subsequent metabolism of the absorbed lipid. This may cause problems with growth and the maintenance of a good nutritional status. An understanding of the mechanisms determining the functional capacity of the gastrointestinal tract to digest and absorb dietary lipid and the subsequent partitioning of the absorbed lipid is required to the management of such diseases and to provide the conditions for growth.

An altered functional capacity of the gastrointestinal tract to digest and absorb dietary lipid is manifested as excessive stool lipid losses compared to those in healthy individuals. The term used in the clinical practice is steatorrhoea, which refers to excessive stool lipid losses but does not take into account whether the lipid losses are due to maldigestion and/or malabsorption. The terminology used to describe mechanisms of stool lipid losses in the clinical practice and in the literature requires clarification. It is inappropriate to use steatorrhoea, malabsorption and maldigestion inter-changeably, because the physiological

causes underlying digestion and absorption are different. In this thesis a clear differentiation between these terms is applied. The term maldigestion is used to describe reduced hydrolysis of lipid, which results in excess stool lipid losses due to the presentation of lipid in a form that cannot be absorbed by the intestinal mucosa. The term malabsorption is used to describe lipid that has been digested but has failed to be absorbed due to a failure in the absorptive process itself. When the expression total lipid excretion in stools is used, no differentiation is made between stool lipid due to maldigestion and/or malabsorption. Excessive lipid losses in stools refer to the excretion of lipid in stools in higher amount than that observed in healthy individuals.

Patients with cystic fibrosis commonly have excessive lipid losses in stools, which may be due to several factors such as pancreatic exocrine insufficiency, altered liver metabolism, especially that of bile salts, altered luminal environment or properties of the gastrointestinal mucosa [Kopelman 1991]. Pancreatic insufficiency is thought to be the main cause of excessive lipid excretion in stools and most attention has therefore been focused on the digestive capacity of the gastrointestinal tract. Excessive lipid losses in stools in cystic fibrosis have been clinically managed by pancreatic enzyme replacement therapy (PERT) and recent advances in PERT, such as the development of acid resistant microsphere preparations have reduced the occurrence and severity of lipid losses in stools. However, the development of PERT has led to a general belief that gastrointestinal dysfunction in cystic fibrosis is manageable by the PERT and the development of other treatments to manage stool lipid losses have been of secondary interest. Patients often regulate the dose of enzyme preparations ingested according to the gastrointestinal symptoms and the appearance of their stools. In some cases this has resulted in increased doses of enzyme preparations ingested by the patients. However, high-dose, high-strength enzyme preparations have been associated with fibrosing colonopathy [Smyth *et al.* 1994]. Concern over possible adverse effects of PERT led to the recommendation of the maximum daily enzyme intake. However, despite PERT excessive lipid losses in stools remain a problem in some patients and clearly PERT is not sufficient to correct stool lipid losses in all cystic fibrosis patients. Furthermore, attempts to improve clinical management of cystic fibrosis patients have focused on respiratory function, instead of investigating the causes of continuing excessive lipid losses in stools and the failure of PERT to correct the excessive losses in some patients. Without a better understanding of the extent to which dietary lipid is maldigested and/or malabsorbed in cystic fibrosis and which factors influence the

digestion and absorption of lipids, the development of alternative approaches for the management of excessive lipid losses in stools will not be achievable.

Excessive lipid losses in stools may contribute to energy deficit and nutrient deficiencies, which may be sufficient to limit growth and cause weight loss in cystic fibrosis [Wootton *et al.* 1991]. Furthermore, poor nutritional status may be related to a decreased pulmonary function [Kraemer *et al.* 1978] and may affect the overall survival of the patients. Therefore, the nutritional and gastrointestinal problems of patients need to be considered in the clinical management of the patients. In the Wessex Region, 140 children and 90 adults are currently managed for cystic fibrosis. All children receive dietetic advice at 2 month intervals and all adults at 6 month intervals to support or to increase dietary intake. A third of the patients at any given time, receive dietary supplements and 10% receive overnight supplementary feeding via a nasogastric tube or a gastrostomy. Most patients, 90%, receive PERT. Patients who already manifest or are most at risk of developing poor nutritional status and growth are those patients who are receiving dietary supplements or overnight supplementary feeding or PERT and have a history of nutritional problems, mainly inadequate dietary intake or gastrointestinal symptoms.

The extent to which dietary lipid is digested and absorbed in cystic fibrosis patients and the proportional contribution of factors affecting these two processes are poorly understood. This is principally due to the lack of a specific and clinically applicable methods for the investigation of the functional capacity of the gastrointestinal tract to digest and absorb dietary lipid. The best approaches so far have been balance studies, where the availability of dietary lipid is assessed by determining dietary lipid intake and stool lipid losses. However, balance studies are not capable of determining the origin of lipid in stools, which may be derived from either dietary residue or endogenous losses and bacteria [Gompertz & Sammons 1963]. Balance studies do not differentiate between lipid maldigestion and malabsorption either. The ability to distinguish between and quantify the separate processes of maldigestion and malabsorption would be of benefit for the treatment of excessive lipid losses in stools and would enable development of new therapies. It is of importance to manage the cause of the excessive lipid losses in stools, whether due to maldigestion and/or malabsorption.

Equally important to the gastrointestinal handling of dietary lipid is the metabolism of lipid after absorption. The way lipid is partitioned after absorption influences whether lipid is stored or oxidised for immediate energy production. In cystic fibrosis very little is known

about the metabolism of the absorbed lipid and it has generally been assumed not to differ from that of healthy individuals. However, it has been suggested that basal metabolic rate (BMR) is elevated in cystic fibrosis patients compared to healthy individuals [Vaisman *et al.* 1987; Buchdahl *et al.* 1988] and that carbohydrates contribute a greater proportion to the BMR in cystic fibrosis patients compared to healthy individuals [Bowler *et al.* 1993]. Nevertheless, postprandial substrate metabolism and factors that influence substrate utilisation in cystic fibrosis is poorly understood.

To better manage cystic fibrosis patients in the clinical practice, further research is required to increase the understanding of the factors that determine the functional capacity of the gastrointestinal tract to digest and absorb dietary lipid and factors that affect the metabolism of the absorbed lipid. Initially it is required to characterise the dietary lipid losses, especially the extent to which lipid is maldigested and/or malabsorbed in cystic fibrosis patients and to investigate whether the postprandial lipid metabolism in cystic fibrosis patients differs from that in healthy individuals. Therefore, the objectives of this thesis were to determine the amount and type of stool lipid losses and to investigate the metabolic disposal of the absorbed dietary lipid in cystic fibrosis patients compared to control subjects. Patients who receive supplementary feeding are most likely to have problems with the gastrointestinal handling and metabolic disposal of dietary lipid and to be at risk of developing poor growth or nutritional status. This patient group has therefore been selected for investigation in this thesis. The findings of these investigations would make it possible to develop a conceptual framework, which is required to improve the nutritional management of cystic fibrosis patients and other patients with gastrointestinal dysfunction. The hypothesis of this thesis is that poor nutritional status or disturbed growth in cystic fibrosis patients is associated with two metabolic disturbances: 1) increased stool lipid losses due to malabsorption of dietary lipid in addition to maldigestion and 2) altered metabolism of absorbed dietary lipid. The investigation of the hypothesis required the development and evaluation of a method which could be used to differentiate between maldigestion and malabsorption of dietary lipid. A combination of novel stable isotope technology and traditional methods for assessing stool lipid losses and indirect calorimetry measurements was applied to investigate the digestion, absorption and metabolic disposal of dietary lipid in cystic fibrosis patients and control subjects.

The thesis is presented in eight sections. Following a brief introduction to the research (Chapter 1), the present knowledge of the gastrointestinal handling and metabolism of absorbed lipid in health and in cystic fibrosis are summarised in the literature review and the hypothesis is defined in Chapter 2. Development and evaluation of the methods to differentiate between maldigestion and malabsorption of dietary lipid are described in Chapter 3. The developed methods were used to investigate digestion, absorption and metabolic disposal of [1,1,1-^{13}C] tripalmitin in cystic fibrosis patients receiving supplementary feeding via a gastrostomy and in a group of healthy control subjects. The general methods are described in Chapter 4, the study groups including anthropometry, BMR and dietary intake of the subjects in Chapter 5, the results of stool lipid losses in Chapter 6 and results of metabolic disposal of dietary lipid in Chapter 7. Finally the results and their implications for the clinical management of cystic fibrosis are discussed in Chapter 8.

Chapter 2: Review of literature

2.1 Introduction

The current understanding of digestion, absorption and metabolic disposal of dietary lipid firstly in health and secondly in cystic fibrosis are discussed in the review of literature. In the first part of the review the dietary intake of lipid in the UK (See 2.2) and the gastrointestinal handling of dietary lipid are summarised (See 2.3). The current practices applied for the assessment of the gastrointestinal handling of dietary lipid both using traditional balance techniques and pancreatic function tests and novel stable isotope techniques are presented (See 2.4). Finally the metabolism of absorbed lipid, especially its transport, storage and oxidation are summarised (See 2.5). Lipid metabolism may be altered in cystic fibrosis due to various factors related to the characteristics of the disease and the nutritional status of the patients and therefore offers an opportunity to investigate the consequences of the altered lipid metabolism. Factors affecting the gastrointestinal handling of lipids and energy metabolism in cystic fibrosis are discussed in section 2.6. In the last section the literature review is summarised and the hypothesis is defined (See 2.7).

2.2 Structure and properties of dietary lipid

Lipids are a heterogeneous group of compounds including fatty acids (FA), triacylglycerols (TAG), phospholipids, steroids and related compounds, which have a common property of being insoluble in water but soluble in non-polar solvents such as chloroform or alcohols [Murray *et al.* 1996]. Lipids have several structural and physiological functions in the human body. Lipids assimilated from the diet serve as a direct energy source needed to satisfy the metabolic demand arising from maintenance of vital body functions, mechanical work and growth in children [Swinburn & Ravussin 1994]. Lipids also provide an indirect energy source when mobilised from the adipose tissue and are structural components as thermal insulators in the subcutaneous tissues and around organs, electrical insulators in myelinated nerves and also cell membrane constituents [Murray *et al.* 1996].

2.2.1 Type of dietary lipid

Lipids in animal tissue and in plants are stored mainly as TAG and therefore the main component of dietary lipid is TAG constituting about 70 to 100g of the daily lipid intake in the UK (Table 2.1). Other dietary lipids such as phospholipids constitute only 2 to 3 g and cholesterol constitute less than 1 g of the dietary lipid intake [Gregory *et al.* 1990]. Also lipid soluble vitamins A, D and E and carotenoids are chemically classified as dietary lipid. The main focus of this thesis is in the metabolism of dietary TAG.

Triacylglycerols are esters of alcohol glycerol and fatty acids [IUPAC-IUB 1967]. In TAG all three glycerol hydroxy groups are esterified with a FA, in diacylglycerol (DAG) and in monoacylglycerol (MAG) two and one positions are esterified, respectively. Figure 2.1 shows the numbering of carbon atoms of glycerol in TAG using -*sn* system.

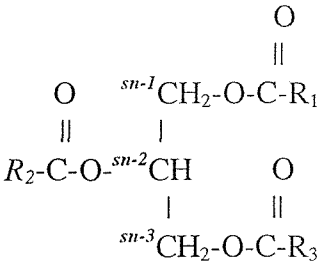


Figure 2.1. Triacylglycerol showing *sn-* (*1,2,3*) positions, R = fatty acid.

Naturally occurring TAG are mixed acylglycerols, where the most abundant FA are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) [Gurr & Harwood 1991]. Fatty acids that make up TAG can be classified chemically as saturated or unsaturated (monounsaturated and polyunsaturated) with one or more double bonds and are usually straight-chain derivatives containing an even number of carbon atoms. A type of geometric isomerism occurs in unsaturated FA depending on the orientation of atom groups around the axes of double bonds [Murray *et al.* 1996]. If the acyl chains are on the same side of the bond, it is called cis-, as in oleic acid, if on the opposite sides, it is called trans-, as in elaidic acid. Most naturally occurring unsaturated FA are of the cis-configuration. Carbon atoms of FA are numbered from the carboxyl carbon, carbon number 1, and the

terminal methyl carbon is known as the n- or ω-carbon [IUPAC-IUB 1967]. The number and position of the double bonds can be indicated in two ways: Δ^9 indicates a double bond between carbon atoms 9 and 10 of the FA counting from carboxyl carbon and ω9 or n-9 indicates a double bond on ninth carbon counting from methyl carbon. Saturated and monounsaturated FA can be synthesised by animal cells but polyunsaturated FA, linoleic acid (18:2, n-6) and α-linolenic acid (18:3, n-3), the essential FA, need to be provided by the diet [Thomson *et al.* 1989].

The physical properties of lipids are affected by their individual FA, the most important property being melting point [Gurr & Harwood 1991]. Generally membranes are incapable of operating with lipids that contain crystalline acyl chains, i.e. acyl chains with melting point above 37°C [Gurr & Harwood 1991]. The melting points of saturated FA are high e.g. palmitic acid 60.7°C and stearic acid 69.6°C. Double bond lowers the melting point of a FA so that melting point of oleic acid is 16°C and linoleic acid is -11°C [Gurr & Harwood 1991]. The overall melting point of lipid is determined by the combination of its constituent FA.

2.2.2 Intake of dietary lipid

Lipids are important dietary constituents because they have a high metabolisable energy value (37 kJ/g) compared to carbohydrates (16 kJ/g) and proteins (17 kJ/g), they provide essential FA and facilitate the absorption of lipid soluble vitamins [Murray *et al.* 1996]. Due to their high energy value, foods containing relatively high amounts of lipid provide a concentrated energy source. High-lipid diet may therefore expose to the development of obesity [Prewitt *et al.* 1991] but may also be beneficial in terms of providing energy in a compact form for children or in certain disease states when problems with adequate food consumption occur.

Table 2.1 shows the average daily intake of total lipid and saturated, monounsaturated and polyunsaturated FA in men and women obtained from the dietary and nutritional survey of British adults [Gregory *et al.* 1990]. The average intake of dietary lipid, especially that of saturated lipid, exceeded the recommended dietary reference values for both men and women in the UK (Department of Health 1991, Dietary reference values for lipid intake as proportion of energy intake: total lipid 30%, saturated FA 10%, cis-polyunsaturated FA 6%, cis-monounsaturated FA 12% and trans-FA 2%). The main lipid sources in the diet

were meat and products (24%), cereals (19%), lipid spreads (16%), milk and products (15%) and vegetables (11%).

Table 2.1. Average daily intake of lipid in British men and women [Gregory *et al.* 1990].

Nutrient	Men		Women	
	g/d	% total energy	g/d	% total energy
Total lipid	102.3	37.6	73.5	39.2
Saturated FA	42.0	16.5	31.1	17.0
n-3 polyunsaturated FA	1.95	0.72	1.35	0.73
n-6 polyunsaturated FA	13.8	5.07	9.60	5.12
Monounsaturated FA	31.4	12.4	22.1	12.2

2.3 Gastrointestinal handling of dietary lipid

It appears from the literature that the individual metabolic pathways of digestion and absorption of dietary lipid are relatively well described. However, the overall physiological process of the gastrointestinal handling of dietary lipid is less well defined. The proportional contribution of the individual factors for the overall digestion and absorption of lipids and the factors that control or limit lipid digestion and absorption are less well understood. A profound understanding of the mechanisms determining the functional capacity of the gastrointestinal tract to digest and absorb dietary lipid in health is necessary to be able to confront and manage problems in digestion and absorption of lipids, which occur in patients with gastrointestinal dysfunction. Figure 2.2 presents a schematic overview of the gastrointestinal handling of dietary lipid. Prior to assimilation, dietary lipid which is mainly TAG is digested by lipases and solubilised by bile salts and subsequently absorbed by enterocytes as MAG and FA. An appropriate luminal environment, such as suitable pH which is mainly regulated by bicarbonate, is required for digestion and absorption of lipids.

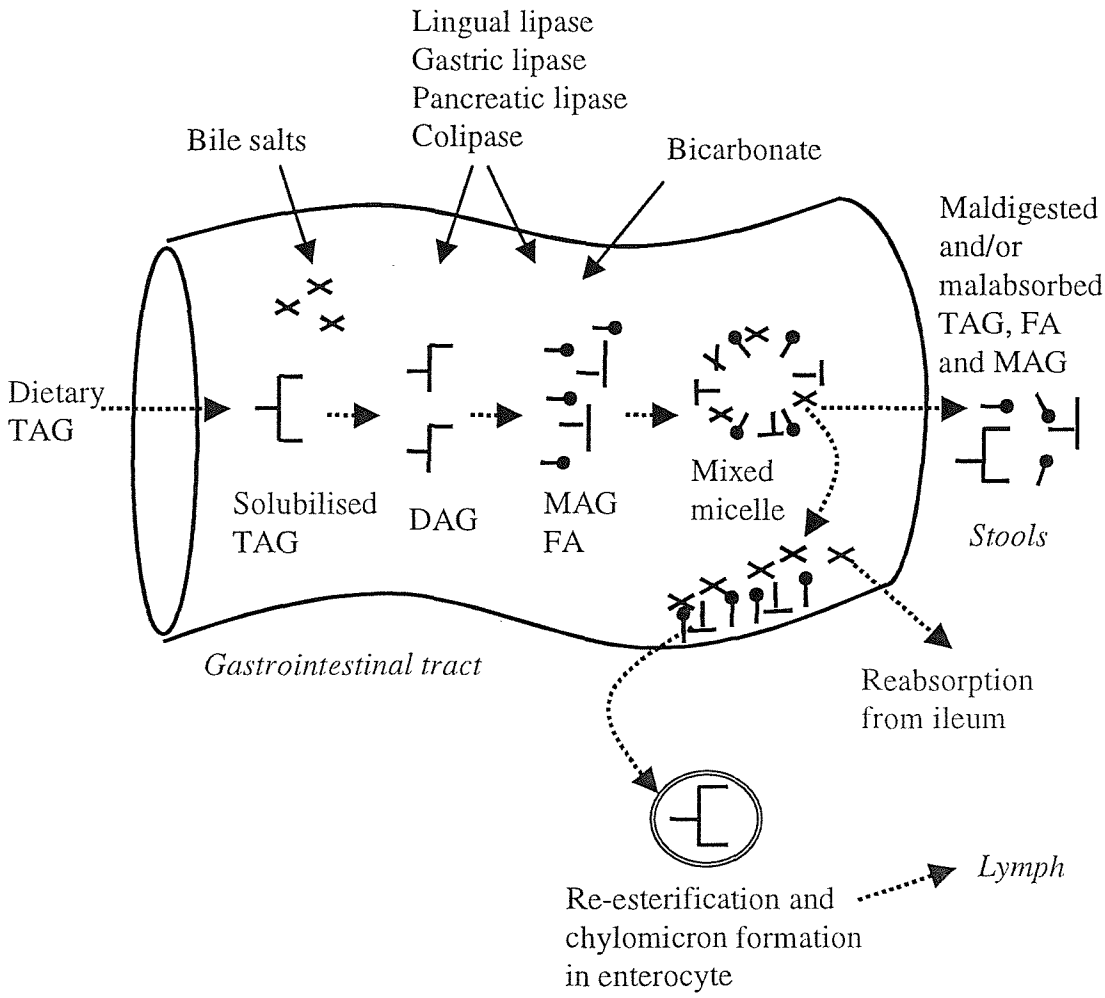


Figure 2.2.Overview of the gastrointestinal handling of dietary TAG.

2.3.1 Digestion

Triacylglycerols are digested (hydrolysed) to MAG and FA by action of lingual, gastric and pancreatic lipases prior to absorption. Digestion begins in mouth by lingual lipase, which is secreted by Ebner's glands in the back of the tongue and in the stomach by gastric lipase secreted by gastric glands [Hamosh *et al.* 1975; Hamosh & Burns 1977]. The pH optimum of the lingual lipase and the gastric lipase is 3.0 to 6.0, below which the lipases are inactivated [Liao *et al.* 1984]. However, both lipases remain active in stomach after food consumption due to the buffering effect of the ingested food [Liao *et al.* 1984; De Nigris *et al.* 1985]. Both lingual and gastric lipase can hydrolyse long-, medium- and short-chain TAG [De Nigris *et al.* 1985; Gargouri *et al.* 1986] and can hydrolyse ester bonds both at

the *sn*-1 and *sn*-3 positions of TAG [Carey *et al.* 1983]. However, it may be that the gastric lipase preferentially cleaves the *sn*-3 position relative to *sn*-1 position [Tso 1985]. It has been estimated that the two lipases hydrolyse approximately 30% of the ingested TAG in healthy individuals during 2 to 4 hours of gastric emptying after a meal [Linscheer & Vergroesen 1994], but the overall significance of the lingual and gastric lipase for the total digestion of dietary lipid is not known.

Gastric chyme that consists of partly digested food is delivered in small quantities to duodenum by gastric peristalsis and intermittent relaxation of pyloric musculature [Hunt 1963]. Fatty acids, MAG, amino acids and acidic pH in the gastric chyme induce the release of cholecystokinin and secretin from the duodenal mucosa into the circulation [Grossman 1968]. Secretin stimulates release of electrolytes and to a minor degree the release of digestive enzymes from pancreas whilst cholecystokinin stimulates the synthesis and release of enzymes and to a lesser extent, the release of electrolytes from the pancreas [Grossman 1968]. Pancreatic bicarbonate and the buffering effects of intestinal mucosa neutralise the gastric chyme and therefore provide a suitable environment for action of lipases [Guyton & Hall 1996].

The majority of TAG digestion occurs in the small intestine by pancreatic lipase which hydrolyses TAG first to *sn*-1,2 DAG and then to both *sn*-1 MAG and *sn*-2 MAG, the hydrolysis to *sn*-2 MAG being the predominant end product [Borgström 1954]. Fatty acids linked to *sn*-2 position may be removed after isomerisation to *sn*-1 position, which however is a relatively slow process and as a result, the major end product of the TAG digestion is *sn*-2 MAG and FA [Borgström 1954]. Further hydrolysis of *sn*-1 MAG or *sn*-2 MAG results in formation of glycerol and FA, although according to Senior (1964) only a relatively small proportion of TAG is completely hydrolysed to glycerol.

Pancreatic lipase is activated in the presence of colipase [Borgström & Erlanson 1973] and mixed micelles (See 2.3.2) resulting in a conformational change of the lipase molecule, whereby hydrophobic sites of the lipase are exposed to hydrolyse TAG in the lipid-water interface [Guerciolini 1997]. Colipase is secreted by pancreas to the duodenum in a precursor form, procolipase, which is transformed into colipase by trypsin [Larsson & Erlanson-Albertsson 1991]. Pancreatic lipase is usually considered the major lipase digesting dietary lipid. It seems that in healthy individuals, pancreatic lipase is excreted in excess to digest the ingested lipid, since very little residual pancreatic function, about 1% to 2%, is required to prevent lipid maldigestion [Durie & Forstner 1989]. Furthermore, the

concentration of lipase in the ileum is reduced compared to the concentration in the duodenum and only 1% of the lipase activity survives small intestinal transit [Layer *et al.* 1986]. It is not well known what causes the inactivation of lipase in the gastrointestinal tact, but it has been suggested that proteolytic enzymes, particularly chymotrypsin, inactivate lipase by altering the colipase-lipase complex [Thiruvengadam & DiMagno 1988]. Bile salts may prevent the inactivation of lipase by unfolding at the surface of the substrate, which stabilises the structure of the lipase [Borgström & Erlanson 1973]. Thiruvengadam and DiMagno (1988) suggested that the loss of lipase activity during small intestinal transit may be of physiological significance, since lipid digestion is delayed so that ingested lipid is dispersed over a larger segment of intestine for absorption. However, the loss of lipase activity during transit may impede with lipid digestion in patients with exocrine pancreatic insufficiency who are likely to have a reduced lipase secretion and activity in the first place.

2.3.2 Solubilisation

The size of ingested lipid particles is reduced (less than 1 μm in diameter when entering duodenum) and therefore the surface area increased by mechanical mixing due to chewing and gastric contractions [Senior 1964]. This process is also called solubilisation, which is further enhanced by dietary phospholipids and release of MAG from TAG [Senior 1964]. The solubilised lipid particle has TAG and DAG in the centre and a monolayer of phospholipids and FA on the outside of the particle. Bile salts have a significant role in the solubilisation of lipids. Firstly bile salts reduce the size of lipid particles (increasing lipid-water interface area) and secondly bile salts form micelles (mixed micelles) with lipid digestion products and therefore facilitate their absorption in enterocytes [Guyton & Hall 1996]. Bile acids are synthesised in the liver from cholesterol leading to two primary bile acids cholyl-CoA and chenodeoxycholyl-CoA, which are conjugated through a peptide bond with glycine or taurine (glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid) and are secreted in bile to duodenum [Senior 1964]. Due to alkaline pH and the presence of sodium and potassium in the bile, the bile acid conjugates appear as bile salts [Murray *et al.* 1996]. The total bile salt pool is approximately 3 to 5 g and circulates 6 to 10 times per day. The synthesis and secretion of bile is induced by cholecystokinin [Ertan *et al.* 1971] and when a certain concentration of bile salts has been reached in the gastrointestinal tract, the critical micellar concentration, bile salts form mixed

micelles with the lipid digestion products [Borgström 1985]. The optimal pH for the stability of the mixed micelles is between 6.5 and 8 [Shiau & Levine 1980]. Absorption of dietary lipid takes place from a micellar solution [Hofmann & Borgström 1964] and the bile salts are passed on to the ileum where they are absorbed and recirculated in the portal blood to the liver for re-entry at the duodenum [Borgström *et al.* 1963]. The absorption of bile salts occurs by active transport and by passive diffusion [Dietschy 1968]. In a healthy individual approximately 0.5 g of bile salts are excreted in stools each day.

2.3.3 Absorption

The MAG and FA digested from TAG are absorbed from the mucosa of small intestine, mainly in jejunum [Borgström *et al.* 1963]. No absorption occurs by gastric mucosa, despite partial hydrolysis of TAG in the stomach [Senior 1964]. Lipid digestion products form mixed micelles with bile salts (size 4 to 6 nm), which facilitate the movement of MAG and FA through aqueous environment of the gastrointestinal lumen to the absorptive site [Hofmann & Borgström 1964]. Lipids need to traverse through an unstirred water layer at the surface of the microvillus membrane before absorption [Wilson *et al.* 1971]. The rate of lipid absorption is inversely related to the size of the mixed micelles and the thickness of the unstirred water layer, which according to Wilson *et al.* (1971) primarily determine the rate of absorption. It has been suggested that acidic pH of the microclimate in the unstirred water layer and brush border membrane of enterocytes regulates the dissociation of mixed micelles and therefore absorption of lipids [Shiau & Levine 1980]. In this process the mixed micelles migrate close to the absorptive site where an acidic pH (about 5 to 6.5) facilitates dissociation of the micelles [Shiau & Levine 1980]. Subsequently MAG and FA move from the micelles into enterocytes by passive diffusion [Tso 1985]. Bile salts from the dissociated mixed micelles pass on to the ileum where they are absorbed [Borgström *et al.* 1963]. Glycerol formed in the digestion of TAG is absorbed by passive diffusion, and mostly enters blood circulation [Senior 1964]. For efficient absorption to occur an inward diffusion gradient of digestion products is needed in the enterocytes, which is ensured by binding FA to fatty acid binding proteins and subsequently by re-esterification of the absorbed FA into TAG [Ockner *et al.* 1972; Ockner & Manning 1974]. According to Shiau (1981) the re-esterification occurs mainly by monoacylglycerol pathway beginning from MAG (more than 70% of the esterification), but also by phosphatidic acid pathway beginning from

glycerol-3-phosphate, which is derived from glucose metabolism. The reformed TAG is packaged to chylomicrons and transported to circulation through lymphatic system. The metabolism of absorbed lipid is reviewed in 2.5.

Dietary lipid that fails to be digested and/or absorbed is excreted in stools. The total stool lipid losses in healthy adults and children are normally less than 7 g per day [Wollaeger *et al.* 1947; Fourman *et al.* 1948; Shmerling *et al.* 1970; Murphy *et al.* 1991]. Gompertz and Sammons (1963) suggested that the stool lipid originates from dietary lipid, bacterial lipid and endogenous lipid losses, such as bile and desquamated cells. A contribution of endogenous lipids or bacterial lipid to the total stool lipid has been shown by feeding diets of very low lipid content to healthy individuals and by measuring the total stool lipid losses that have exceeded the lipid intake [Wiggins *et al.* 1969]. Leroy *et al.* (1986) showed that lipid losses in stools were in average 5 g/day when subjects received lipid-free and residue-free elemental diet. Bacteria content of stools is on average 55% in healthy adults [Stephen & Cummings 1980] and 27% in healthy children [Murphy *et al.* 1991] of the stool dry weight and approximately 10% to 15% of the bacterial weight is lipid [Stephen & Cummings 1980].

2.4 Assessment of gastrointestinal handling of dietary lipid

Techniques to assess the gastrointestinal handling of dietary lipid have been used as a research tool and also in clinical practice to diagnose patients who have problems with either digestion or absorption of dietary lipid and to define the extent of maldigestion and/or malabsorption of lipids. The most widely used method for assessment of the capacity of the gastrointestinal tract to digest and absorb dietary lipid is to measure total lipid excretion in stools (See 2.4.1). The stool total lipid excretion is used to diagnose whether patients have excessive lipid losses in stools, compared to healthy subjects. However, the total lipid losses in stools do not take into account the origins of the stool lipid, whether due to maldigestion or malabsorption of lipid and therefore the causes of excessive lipid losses in stools remain unknown. Pancreatic function tests are used to determine the capacity of the pancreas to secrete digestive enzymes and therefore digestive capacity of the gastrointestinal tract (See 2.4.1). However, the pancreatic function tests are often referred to as a technique, which may be used to assess the overall capacity of the gastrointestinal tract to handle dietary lipid. A novel stable isotope technique that applies ^{13}C-labelled lipid substrates may be used

to trace the gastrointestinal handling of dietary lipid (See 2.4.2). In principle two approaches have been used 1) analysis of stool losses of the labelled substrate, which serves as an estimate for maldigested and/or malabsorbed lipid or 2) analysis of the label excretion on breath, which serves as an indirect measure of maldigestion and/or malabsorption of dietary lipid. These techniques and their limitations in the assessment of the gastrointestinal handling of dietary lipid are discussed below.

2.4.1 Determination of lipid excretion in stools and pancreatic function tests

Methods used to assess total lipid excretion in stools and pancreatic function tests are summarised in Table 2.2. The most common technique to diagnose excessive lipid losses in stools, is to measure stool total lipid and compare it to that of healthy individuals. Primarily three types of tests have been used 1) Determination of stool total FA [Van de Kamer *et al.* 1949], 2) acid steatocrit [Tran *et al.* 1994] and 3) Sudan stain [Khouri *et al.* 1989a]. It has also been suggested that instead of measuring the actual stool lipid, the extent of stool lipid losses may be assessed indirectly from stool weight using a regression equation [Bijleveld *et al.* 1986]. These techniques may be used to differentiate between subjects with substantive lipid excretion in stools and subjects with normal lipid excretion in stools. However, these methods do not determine the origin of stool lipid (dietary, bacterial or endogenous losses) or differentiate between maldigestion and malabsorption of lipid. Lipids of non-dietary origin may contribute to the stool total lipid losses in substantive amounts [Murphy *et al.* 1991] and dietary lipid losses in stools may be due to maldigestion of lipid in which case the lipid is not presented in a form that could be absorbed from the intestinal enterocytes or malabsorption of lipid due to a failure in the absorptive process itself.

Determination of the form of stool lipid could provide further insight into the differentiation between maldigestion and malabsorption of lipid. Therefore detection of TAG in stools would suggest maldigestion and detection of lipid digestion products FA and MAG in stools would suggest malabsorption. This approach was first applied by Thompson and co-workers (1969a and 1969b) who measured a higher absolute amount of TAG in stools (g/d) and a higher concentration of TAG in stools (g/100 g stools) in patients with steatorrhoea of pancreatic origin compared to patients with steatorrhoea of intestinal origin. However, Khouri and co-workers (1989b) found no difference in the concentration of TAG in stools between patients with pancreatic insufficiency and control subjects. Instead they

observed an increased concentration of FA in stools in patients with pancreatic insufficiency [Khouri *et al.* 1989b], which could have been due to hydrolysis of TAG beyond absorptive site, as postulated by the authors, or due to the sample processing and analysis which could have caused the hydrolysis of TAG to FA. Although the results of the two studies were not in an agreement, the approach of measuring TAG and FA in stools may be amenable to differentiate between lipid maldigestion and malabsorption if the analytical methods are optimised to measure TAG and FA in stools that originate from diet.

Despite pancreatic functions tests determine the capacity of the pancreas to secrete digestive enzymes (Table 2.2), they are often referred to as tools for the assessment of the overall capacity of the gastrointestinal tract to handle dietary lipid. The most direct method to determine the secretory capacity of the pancreas is to measure pancreatic enzyme activities in duodenal juice following stimulation of the pancreas by hormones (pancreozymin secretin test) [Hadorn *et al.* 1968]. The test is invasive and therefore several indirect tests have been developed. The indirect tests typically evaluate pancreatic enzymes in stools such as chymotrypsin [Bonin *et al.* 1973] or elastase [Stein *et al.* 1996] or excretion of digestion products (fluorescein, p-aminobenzoic acid) in urine or blood following administration of synthetic peptides, which are hydrolysed by pancreatic enzymes [Arvanitakis & Greenberger 1976; Malis *et al.* 1979; Barry *et al.* 1982; Weizman *et al.* 1985]. The indirect tests are relatively easy to perform but are less accurate compared to direct pancreatic function test. Indirect tests also typically measure the activity or concentration of proteases, which may not directly reflect the lipase activity or concentration in the gastrointestinal tract.

Analysis of total lipid excretion in stools may be used to diagnose excessive lipid losses in stools and pancreatic function tests may be used to diagnose pancreatic insufficiency. However, there is a need to develop a method that may be used as a research tool and also in the clinical practice to assess the overall functional capacity of the gastrointestinal tract to digest and absorb dietary lipid and to differentiate between maldigestion and malabsorption of dietary lipid. Stable isotope techniques make it possible to quantitatively trace the metabolism of dietary lipid. These techniques are reviewed in the next section (2.4.2).

Table 2.2. Methods for assessment of lipid excretion in stools and pancreatic function.

Method [Reference]	Description	Properties
Lipid excretion in stools		
Stool total FA [Van de Kamer *et al.* 1949]	Wet stools are hydrolysed and FA are extracted to solvent and determined by titration.	Measures total FA in stools (hydrolysed from TAG, MAG, phospholipids, cholesteryl esters etc.), which is used to diagnose excessive lipid losses in stools (more than 7g lipid in stools/day). The origins of the FA are not determined and therefore differentiation between maldigested and malabsorbed lipid is not possible.
Acid steatocrit [Tran *et al.* 1994]	Wet stools are homogenised, acidified and 75 μl is centrifuged in a capillary tube. Steatocrit is calculated as: Fatty layer/(fatty layer + solid layer) length.	Estimates total stool lipid, which has been found to correlate ($r = 0.81$) with the method of Van de Kamer *et al.* (1949). May be used to diagnose excessive lipid losses in stools, but does not differentiate between maldigested and malabsorbed lipid.
Sudan stain [Khouri *et al.* 1989a]	A stool sample is transferred to a glass slide and is treated with Sudan stain solution. The sample is examined by light microscopy to quantify the amount of lipid by the number and size of lipid droplets.	Estimates lipid losses in stools by the number and size of lipid droplets. May be used as a semi-quantitative measure of excessive lipid losses in stools, but does not differentiate between maldigested and malabsorbed lipid.
Regression equation [Bijleveld *et al.* 1986]	Stool lipid (g/day) = 0.07 × wet stool weight (g) + 3.6. (Children with cystic fibrosis)	Estimates stool lipid losses from stool wet weight. An indirect method assuming a uniform lipid concentration in all stool samples.

Table 2.2. Continued.

Method [Reference]	Description	Properties
Pancreatic function test		
Pancreozymin secretin [Hadorn *et al.* 1968]	Duodenal juice is collected before and after intravenous injection of pancreozymin and secretin, which stimulate pancreatic secretion. Enzyme activities (lipase, bicarbonate, trypsin and chymotrypsin) are determined from the juice.	Determines pancreatic enzyme activities in duodenal juice, which enables quantitative discrimination between normal and reduced exocrine pancreatic secretory capacity. Invasive due to duodenal intubation.
Fluorescein dilaurate [Barry *et al.* 1982]	Fluorescein dilaurate is ingested orally with a standardised meal. Fluorescein dilaurate is digested by pancreatic esterase to liberate lauric acid and fluorescein. Fluorescein is absorbed and excreted in the urine and is measured spectrophotometrically.	Reduced excretion of fluorescein in urine indicates pancreatic insufficiency. Indirect test of pancreatic digestive (proteolytic) capacity. Assumes that digestion of fluorescein dilaurate describes the overall digestive capacity of the pancreas and may therefore be non-specific for assessing the lipolytic capacity of the pancreas.
Bz-Ty-PABA [Arvanitakis & Greenberger 1976] Ac-Ty-PABA [Malis *et al.* 1979]	N-benzoyl-L-tyrosyl-p-aminobenzoic acid or N-acetyl-L-tyrosyl-p-aminobenzoic acid is ingested orally with a standardised meal. Bz-Ty-PABA or Ac-Ty-PABA is digested by pancreatic chymotrypsin to Bz-Ty or Ac-Ty and p-aminobenzoic acid (PABA), which is excreted in urine.	Reduced recovery of PABA in urine indicates pancreatic insufficiency. Indirect test of pancreatic digestive (proteolytic) capacity (see above; Barry *et al.* 1982). Has been found to correlate ($r = 0.59$) with stool FA content by the method of Van de Kamer (1949).
Bz-Ty-PABA [Weizman *et al.* 1985]	As above, except instead of urine samples, blood samples are obtained for determination of the PABA recovery in plasma.	Reduced recovery of PABA in plasma indicates pancreatic insufficiency. Indirect test of pancreatic digestive (proteolytic) capacity (see above; Barry *et al.* 1982). Has been found to correlate ($r = 0.80$) with duodenal colipase secretion.
Stool elastase [Stein *et al.* 1996]	Stool elastase concentration is measured immunologically from wet stools.	Reduced concentration of elastase in stools indicates pancreatic insufficiency. Indirect test of pancreatic digestive (proteolytic) capacity. Has been shown to correlate ($r = 0.82$) with duodenal lipase secretion.

2.4.2 Stable isotope technique

Stable isotope technique may be used to quantitatively determine the gastrointestinal handling and metabolic disposal of dietary lipid. Stable isotopes make it possible to trace the dietary lipid metabolism in both the gastrointestinal tract and after absorption as depicted in Figure 2.3. The principle of the technique is that a ^{13}C-labelled lipid substrate is orally administered to subjects and the ^{13}C-label excretion in stools and on breath is measured. The total excretion of ^{13}C-labelled substrate in stools serves as an estimate for the amount of dietary lipid that is maldigested and/or malabsorbed and the ^{13}C excretion on breath ($^{13}CO_2$) serves as an estimate for the amount of lipid that has been oxidised. The concept of stable isotope techniques and their use in nutrition research is discussed and the studies that have used ^{13}C-labelled lipids for the assessment of the gastrointestinal handling of dietary lipid are reviewed in this section.

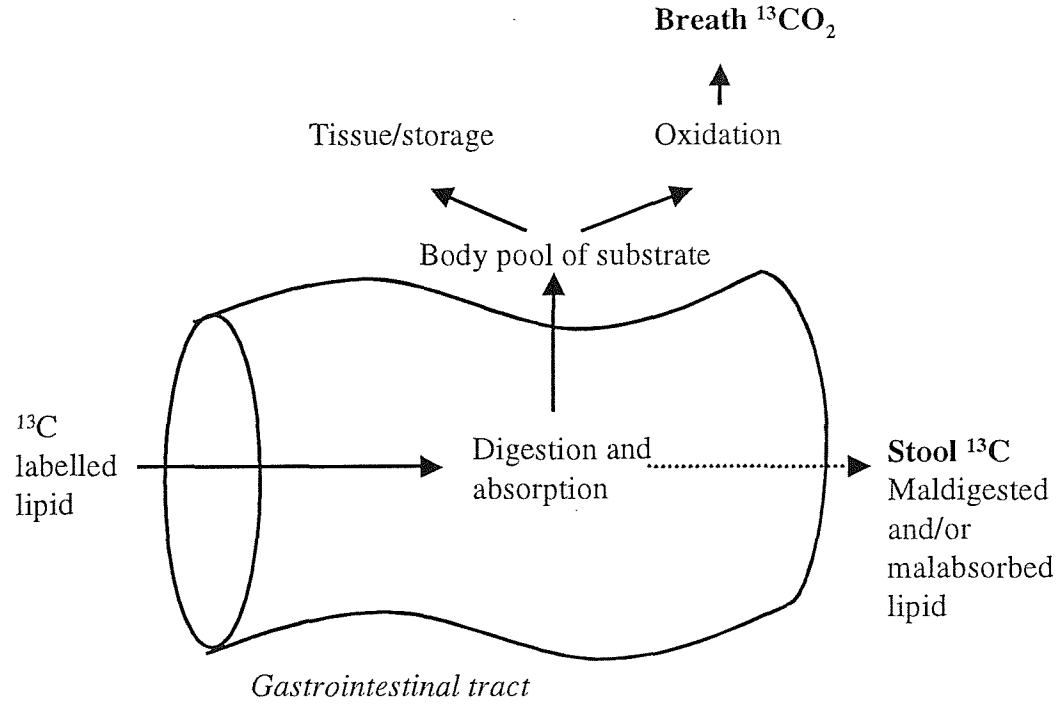


Figure 2.3. Principle of ^{13}C-labelling studies to determine the gastrointestinal handling and metabolic disposal of dietary lipid.

2.4.2.1 Basic concept of stable isotope technique

Carbon is an essential constituent of lipids and therefore stable isotope of carbon, ^{13}C, may be used to trace the metabolism of dietary lipid. Investigation of the gastrointestinal handling of dietary lipid by tracing the administered stable isotope labelled lipid makes it possible to differentiate between dietary lipid and that of bacterial lipid and endogenous lipid within stools. Label excretion on breath makes it possible to differentiate between oxidation of dietary lipid (exogenous lipid) and endogenous lipid (mobilised from storage lipid). Lipids labelled with radioactive isotope of carbon, ^{14}C, were previously used to trace metabolism of dietary lipid [Thorsgaard Pedersen 1985] but radioactive isotopes are not considered suitable for studies in children, women of childbearing age or pregnant women and nowadays stable isotopes are preferred over radioactive isotopes. Stable isotopes present no radiation hazard and they are non-toxic in quantities used in biological research [Jones 1990].

Principally the technique involves an oral administration of ^{13}C-labelled lipid and collection of body excretions, fluids and tissues such as stools, breath and blood for tracing the metabolism of the administered lipid. The isotope of an element is defined according to the atomic mass of its nucleus, which consists of protons and neutrons. The nuclei of different isotopes of an element have the same number of protons but vary in the number of neutrons resulting in different atomic masses for the isotopes of the element. Carbon has three isotopes, each of which has six protons and different number of neutrons: ^{12}C, (stable isotope, six neutrons), ^{13}C (stable isotope, seven neutrons) and ^{14}C, (radioactive isotope, eight neutrons).

Stable isotopes of carbon occur in the nature with a natural abundance of approximately 98.9% and 1.1%, ^{12}C and ^{13}C respectively [Matwiyoff & Ott 1973]. The unit of notation for isotopic measurements is an isotopic ratio, which is generally reported as abundance (atom %). Atom % ^{13}C is an absolute measurement of atoms of the isotope (^{13}C) in 100 atoms of the element ($^{12}C + {}^{13}C + {}^{14}C$). In practice the abundance of ^{13}C is usually compared to total stable isotopes:

$$\text{Atom \% } {}^{13}C = \frac{{}^{13}C}{({}^{12}C + {}^{13}C)} \times 100$$

Enrichment is expressed as atom percent excess (APE), which represents the abundance above the natural background level. When ^{13}C is used as a tracer, very small changes in its abundance relative to ^{12}C are measured, therefore for practical reasons unit δ (‰ = part per mil) is commonly used. The δ value is a relative measurement, which is determined as:

$$\delta \text{ value} = \frac{(^{13}C/^{12}C_{\text{sample}}) - (^{13}C/^{12}C_{\text{PDB}})}{(^{13}C/^{12}C_{\text{PDB}})} \times 1000$$

Where PDB refers to the ^{13}C abundance of an international standard Pee Dee Belemnite, South Carolinian carbonate, which has $^{13}C/^{12}C$ ratio of 0.0112372, atom % 1.1112328 and δ value 0 [Barrie *et al.* 1989]. Most natural samples contain less ^{13}C than the standard PDB, so the δ values are usually negative with respect to PDB. Very little PDB is left in the world and therefore laboratories use other references, such as beet sugar, which are set against secondary references that have been set against PDB.

The ^{13}C abundance of a sample (stools, blood, urine or their components) is measured following combusting into CO_2 and separation of the masses of three CO_2 ions, 44 [$^{12}C^{16}O^{16}O$], 45 [$^{13}C^{16}O^{16}O$] or 45 [$^{12}C^{17}O^{16}O$] and 46 [$^{12}C^{18}O^{16}O$] in the magnetic field of the isotope ratio mass spectrometry (IRMS) [Klein & Klein 1985]. The ^{13}C abundance is expressed as atom % or δ value by the software provided by the manufacturer.

Because ^{13}C is a naturally occurring stable isotope of carbon, depending on the photosynthetic pathway by which CO_2 in the atmosphere is converted to nutrients the natural ^{13}C abundance of foods varies. Calvin-Benson photosynthetic pathway [Calvin 1962], C_3 pathway, incorporates less $^{13}CO_2$ than is present in the atmosphere producing carbohydrates, proteins and lipids that are relatively depleted in ^{13}C. Whereas Hatch-Slack photosynthetic pathway [Hatch & Slack 1970], C_4 pathway, incorporates relatively more $^{13}CO_2$ resulting in nutrients enriched with ^{13}C in comparison to the products of C_3 pathway. Relative ^{13}C abundance of some foods is shown in Table 2.3.

Table 2.3. Relative ^{13}C abundance of some foods [Barrie *et al.* 1989].

Food	δ value	Atom %
Cane sugar (C_4) [1]	-11.65	1.0984
Beet sugar (C_3) [1]	-25.96	1.0827
Maize flour (C_4)	-10.38	1.0998
Rice flour (C_3)	-23.61	1.0853

[1] Photosynthetic route: C_3 = Calvin-Benson pathway and C_4 = Hatch-Slack pathway.

Due to the natural abundance of ^{13}C, the starting or baseline abundance of the sample to be measured must be known. For example the ^{13}C enrichment of breath sample is the $^{13}CO_2$ abundance in excess of the $^{13}CO_2$ abundance before the labelled substrate was administered [Schoeller *et al.* 1980]. Depending on the diet consumed the background level of the expired $^{13}CO_2$ varies both between-individuals and within-individual and also at different regions depending on the diet consumed [Schoeller *et al.* 1980]. The ^{13}C abundances of carbohydrates and lipids are different and therefore a shift in the relative rate of the metabolism of these substrates in the body changes the background abundance of the expired CO_2 [Wolfe *et al.* 1984]. The change in the breath ^{13}C abundance according to the metabolised nutrients was demonstrated in infants receiving total parenteral nutrition. During a glucose-amino acid solution (10% glucose, 2% amino acids; δ value -14) the average $^{13}CO_2$ abundance in breath closely approximated that of the intravenous solution and when the preparation was changed to a lipid solution (10% lipid; δ value -29), the ^{13}C abundance of the expired CO_2 began to change towards the lower ^{13}C abundance [Schoeller *et al.* 1980]. Also exercise changes the metabolic state and therefore alters the background ^{13}C abundance of the expired CO_2 [Wolfe *et al.* 1984]. During a metabolic study, a constant baseline ^{13}C abundance can be maintained by avoiding excessive intake of C_4 foods, such as corn and tropical fruits for three days prior to the study and during the entire study period [Klein & Klein 1985]. In addition, it has been shown that resting and withholding food for 8 hours before the study and throughout the study reduces the baseline variability of the ^{13}C abundance [Schoeller *et al.* 1980]. Fasting throughout the study is not always possible, especially when children are studied and if feeding is required, foods of C_3 origin may be offered without causing an increase in the

label excretion on breath. A typical test meal of low ^{13}C abundance consists of cheese sandwich and coffee [Schoeller *et al.* 1980] or of white bread, butter and orange juice [Murphy *et al.* 1995]. Calculations of the breath ^{13}C enrichment require information on the amount of carbon excreted on breath in addition to the ^{13}C abundance of the breath CO_2 and therefore the rate of CO_2 excretion on breath ($\dot{V}CO_2$) should be measured by indirect calorimetry [Jones 1996; Amarri *et al.* 1998]. Stable isotope techniques offer at their best, a noninvasive and nonhazardous method for investigation the metabolism of dietary lipid in children and adults. However, the studies require careful planning and controlling the background ^{13}C abundance.

2.4.2.2 Stable isotope technique in assessment of gastrointestinal handling of dietary lipid

Stable isotopes have been used in increasing amounts for assessment of gastrointestinal handling of dietary lipid during recent years. The most commonly used approach is to indirectly assess the digestion and absorption of lipids by measuring the label excretion on breath following an oral administration of a ^{13}C-labelled lipid [Schoeller *et al.* 1977; Ghoos *et al.* 1985; Klein & Klein 1985; Weaver *et al.* 1998]. These studies have also been used in a clinical setting to differentiate between patients with lipid maldigestion or lipid malabsorption and healthy subjects. These so called breath tests are based on a principle that the label excretion on breath following administration of a ^{13}C-labelled lipid substrate is limited only by one physiological step, which is called the rate-limiting step. Maldigestion of lipids has been evaluated by measuring label excretion on breath following an oral administration of a ^{13}C-labelled TAG and malabsorption by measuring the label excretion on breath following an oral administration of a ^{13}C-labelled FA. Breath tests using TAG assume that the rate-limiting step is digestion of TAG to FA and MAG through action of pancreatic lipase and that the absorption of digestion products is complete. Whereas in FA breath tests the rate-limiting step is absorption, since FA do not require digestion. Both approaches assume that the label excretion on breath is not affected by the events occurring after absorption and for example the oxidation of the absorbed FA occurs to a similar extent in all subjects. The main substrates that have been used in breath tests are ^{13}C-labelled TAG, trioctanoin ([1,1,1-^{13}C] trioctanoin), triolein ([1,1,1-^{13}C] triolein), hiolein (uniformly ^{13}C-labelled TAG with various FA) and mixed

TAG (1,3 distearoyl, 2[^{13}C]octanoyl glycerol), and FA such as palmitic acid. Studies that have applied breath tests for the assessment of the gastrointestinal handling of dietary lipid are reviewed below.

One of the first ^{13}C-labelled lipid substrates used to evaluate the gastrointestinal handling of dietary lipid was trioctanoin. It was shown in three studies that the label excretion on breath was lower in patients with pancreatic insufficiency due to cystic fibrosis compared to control subjects after oral administration of ^{13}C-labelled trioctanoin [Watkins *et al.* 1977a; Murphy *et al.* 1990; McClean *et al.* 1993]. The lower label excretion on breath was interpreted as resulting from maldigestion of trioctanoin. Despite a difference in the label excretion on breath was found between the group averages, the label excretion on breath of some patients was similar to the label excretion on breath in controls. Overlap between the groups may have occurred due to insensitivity of the label excretion on breath to detect mild maldigestion of lipid or the label excretion on breath may not be solely determined by the digestion of the administered trioctanoin in the cystic fibrosis patients. Medium chain TAG do not occur in common foods, therefore trioctanoin may not be the best substrate to trace the metabolism of dietary lipid. However, repeating the studies in the same patients with pancreatic enzyme preparations resulted in an increase in the label excretion on breath [Watkins *et al.* 1977a; Murphy *et al.* 1990; McClean *et al.* 1993] suggesting that the label excretion on breath was at least partially determined by the gastrointestinal handling of trioctanoin.

Mixed TAG (1,3 distearoyl, 2[^{13}C]octanoyl glycerol) has also been used to assess the digestive capacity of the gastrointestinal tract [Swart & Van Den Berg 1998; Weaver *et al.* 1998]. The studies are based on the assumption that the long-chain FA stearic acid in the *sn*-1 and *sn*-3 positions is hydrolysed by pancreatic lipase and the resulting medium chain FA octanoic acid or MAG containing octanoic acid is completely absorbed and therefore the label excretion on breath reflects the digestion of the administered mixed TAG. Vantrappen *et al.* (1989) found a lower recovery of label on breath in patients with chronic pancreatitis compared to either control subjects or patients with steatorrhoea of nonpancreatic origin following an oral administration of the mixed TAG. Similarly to the trioctanoin studies, in this study the label excretion on breath in patients with chronic pancreatitis extended over the label excretion on breath in patients with excessive lipid losses in stools due to nonpancreatic causes suggesting that mixed TAG breath test may not be specific for detection of lipid maldigestion or may not detect mild maldigestion.

Amarri *et al.* (1997) found a lower label recovery on breath in cystic fibrosis patients compared to both control subjects and patients with mucosal disease (short bowel syndrome and coeliac disease) following administration of mixed TAG, but again an overlap between the two groups in the breath label recovery was observed. Both studies [Vantrappen *et al.* 1989 and Amarri *et al.* 1997] found an increase in the label recovery on breath when the studies were repeated with pancreatic enzyme preparations. The label recovery on breath following an oral administration of mixed TAG has been found to correlate with duodenal lipase output suggesting that the test reflects duodenal lipolytic activity [Vantrappen *et al.* 1989]. However, Kalivianakis *et al.* (1997) found a poor repeatability in the label recovery on breath when the mixed TAG test was carried out in healthy adults on two occasions under similar conditions. Repeatability coefficient of the cumulative label excretion on breath over 9 hours was found to be 23% (second test performed may give on average 23% higher or lower label excretion on breath compared to the first test).

The FA composition of hiolein more closely resembles the dietary FA composition than either trioctanoin or mixed TAG and is therefore a more physiological substrate for investigation of the gastrointestinal handling of dietary lipid. Hiolein is a mixture of uniformly ^{13}C-labelled TAG consisting mainly of oleic acid, palmitic acid and linoleic acid and has been used to assess the lipid digestion in the gastrointestinal tract. A lower label excretion on breath (below 95% confidence interval of the controls) was found in patients with pancreatic insufficiency (abnormal pancreozymin secretin test), compared to control subjects [Lembcke *et al.* 1996]. However, the label excretion on breath of patients with mild or moderate exocrine pancreatic insufficiency did not differ from that of the controls.

Watkins *et al.* (1982) used a combination of ^{13}C-labelled lipids, trioctanoin, triolein and palmitic acid, to differentiate between patients with pancreatic insufficiency, patients with mucosal disease and patients with solubilisation defects and control subjects by measuring the label excretion on breath. Following administration of trioctanoin, the label recovery on breath was lower in patients with pancreatic insufficiency compared to control subjects or other patient groups suggesting that trioctanoin absorption was limited only by digestion. Following administration of palmitic acid, the label recovery on breath was similar in patients with pancreatic insufficiency and controls, but was lower in patients with mucosal disease or solubilisation defects. All three patient groups had a lower label recovery on breath compared to controls following triolein administration, which suggests

that the patients with pancreatic insufficiency maldigested and therefore also malabsorbed the administered triolein and patients with mucosal disease or solubilisation defect malabsorbed the digestion product, oleic acid or mono-olein (MAG). This study shows that a combination of the ^{13}C-labelled lipids may be useful to detect the origins of the altered gastrointestinal handling of lipids. However, also the combination of breath tests assumes that only one rate-limiting physiological step in each test determines the label excretion on breath. Prior to applying these studies in a clinical setting the gastrointestinal handling of the labelled lipids should be investigated directly by measuring the label excretion in stools in order to confirm that no alterations occurs in the metabolism of the absorbed lipid in the patient group studied.

Kalivianakis *et al.* (1999) also applied a combination of two ^{13}C-labelled lipids, mixed TAG and linoleic acid to detect lipid maldigestion and malabsorption in cystic fibrosis patients. The label recovery on breath was compared to stool total lipid losses and to plasma ^{13}C-label recovery. No association was found between the label recovery on breath following an oral administration of mixed TAG and the stool total lipid losses and the possibility of lipid maldigestion was excluded. Whereas a negative association was found between 8 hour plasma ^{13}C-linoleic acid concentration and the stool total lipid losses and the low label recovery on plasma was interpreted to result from malabsorption of the administered FA. However, the measured stool ^{13}C-label losses of 0% to 1.8% of the administered dose after administration of the linoleic acid in the cystic fibrosis patients were disregarded in the interpretation of the results [Kalivianakis *et al.* 1999; Murphy *et al.* 1999; Verkade 1999]. It is also interesting to note that the cumulative label recovery on breath over 6 hours in cystic fibrosis patients by Kalivianakis *et al.* (1999) was of similar range (2.4% to 40.2% of the administered dose) compared to study by Amarri *et al.* (1997) also with cystic fibrosis patients (0% to 31.7% of the administered dose) and was in average lower than the label recovery on breath in control subjects. In previous studies a low label recovery on breath after administration of mixed TAG was interpreted to originate from reduced digestion of mixed TAG.

Based on observed differences in the label excretion on breath between for example patients with confirmed pancreatic insufficiency and control subjects, it seems that the label recovery on breath following administration of various ^{13}C-labelled lipids may be useful in the clinical setting to determine disorders in digestion or absorption of dietary lipid and also to assess the efficacy of pancreatic enzyme replacement therapy (PERT).

However, despite differences between the group averages, most studies reported an overlap in the label excretion on breath in individual subjects, which suggests that it may not be possible to detect mild disorders in the gastrointestinal handling of dietary lipid using these studies or that the label excretion on breath is not solely determined by the rate-limiting step. All the breath tests assume that the metabolic partitioning and oxidation of the absorbed lipid is uniform and consistent. Although there is some evidence of the oxidation of octanoic acid in healthy individuals [Schwabe *et al.* 1964; Metges & Wolfram 1991], the uniform oxidation of the substrates used in the breath test has not been confirmed by systematic studies firstly in healthy individuals and secondly in the patient group the test is aimed at. Different FA may be oxidised to different extent even in healthy individuals [Jones *et al.* 1985b; Jones *et al.* 1999] and therefore it is important to confirm that no alterations in the oxidation of the administered lipid occurs in the patient group of interest. Some studies have shown an association between label excretion on breath and pancreatic lipase secretion [Vantrappen *et al.* 1989, Lembcke *et al.* 1996], but the pancreatic lipase activity on its own may not describe the overall digestive capacity of the gastrointestinal tract for example if malabsorption occurs in addition to maldigestion.

The most direct way to assess the gastrointestinal handling of dietary lipid is to measure label losses in stools following administration of ^{13}C-labelled lipid. Schoeller *et al.* (1981) and Jones *et al.* (1985a) were the first groups to measure the ^{13}C-label excretion in stools following on oral administration of ^{13}C-labelled lipid. The natural variability of the ^{13}C abundance in stools was found to be small, 0.6 δ units (SD) [Schoeller *et al.* 1981]. However, the label losses in stools varied even in healthy individuals depending on the FA studied (SD 0.1% to 4.4 % of the administered dose) [Jones *et al.* 1985b; Jones *et al.* 1999] highlighting the importance of measuring stool losses of the label when assessing the gastrointestinal handling of dietary lipid.

Total ^{13}C-label excretion in stools has been investigated in cystic fibrosis patients compared to control subjects following administration of ^{13}C-labelled palmitic acid [Murphy *et al.* 1997] and tripalmitin [Murphy *et al.* 1998]. The label excretion in stools was greater in the control subjects compared to cystic fibrosis patients after administration of palmitic acid, whereas the label excretion in stools was greater in the cystic fibrosis patients following administration of tripalmitin. The poor availability of the palmitic acid in the control subjects [Murphy *et al.* 1997] may be due to the form of the substrate administered (label sprinkled on the butter and served with toast). Dietary lipids are

emulsions whereas synthetically produced lipid substrates are in a crystalline form, which may reduce their availability to the body [Emken *et al.* 1993]. It may be that gastrointestinal luminal environment in cystic fibrosis patients was more favourable for the absorption of free FA and that in a more alkaline gastrointestinal tract in controls the administered FA formed salts which were subsequently lost in stools. However, it remains unresolved why the stool losses of the label were greater in the control subjects compared to the cystic fibrosis patients following palmitic acid ingestion. The ^{13}C-labelled tripalmitin was administered as an emulsion drink and was well digested and absorbed in the controls indicating that it is important to administer the lipid substrate in a physiological form that may be handled in the gastrointestinal tract by the healthy individuals. The total label losses in stools may be used to determine the bioavailability of the administered substrate but further development of the technique is required to differentiate quantitatively between maldigestion and malabsorption of lipids.

2.5 Metabolism of absorbed dietary lipid and endogenous lipid

The principle metabolic pathways and regulation of transport, storage and oxidation of dietary lipid and endogenous lipid are reviewed in this section with the main focus in the metabolism of TAG and FA. The way absorbed dietary lipid is partitioned towards storage or oxidation and proportions of macronutrients utilised for energy production may influence weight gain, body composition and growth rate. Subsequent metabolism of the absorbed lipid may also have implications for studies that apply stable isotope tracers indirectly (^{13}C excretion on breath) to investigate the gastrointestinal handling of dietary lipid. Alterations in the metabolism of the absorbed lipid may be reflected in the ^{13}C excretion on breath, which could erroneously be interpreted as resulting from the gastrointestinal function.

An overview of the metabolism of the absorbed dietary lipid and endogenous lipid is presented in Figure 2.4. After absorption from the gastrointestinal tract to enterocytes, dietary TAG is transported as chylomicron to tissues and organs for energy production or for storage. The stored lipid (endogenous lipid) is mobilised and transported between body tissues and organs as lipoproteins or as non-esterified fatty acids bound to albumin (NEFA) as required by the metabolism. The FA metabolism interacts with the glucose metabolism in liver, muscle and adipose tissue, which are regulated in such a way that FA

and glucose concentrations in the circulation are carefully controlled [Randle *et al.* 1963]. How this regulation occurs and to what extent the regulation may or may not be disturbed prior to development of diseases or for disturbance of growth are not fully understood.

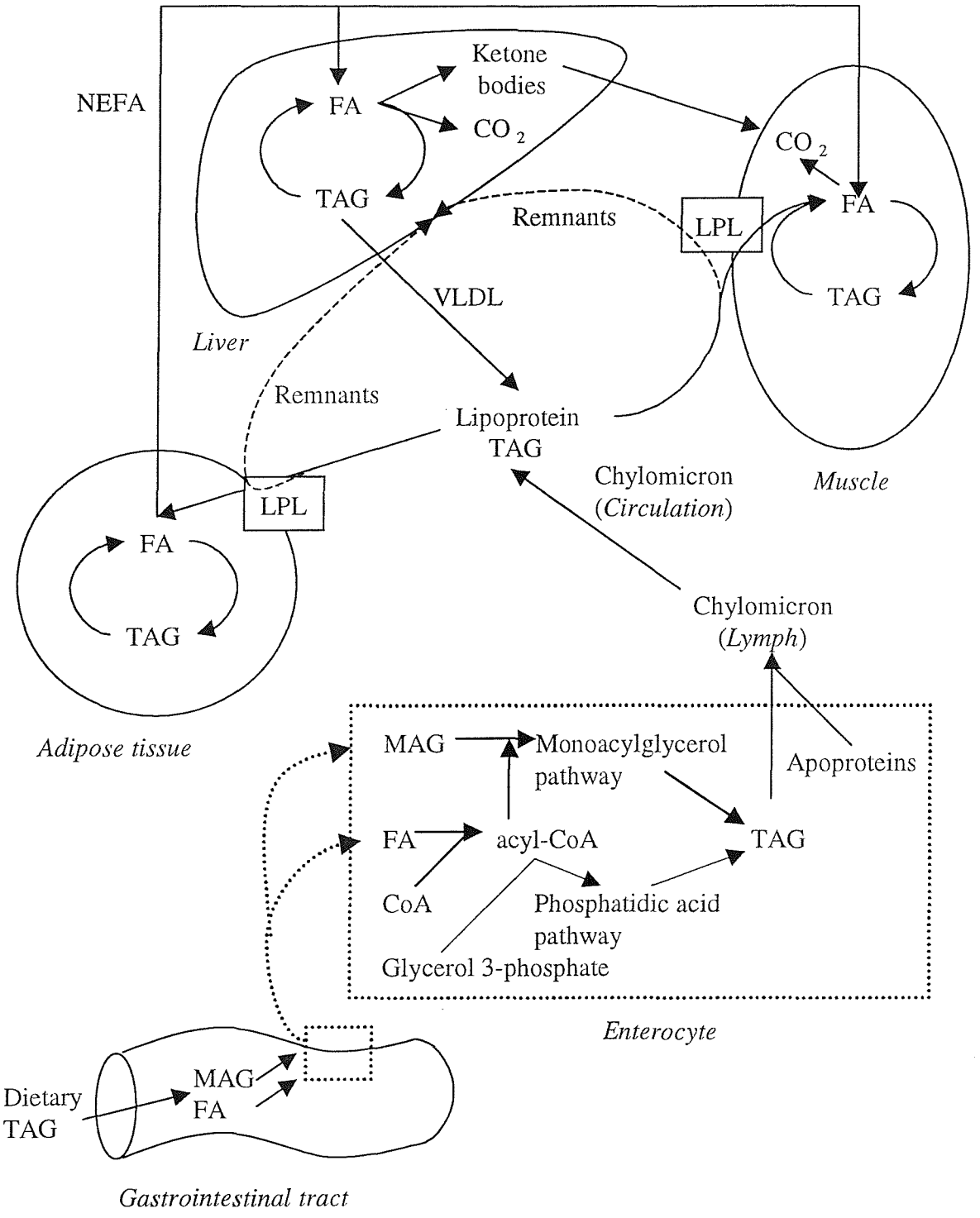


Figure 2.4. Overview of the metabolism of absorbed dietary lipid and endogenous lipid [Modified from Murray *et al.* 1996]. LPL = lipoprotein lipase, NEFA = non-esterified fatty acids, VLDL = very low-density lipoprotein.

2.5.1 Transport

Lipids are water-insoluble molecules and are therefore transported between tissues and organs in lipid-protein complexes, lipoproteins (TAG) or bound to albumin (FA). Lipoprotein metabolism and especially disturbances in the regulation of the postprandial lipoprotein metabolism may have an important role in the development of diseases such as coronary heart disease and obesity [Griffin 1997; Williams 1997]. The detailed discussion of the lipoprotein metabolism is beyond the scope of this review but the main pathways of the TAG and FA transport will be presented to give an overview of the metabolic partitioning of the absorbed lipid.

Lipoproteins consist of lipid core, mainly TAG, cholesterol esters and phosholipids, which is surrounded by a layer of phospholipids and cholesterol and integral or peripheral apoproteins [Murray *et al.* 1996]. Absorbed lipids from the gastrointestinal tract are transported in blood circulation to muscle and adipose tissue as chylomicrons, and lipids from liver are transported to other organs and tissues as VLDL. The FA from adipose tissue are transported as NEFA bound to albumin. In addition to chylomicrons and VLDL, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) transport lipids in the circulation [Sethi *et al.* 1993]. Triacylglycerol is the predominant lipid in chylomicrons and VLDL and cholesterol in LDL and phospholipid in HDL. The major apoproteins in chylomicrons are B-48, A1, A2, C and E, in VLDL B100, C and E, in LDL B100 and in HDL A1, A2, C and E as cited by Murray *et al.* (1996).

Absorbed dietary lipid is transported as chylomicrons from the enterocytes, where nascent chylomicrons are formed. Firstly apoprotein B-48 is synthesised by ribosomes in the rough endoplasmic reticulum and is incorporated with re-esterified TAG (See 2.3.3) in the smooth endoplasmic reticulum. Nascent chylomicrons pass through Golgi apparatus, where more lipid and apoproteins are added and finally chylomicrons are released to the lymphatic vessels and passed to the systemic blood via the thoracic duct [Shiau 1981]. Fatty acids with less than 14 carbon atoms are bound to albumin and transported directly to liver by portal vein. Nascent chylomicrons take up apoproteins C and E from HDL in the circulation and thereby form chylomicrons [Shiau 1981]. Chylomicrons bind to LPL on the endothelial surface of blood capillaries in extrahepatic tissues, mainly adipose tissue and muscle. The LPL catalyses the hydrolysis of TAG from the chylomicrons releasing FA, which are mainly taken up by tissues but some FA are bound to albumin and released

to the circulation as NEFA [Griffiths *et al.* 1994]. The LPL is expressed in different tissues to different extent depending on the nutritional state (fasting vs. fed state) as reviewed by Fielding and Frayn (1998). The activity of LPL is associated with the requirements for FA and therefore LPL activity increases in the fed state. Although the activity of the LPL is important for delivery of FA to tissues, in the absence of LPL the normal tissue metabolism can be maintained but the clearance of TAG from the circulation will be disturbed [Fiedling & Frayn 1998]. After hydrolysis of TAG from the chylomicrons, apoproteins A and C, phospholipids and cholesterol are transferred to HDL [Tall 1990]. The remaining particles, chylomicron remnants, are taken up by apoprotein E specific receptors in the hepatocytes [Sethi *et al.* 1993]. The clearance of chylomicrons is usually fast in humans with half-life of 5 to 10 min, but due to the continuing entrance of chylomicrons to the circulation following consumption of a meal, the peak chylomicron concentration in the circulation occurs about 2 hours after a meal [Griffiths *et al.* 1994; Frayn 1996].

The formation of VLDL is similar to that of chylomicrons, except it occurs in the hepatocytes and the apoprotein composition of VLDL is different to that of chylomicrons [Gibbons 1990]. The VLDL particles transport endogenous lipid, mainly TAG, from liver to other tissues where VLDL binds to LPL in blood capillaries for release of FA. Subsequently, phospholipids and apoprotein C are transferred to HDL, whilst apoproteins B and E are retained [Tall 1990]. These VLDL remnants result in the formation of LDL. The LDL particles are the major carriers of cholesterol to tissues where they are used for membrane structure and conversion into metabolites such as steroid hormones [Dietschy *et al.* 1993]. Disturbances in LDL metabolism and especially its subclasses may be associated with the increased risk of coronary heart disease [Griffin 1997]. The HDL particles transport excess cholesterol from peripheral cells to liver for degradation and for synthesis of bile salts [Tall 1990].

2.5.2 Storage and oxidation

Detailed discussion of what determines the energy metabolism and substrate mix utilised for energy production (oxidation) is beyond this review, but the principal elements and metabolic pathways involved in the storage and oxidation of lipids and energy metabolism will be presented. It would seem that the metabolic demand sets the energy and nutrient

requirements, but at the same time the availability of the substrates and energy from the diet and/or endogenous stores may regulate their utilisation. Interactions between body tissues are set to maintain a control of FA and glucose concentrations in the circulation [Randle *et al.* 1963]. This balance is disturbed by the entry of energy and nutrients from the diet and the various regulative processes of energy and macronutrient metabolism operate to maintain the balance in order to ensure tissue integration, vital body functions and growth. Despite a fair amount of research in this area has been carried out, the effects of dietary nutrient composition on the metabolic partitioning of lipids and the overall regulation of the lipid metabolism is poorly understood.

Fatty acids released from TAG in chylomicrons or in VLDL may be taken up by adipose tissue, muscle and other extrahepatic tissues and NEFA may be taken up by liver and muscle. Fatty acids may be re-esterified to form TAG, mainly in the adipose tissue and in relatively small amounts in muscle or oxidised mainly in the muscle and liver to provide energy. Triacylglycerols may also be formed from glucose through lipogenesis, however the interconvertibilities of protein, carbohydrate and lipid are limited [Stubbs 1998]. Under extreme conditions, such as overfeeding with a single substrate lipogenesis may be induced [Schutz *et al.* 1985; Acheson *et al.* 1988]. Hormone sensitive lipase (HSL) hydrolyses TAG within the adipose tissue and other extrahepatic tissues to form FA and glycerol [Frayn *et al.* 1995]. The released FA may be reconverted to acyl-CoA and re-esterified with glycerol-3-phosphate (from glycolysis) to form TAG [Langin *et al.* 1996]. The re-esterification and lipolysis of TAG are continuing processes in the adipose tissue and when the rate of lipolysis exceeds re-esterification, the FA are mobilised to the circulation, where they are bound to albumin (NEFA) and are transported to other tissues. Hormones regulate lipolysis of TAG and re-esterification of TAG by stimulation or inhibition of LPL and HSL as cited by Murray *et al.* (1996). Insulin inhibits HSL and therefore regulates FA release from the adipose tissue. Epinephrine, norepinephrine, glucagon, thyroid stimulating hormone, adrenocorticotropic hormone and growth hormone stimulate lipolysis. In liver, the release of FA from TAG and re-esterification are regulated by insulin and glucagon (increases release of FA for oxidation). In fed state the LPL activity is high relative to that of HSL whereas in fasted state the HSL is active and the LPL activity is suppressed [Frayn *et al.* 1995, Langin *et al.* 1996]. Therefore LPL and HSL are regulated in a co-ordinated manner and govern FA mobilisation and re-esterification to TAG.

The rate of utilisation of NEFA from the circulation is believed to depend on its

concentration, which is regulated by the re-esterification and lipolysis of TAG in the adipose tissue [Frayn 1996]. Therefore, the uptake of NEFA by muscle for oxidation is dependent on the FA delivery regulated by blood concentration of NEFA and blood flow [Frayn *et al.* 1995]. After fasting, the concentrations of insulin and glucose are low at circulation, FA are liberated from the adipose tissue and therefore the concentration of the NEFA increased compared to the fed state [Coppack *et al.* 1989]. The released FA are taken up by tissues, mainly muscle and liver where the FA are either oxidised or re-esterified to TAG and stored. However, in the fasted state the re-esterification of FA is low and only about 3% of the released FA are re-esterified [Frayn *et al.* 1995].

Following a meal, glucose concentration in the circulation rises, which stimulates secretion of insulin [Coppack *et al.* 1992]. As a result HSL is suppressed and the concentration of the NEFA in the circulation falls [Langin *et al.* 1996]. Increase in insulin concentration stimulates LPL in adipose tissue, which increases clearance of circulating TAG and therefore the uptake of FA from chylomicrons [Coppack *et al.* 1992]. The FA taken up by the adipose tissue are mainly esterified to form TAG [Coppack *et al.* 1989, Frayn *et al.* 1995]. Fatty acids taken up by the muscle mainly from the chylomicrons are primarily used for energy production, although muscle has small TAG stores for local energy production at a later stage [Elia *et al.* 1988, Coppack *et al.*1992].

Fatty acids entering muscle through action of LPL or liver through carrier mediated process or FA released from TAG within tissue may be oxidised in mitochondria to generate energy [Baldwin & Krebs 1981]. In FA oxidation two carbon fragments (acetyl-CoA) are liberated at the time whereby FA chain is broken between α (2^{nd}) and β (3^{rd}) carbon atoms and therefore the process is called β-oxidation. Prior to oxidation FA must be converted to an active intermediate, acyl-CoA. In the endoplasmic reticulum and on the outer membrane of mitochondria acyl-CoA synthetase catalyses conversion of FA to fatty acyl-CoA in the presence of ATP and coenzyme A [Schulz 1991]. Both NEFA and fatty acyl-CoA bind in cytosol to fatty acid binding proteins, which aid intracellular transport of FA [Ockner *et al.* 1972]. Long-chain FA require carnitine (β-hydroxy-γ-trimethylammonium butyrate) to penetrate the inner membrane of mitochondria [Schulz 1991]. Carnitine palmitoyltransferase I in the outer mitochondrial membrane converts fatty acyl-CoA to acylcarnitine, which can penetrate the inner membrane of the mitochondria. Carnitine acylcarnitine translocase in the inner membrane of the mitochondria exchanges carnitine to acylcarnitine, which reacts with CoA catalysed

by carnitine palmitoyltransferase II generating fatty acyl-CoA. It is believed that fatty acids of less than ten carbons can enter mitochondria as free acids independent of carnitine [Gurr & Harwood 1991].

Fatty acyl-CoA enters β-oxidation in mitochondria where two-carbon units, acetyl-CoA, are liberated in a sequential of reactions starting from carboxyl end as cited by Murray *et al.* (1996). Fatty acyl-CoA is transformed to Δ2-trans-enoyl-CoA catalysed by acyl-CoA dehydrogenase in the presence of flavoprotein containing FAD (flavin adenine dinucleotide), as a coenzyme (liberates $FADH_2$). Next, 3-hydroxyacyl-CoA is formed in the presence of water and Δ2-enoyl-CoA hydratase. Further dehydrogenation occurs by L(+)-3-hydroxyacyl-CoA dehydrogenase, NAD+ (nicotinamide adenine dinucleotide) as a coenzyme (liberates NADH), generating 3-ketoacyl-CoA. Finally in the presence of coenzyme A, thiolase catalyses formation of fatty acyl-CoA and acetyl-CoA. Resultant fatty acyl-CoA, two carbons shorter, re-enters β-oxidation and may be degraded completely to acetyl-CoA. Acetyl-CoA enters citric acid cycle where it is oxidised to CO_2 and water and reducing equivalents are released [Baldwin & Krebs 1981]. The unsaturated FA are oxidised through modified β-oxidation where additional enzymes are required to change the isomeric positions of FA and to move double bonds to required positions [Schulz 1991]. Modified form of β-oxidation occurs also in peroxisomes and α-oxidation in brain tissue [Murray *et al.* 1996]. Reducing equivalents (NADH and $FADH_2$) generated both in β-oxidation and citric acid cycle are oxidised by the respiratory chain with release of ATP, which can transfer energy to the required physiological processes. High rate FA oxidation for example during starvation or insulin deficiency results in production of ketone bodies, β-hydroxybutyrate, acetoacetate and acetone (spontaneously formed from acetoacetate), in the liver via hydroxymethylglutaryl-CoA cycle [McGarry & Foster 1980]. Ketone bodies may be used as a fuel in the extrahepatic tissues.

The total energy expenditure of an individual consists of basal metabolic rate (BMR), thermic effect of food and physical activity [Swinburn & Ravussin 1994]. Basal metabolic rate contributes 50% to 70% of the daily total energy expenditure and its main predictor is lean body mass (LBM) [Cunningham 1980]. The thermic effect of food (energy cost of the metabolism of nutrients) represents 5% to 10% of the total energy expenditure [Elia *et al.* 1988; Schutz *et al.* 1989]. The physical activity is the most variable component of total energy expenditure and is determined by the intensity and duration of the activity [Swinburn & Ravussin 1994]. In addition to energy requirements

for maintenance of the basal body functions and physical activity, children require energy for growth. To estimate energy requirements of individuals, World Health Organization (1985) expert committee recommended, based on growth studies, an extra allowance for growth of 21 kJ/g weight gain.

The overall energy production is ultimately regulated by the energy status of the cell (energy requirements) whereby an increase in ATP/ADP ratio reduces substrate oxidation and therefore energy production [Frayn 1995]. When weight is maintained the energy intake equals energy expenditure and therefore an imbalance between energy intake and energy expenditure results in either weight gain or weight loss [Swinburn & Ravussin 1994]. The role of individual substrates (lipids, protein and carbohydrate) in the energy balance is less well defined as reviewed by Frayn (1995). According to Stubbs (1998) the profile of substrates utilised for energy metabolism is mainly determined by the dietary macronutrient composition. The oxidation of the substrates is shifted towards the dietary substrate composition, so that the lipid oxidation increases on a high-lipid, low-carbohydrate diet and carbohydrate oxidation increases on a high-carbohydrate diet [Hill *et al.* 1991]. However, studies measuring substrate oxidation rates by indirect calorimetry or using respiratory chamber have shown that an increase in the lipid content of a meal does not seem to affect the extent of lipid oxidised [Flat *et al.* 1985; Schutz *et al.* 1989]. These observations suggest that following a mixed meal, carbohydrates and proteins are preferentially oxidised and the energy requirements are met by the lipid oxidation and any excess energy is stored. Combining stable isotope tracer technique with indirect calorimetry measurements showed that an increase in the amount of lipid in a meal increased the dietary lipid oxidation and suppressed the endogenous lipid oxidation, whilst the net lipid oxidation rates were similar following both meals [Bennoson *et al.* 1998]. However, the increase in the dietary lipid oxidation was not directly proportional to the increase in the amount of dietary lipid in the meal. Although it is debatable whether a change in a lipid content of a meal affects lipid oxidation, the lipid balance is altered as a result of addition of lipid in a meal. Excess lipid intake compared to the energy requirements results in a positive lipid balance (storage) and therefore in a positive energy balance [Flatt *et al.* 1985; Diaz *et al.* 1992] whereas negative energy balance promotes lipid oxidation [Abbott *et al.* 1988]. It seems that the capacity to oxidise substrates beyond the requirement is limited and consequently the excess energy intake compared to the requirement is stored. The storage capacity of lipids is large whereas protein and

carbohydrate stores are smaller causing a hierarchy in the extent to which macronutrients may be stored [Stubbs 1998]. Elia *et al.* (1988) showed that following a mixed meal the net carbohydrate oxidation increased whilst the net lipid oxidation decreased. Similarly increase in the intake of carbohydrates and proteins results in increased oxidation of these substrates [Flatt *et al.* 1985; Abbott *et al.* 1988; Stubbs 1998]. However, the oxidative capacity of carbohydrates is limited and the excess is stored as glycogen and when glycogen stores are saturated as lipids (lipogenesis) [Schutz *et al.* 1985; Acheson *et al.* 1988]. Lipids have large body stores and therefore they may act as an energy buffer by releasing or storing energy as required. It seems that the lipid balance reflects the energy balance, which suggests that lipid balance is not directly regulated [Frayn 1995]. Although, the regulation is not well understood, an overall metabolic response to a meal (oxidation vs. storage) seems to be influenced by the composition and quantities of macronutrients in the ingested meal and also the nutritional status of the individual.

2.6 Cystic fibrosis

Lipid metabolism may be altered in cystic fibrosis due to several factors either related to the disease characteristics or nutritional status of patients and therefore offers an opportunity to investigate the consequences of various altered processes of lipid metabolism. The digestion and absorption of dietary lipid may be altered in cystic fibrosis due to disturbances in pancreatic and liver function and intestinal luminal environment and properties of intestinal mucosa [Kopelman 1991]. Reduced digestion and absorption of lipids result in excessive lipid losses in stools, which may contribute to an energy deficit sufficient to limit growth and cause weight loss [Wootton *et al.* 1991]. Very little is known about energy metabolism and metabolism of the absorbed dietary lipid in cystic fibrosis, which could however influence growth and body composition of the patients. The present understanding of the pathogenesis and symptoms of cystic fibrosis, gastrointestinal handling of dietary lipid and energy metabolism and growth in cystic fibrosis are summarised.

2.6.1 Pathogenesis and symptoms

Cystic fibrosis is inherited as an autosomal recessive way resulting in a gene defect and therefore protein defect, cystic fibrosis transmembrane conductance regulator (CFTR), which produces an abnormality in the regulation of chloride channel in the cell membranes [Cuthbert 1992; Kumar & Clark 1994]. In exocrine glands such as pancreas the basic defect results in thick viscoid secretions. Over 100 genetic defects in the CFTR gene have been identified, the most common defect being ΔF508 which in the UK cystic fibrosis population accounts for about 76% of the cases [Super 1992]. The prevalence of cystic fibrosis is 1 in 2000 in Caucasians [Forstner 1986]. Over 90% of the cystic fibrosis children survive into their teens and the mean survival age is 29 years [FitzSimmons 1993]. The management of cystic fibrosis involves principally PERT, medication (aerosol inhalation and antibiotics), vitamin and mineral supplementation, dietary advice (high-lipid and high-energy diet) and chest physiotherapy [Nelson *et al.* 1994, Hill 1998].

The main symptoms are a high sweat sodium and chloride concentration, which is used in diagnosis, pulmonary infections and gastrointestinal symptoms. Several pancreatic, intestinal and hepatic manifestations have been described in cystic fibrosis. Especially alterations in the pancreatic and liver function affect the gastrointestinal handling of dietary lipid, since their secretions (lipase, bicarbonate and bile salts) are necessary for lipid digestion and absorption. Gastroesophageal reflux, vomiting, constipation, and diarrhoea have been reported in cystic fibrosis [Littlewood 1992]. Various obstructions, such as meconium ileus (relative incidence 10% to 15%) which is a result of a failure to pass meconium by a newborn infant and distal intestinal obstruction syndrome (relative incidence 10% to 20%) in older patients resulting in cramping abdominal pain, tenderness and vomiting are also typical gastrointestinal symptoms in cystic fibrosis [Kopelman 1991, FitzSimmons 1993].

2.6.2 Gastrointestinal handling of dietary lipid

The functional capacity of the gastrointestinal tract to digest and absorb dietary lipid may be altered in cystic fibrosis as a result of abnormalities in the exocrine secretory capacity of the pancreas, liver and intestinal glands [Kopelman 1991]. The specific features of the pancreatic function, bile salt metabolism and luminal environment in the cystic fibrosis are

reviewed.

2.6.2.1 Pancreatic function

It has been estimated that approximately 85% of the cystic fibrosis patients are pancreatic insufficient [Gaskin *et al.* 1991]. The pathological features of the pancreas include reduction or lack of pancreatic secretions, resulting from thickening of the secretions in the pancreatic ducts, dilatation of acini and cell atrophy [Sturgess 1984]. The normal development of exocrine pancreatic tissue may be altered already in foetus and is presented as a decrease of acinar to connective tissue ratio in cystic fibrosis patients compared to healthy individuals [Imrie *et al.* 1979]. Degenerative process of the pancreas may proceed after birth suggesting that there may be a lack of early normal maturation or a persistence of the foetal pattern in the development of pancreatic exocrine tissue [Imrie *et al.* 1979]. Gaskin *et al.* (1991) found that approximately half of the cystic fibrosis infants (31 of 64) studied were pancreatic sufficient at the time of the diagnosis and during 9 years of screening 28% (11 of 39) of the patients developed pancreatic insufficiency as assessed by decline in colipase secretion or increased stool lipid losses. Some patients showed improvement in colipase secretion, which suggests that the development of pancreatic insufficiency may not be inevitable in cystic fibrosis.

Alterations in the pancreatic function result in several manifestations that may have implications for the clinical management of the patients. The reduced secretion of pancreatic lipase may disturb lipid digestion. Reduced secretion of bicarbonate may alter gastrointestinal pH, which may cause inactivation of residual lipase activity or precipitation of bile salts. Consequently, pancreatic insufficient patients generally have excessive lipid losses in stools, which may also reduce the availability of lipid soluble vitamins from diet and increase gastrointestinal symptoms. Furthermore reduced energy availability may limit growth and therefore contribute to the development of malnutrition. It has been suggested that meconium ileus, distal obstruction syndrome and rectal prolapse are related to the pancreatic dysfunction [Davidson 1995]. Stool lipid losses in cystic fibrosis and PERT, which is used to manage pancreatic insufficiency are discussed in 2.6.2.4.

2.6.2.2 Bile salt metabolism

Bile salts have two major functions in digestion and absorption of dietary lipid, they solubilise large lipid particles of the food into smaller particles so that lipase can act on them and they facilitate movement of the digested lipid to the intestinal mucosa for absorption [Hofmann & Borgström 1964]. In healthy individuals the liver synthesises approximately 0.6 g bile salts daily of which more than 90% is reabsorbed in the ileum and enters enterohepatic circulation [Borgström *et al.* 1963]. In cystic fibrosis the bile salt losses in stools are commonly elevated compared to healthy individuals [Weber *et al.* 1973; Fondacaro *et al.* 1982; Goodchild *et al.* 1975; Jonas & Diver-Haber 1988]. The studies that have investigated bile salt excretion in stools in cystic fibrosis patients are summarised in Table 2.4. The mean bile salt losses in stools were 1.4 to 6.8 times higher in the cystic fibrosis patients compared to the control subjects. Increased bile salt losses in stools may reduce the body bile salt pool and therefore inhibit digestion of lipids.

Several mechanisms have been proposed for increased bile salt losses in stools in cystic fibrosis. Unhydrolysed lipid or other products of maldigestion may bind bile salts, which fail to be absorbed and are excreted in stools [Weber *et al.* 1973; Watkins *et al.* 1977b]. Therefore, patients with pancreatic insufficiency may have higher bile salt losses than patients with pancreatic sufficiency [Colombo *et al.* 1984] and accordingly PERT may reduce stool bile salt losses in patients with pancreatic insufficiency. However, no difference was found when stool bile salt losses of patients with cystic fibrosis were compared with and without PERT [Watkins *et al.* 1977b]. A more likely cause for excessive bile salt losses in stools is the reduced pH of the gastrointestinal tract, which may precipitate bile salts and therefore causes loss of bile salts in stools [Regan *et al.* 1979]. Bile salt malabsorption may also result from the mucosal cell defect or dysfunction of ileal transport processes which prevent bile salt reabsorption [Harries *et al.* 1979].

Excessive bile salt losses in stools in the cystic fibrosis patients may be compensated to some extent by an increased synthesis of bile salts in the liver. This capacity has been estimated to be 3 to 4 times the bile salt pool size [Small *et al.* 1972]. However, due to persistent bile salt losses in stools, the size of the bile salt pool may diminish and the intraluminal bile salt concentration may be insufficient to solubilise lipid. Furthermore bile salt secretion from the liver may be reduced in cystic fibrosis compared to controls [Weizman *et al.* 1986]. It has been suggested that the ratio of glycine to taurine

conjugated bile salts is increased in cystic fibrosis, which could contribute to the bile salt malabsorption [Roy *et al.* 1977]. Tauroconjugates are more soluble in the duodenum than glycoconjugates affecting the micelle formation properties of the bile salts [Darling *et al.* 1985]. Taurine supplementation has been used in cystic fibrosis to alter the ratio of glycine to taurine conjugated bile salts to resemble that of healthy individuals [Darling *et al.* 1985; Belli *et al.* 1987]. However, the beneficial effects of the intervention in reducing excessive lipid losses in stools are disputed [Thompson *et al.* 1987; Thompson 1988; De Curtis *et al.* 1992].

Table 2.4. Bile salt excretion in stools in cystic fibrosis patients.

Reference	*n*		Age, yr Mean (Range)				Bile salt excretion in stools Mean ± SD	
	Cystic fibrosis	Controls	Cystic fibrosis		Controls		Cystic fibrosis	Controls
Weber *et al.* 1973	24	18	2.5	(0.2 - 9)	2.4	(0.3 - 5)	743.2 ± 55.3 mg/m^2/d [a, 1]	110.0 ± 11.0 mg/m^2/d [1]
Fondacaro *et al.* 1982	5	5	16.7	-	31	-	214.0 ± 48.4 mg/m^2/d [a]	90.8 ± 21.9 mg/m^2/d
Goodchild *et al.* 1975	29	11	8.8	(0.3 - 20.6)	6.3	(0.5 - 13.8)	47.6 ± 37.0 μmol/kg/d [a]	18.0 ± 2.8 μmol/kg/d
Jonas & Diver-Haber 1988	5 2	3	-	(3 – 20) (3 – 7)	-	(5 - 15)	62.4 ± 45.0 μmol/kg/d [2,a] 25.2 μmol/kg/d [3]	17.9 ± 1.9 μmol/kg/d
Colombo *et al.* 1984	4 3	4	12.5	-	Adults		11.2 ± 3.8 mg/kg/d [4, a] 2.2 ± 0.45 mg/kg/d [5]	2.8 ± 1.1 mg/kg/d
Watkins *et al.* 1977b	6	-	1.8	(0.3 - 4.5)	-		513 ± 146 mg/d [6] 545 ± 166 mg/d [7]	-
Walters & Littlewood 1998	132	28	9.4	(0.5 - 30)	11.3	(1 - 30)	397.4 ± 381.4 mg/d	194.7 ± 83.1 mg/d

[1] SEM, [2] Severe steatorrhoea, [3] mild steatorrhoea.
[4] Patients with pancreatic insufficiency, [5] patients with pancreatic sufficiency.
[6] With PERT, [7] without PERT.
[a] Statistically significant significantly different from controls.
- Not reported.

2.6.2.3 Luminal environment

Gastrointestinal luminal environment is altered in cystic fibrosis due to changes in pH and viscous intestinal secretions, which may affect the digestion and absorption of lipids. The gastrointestinal luminal pH may be decreased due to gastric acid hypersecretion [Cox *et al.* 1982] and reduced bicarbonate secretion due to pancreatic insufficiency [Sturgess 1984]. The reduced postprandial pH within the small bowel contributes to lipid maldigestion by inactivating lipase [Barraclough & Taylor 1996] and by precipitating bile salts [Regan *et al.* 1979]. In cystic fibrosis the postprandial pH in the duodenum may remain below 5 at which pH, the lipolytic enzymes are inactivated [Barraclough & Taylor 1996]. However, the pH may rise during the transit and reach similar values in the distal intestine in cystic fibrosis patients compared to control subjects [Gilbert *et al.* 1988]. The rise in pH may be sufficient for lipid digestion and absorption to occur, although Zentler-Munro *et al.* (1984) showed that also the jejunal pH was reduced in some cystic fibrosis patients.

The basic gene defect in cystic fibrosis (CFTR) results in altered electrolyte transport by epithelia in various affected organs. These alterations are best known in the sweat glands where the reabsorption of sodium and chloride fails due to a low chloride permeability of the sweat duct and results in increased salt content of the sweat [Cuthbert 1992]. In contrast, in the pancreas, CFTR causes decreased secretion of chloride which results in a low water and bicarbonate content of the pancreatic secretions [Wilschanski & Durie 1998]. Consequently the protein concentration in the secretions increases and causes precipitates which may obstruct the pancreatic ducts. Thus, the pancreatic secretions to the duodenum are reduced resulting in low lipase, bicarbonate and fluid content. The effects of CFTR on the secretions of the small intestine are less well known. Bouquet *et al.* (1988) and Hardcastle *et al.* (1990) showed that in cystic fibrosis chloride secretion was reduced in biopsy specimens of the small intestine. The resulting dehydration may be further enhanced by possibly increased absorption of glucose [Hardcastle *et al.* 1990]. It has also been shown that mucus of cystic fibrosis patients has an increased viscosity and the carbohydrate content [Wesley *et al.* 1983]. The thick viscous contents of the intestinal lumen may prevent the diffusion of lipid digestion products to the epithelial cells and therefore hinder absorption of dietary lipid. However, these alterations are not well understood.

2.6.2.4 Lipid losses in stools and PERT

Table 2.5 compares lipid losses in stools in cystic fibrosis patients receiving different types of pancreatic enzyme preparations. The stool lipid losses are commonly expressed as mass (g/d) or as a proportion of daily lipid intake or as a coefficient of lipid absorption [(Dietary lipid intake - Stool lipid losses) / Dietary lipid intake × 100]. Expressing stool lipid losses as a proportion of intake or as a coefficient of lipid absorption requires accurate measurement of dietary lipid intake in addition to measurement of lipid losses in stools, therefore a preferable way to report stool lipid losses is as g/day.

Lipid losses in stools are variable in cystic fibrosis patients ranging from normal losses (less than 7 g/d) to extremely high losses (nearly 60 g/d). Lipid losses in stools may be elevated even when the patients receive PERT. It was discussed previously (See 2.3.3) that stool lipid is not only of dietary origin but may also be derived from endogenous secretions, desquamated cells and bacteria. In cystic fibrosis patients stool bacterial mass may be up to three times greater than in control subjects, average losses being 13 g/day [Murphy *et al.* 1991]. Therefore it is likely that a larger proportion of stool lipid is derived from bacteria in cystic fibrosis patients than in healthy controls. Lipid losses in stools may be used as an estimate of dietary lipid losses but do not measure the extent of maldigestion and malabsorption of dietary lipid.

Due to high prevalence of pancreatic insufficiency and excessive lipid losses in stools, cystic fibrosis patients commonly receive PERT, which contain pancreatic enzymes: lipase, amylase and protease. The first conventional pancreatic enzyme preparations were either in powder, tablet or capsule form [George & Mangos 1988]. The conventional preparations reduced lipid losses in stools in cystic fibrosis patients [Lapey *et al.* 1974] but the stool lipid losses remained elevated in most patients. The inadequate improvement of stool lipid losses with the conventional enzyme preparations may have been due to inactivation of the lipase in the acidic environment of the stomach [George & Mangos 1988]. In some patients the administration of gastric acid regulators with the conventional enzyme preparations reduced the stool lipid losses [Chalmers *et al.* 1985].

The most recent type of enzyme preparations are acid resistant enteric coated microspheres. The microspheres resist dissolution of enzymes until the pH in the gastrointestinal tract exceeds 5.5, therefore the inactivation of lipase is prevented in the acidic environment of the stomach [Littlewood *et al.* 1988]. The enteric coated

microspheres have improved the management of excessive lipid losses in stools in cystic fibrosis patients compared to the conventional enzyme preparations [Gow *et al.* 1981; Mitchell *et al.* 1982; Stead *et al.* 1987; Dutta *et al.* 1988]. Improved efficacy of enteric coated microspheres in regulating lipid losses in stools has been attempted by altering gastrointestinal pH using products such as cimetidine (reduces gastric acid output and gastric secretion), famotidine and omeprazole (reduce gastric acid secretion) or misoprostol (reduces gastric acid secretion and stimulates duodenal bicarbonate production). Although some studies have shown reduced lipid losses in stools with enteric coated microspheres and acidity regulators compared to microspheres on their own [Cleghorn *et al.*1988; Carroccio *et al.* 1992], the differences between the compared groups have been modest [Robinson & Sly 1990] or no additional benefits with the acidity regulators have been achieved [Gow *et al.* 1981; Heijerman *et al.* 1993]. Detergents such as Tween 80 (polyoxyethylene sorbitan monoleate) may improve the efficacy of PERT by increasing micelle formation and therefore lipid absorption [King *et al.* 1979], however Bouquet *et al.* (1988) found no additional benefits of detergents administered with enzyme preparations.

The enteric coated microsphere preparations, possibly administered together with an acidity regulator, may normalise lipid losses in stools but stool lipid losses remain elevated in some patients as can be seen from studies summarised in Table 2.5. It is possible that despite administering the enzymes as acid resistant preparations, the enzymes are partially inactivated during the gastrointestinal transit. In pancreatic insufficient cystic fibrosis patients the duodenal pH may reduce to 4 after eating, whereas in healthy individuals the pH in the duodenum stays at 5.5 to 6.5 [DiMagno *et al.* 1977; Guarner *et al.* 1993]. It has also been suggested that the late release of enzymes from the enteric coated microsphere preparations may result in wasting of the enzymes to ileum, where the digestive and absorptive capacity of the lipids is low. Whether the pancreatic enzyme dose in the studies has been adjusted for the individual requirements is also uncertain and the habitual dose, may have been inadequate to correct the stool lipid losses in some patients. Furthermore some cystic fibrosis patients have similar lipid losses in stools compared to healthy individuals and it is unlikely that altering the enzyme therapy in these patients would improve lipid losses in stools. The increased lipid losses in stools in cystic fibrosis patients despite PERT may also be due to factors other than maldigestion of lipids, possibly related to malabsorption of lipids.

Older patients or parents of the younger patients often regulate the dose of enzyme

preparations according to severity of the gastrointestinal symptoms and stool appearance, which may lead to an extensive increase in the amount of enzymes consumed. It has been shown that the dose of enzyme preparations may be reduced in some patients without affecting stool lipid losses [Robinson & Sly 1989]. Furthermore, it has been suggested that excessively high lipase intakes may have adverse effects on the gastrointestinal function. The intake of high-strength pancreatic enzyme preparations in large doses has been shown to associate with thickening of colonic wall and development of colonic strictures known as fibrosing colonopathy [Smyth *et al.* 1994; Mac Sweeney *et al.* 1995]. In a retrospective evaluation, five cystic fibrosis patients had developed histopathological changes in the mucosal cells of colon following a change of the conventional pancreatic enzyme preparations to high-strength preparations 12 to 15 months prior to diagnosis of colonic strictures [Smyth *et al.* 1994]. The relationship between high-strength enzyme preparations and fibrosing colonopathy was subsequently shown in a case-control study [Smyth *et al.* 1995]. However, it has been suggested that the fibrosing colonopathy is related to the surface coating of certain enzyme preparations rather than to the lipase intake [Van Velzen *et al.* 1996]. It was shown that the surface coating, Eudragit L30D55 (methacrylic acid copolymer), which is used in tablet preparations caused fibrosing colonopathy in pigs. Due to the high dose of enzyme preparations consumed by the cystic fibrosis patients, the intake of Eudragit L30D55 can exceed the limit for the safe intake and potentially may cause fibrosing colonopathy. The concern over the association between lipase intake and fibrosing colonopathy led to the recommendation by the Committee on Safety of Medicines (1994) that the daily lipase intake of the cystic fibrosis patients should not exceed 10 000 IU/kg body weight per day. Similarly in the USA it is recommended that the lipase intake should not exceed 2500 IU/kg body weight per meal to prevent the adverse effects of PERT [Borowitz *et al.* 1995].

Table 2.5. Excretion of lipid in stools in cystic fibrosis patients following PERT.

Reference	*n*	Age, yr		PERT [1]	Stool lipid excretion	
		Mean	(Range)		Mean ± SD	(Range)
					g/d	
Leroy *et al.* 1986	8	-	(4 - 12)	Conventional	15.3 ± 25.3 [3]	-
Chalmers *et al.* 1985	13	-	(5 - 19)	Conventional + placebo	40.6	-
				Conventional + 25 mg/kg/d cimetidine [2]	27.0 [a]	-
De Biéville *et al.* 1981	13	-	(6 - 15)	Conventional (Pancreatin Organon®)	17.5 ± 9.2	(5.6 - 35.7)
				Conventional (Pancreatin Organon®)+ 150 - 300 mg cimetidine [2]	20.4 ± 10.7	(5.2 - 43.9)
				Conventional (Pancreatin Organon®)+ 450 - 600 mg cimetidine [2]	19.1 ± 15.2	(4.7 - 58.6)
Mitchell *et al.* 1982	12	9.6	-	Conventional (Viokase®), 88 000 units lipase	14.5 ± 7.5	-
				Conventional (Viokase®), 176 000 units lipase	17.3 ± 9.1	-
				ECM (Pancrease®), 44 000 units lipase	11.5 ± 6.9	-
				ECM (Pancrease®), 88 000 units lipase	8.7 ± 4.1 [b]	-
Stead *et al.* 1987	23	24.8	-	Conventional (Pancrex V Forte®)	27.1	-
				ECM (Creon®)	15.2 [b]	-
Bouquet *et al.* 1988	12	-	(8 - 15)	ECM (Pancrease®)	21.3	-
Brady *et al.* 1991	8	-	(7 - 10)	ECM (Pancrease®), usual dose	7.9 ± 1.8 [3]	(1.2 - 16.6)
				ECM (Pancrease®), ¼ dose	11.9 ± 2.4 [3]	(3.2 - 23.8)
Cleghorn *et al.* 1988	11	9.6	(2 - 17)	ECM (Pancrease®)	14.7 ± 1.7	(1.4 - 41.0)
				ECM (Pancrease®) + misoprostol [2]	7.5 ± 3.3	(5.4 - 13.5) [c]

Table 2.5. Continued.

Reference	*n*	Age, yr		PERT [1]	Stool lipid excretion	
		Mean	(Range)		Mean ± SD	(Range)
					% of daily lipid intake	
Lapey *et al.* 1974	20	-	(12 - 17)	Without enzymes	38.7 ± 20.0	(5.6 - 75.3)
				Conventional (Cotazym®)	25.4 ± 16.5 [d]	(4.3 - 59.2)
Dutta *et al.* 1988	8	24	(18 - 29)	Conventional (Cotazym®)	5.9 ± 2.0 [3]	-
				ECM (Pancrease®)	19.2 ± 5.7 [3,b]	-
Gow *et al.* 1981	11	-	(6 - 13)	Conventional (Cotazym®)	29.5 ± 4.6 [3]	-
				ECM (Pancrease®)	9.6 ± 1.9 [3,b]	-
				ECM (Pancrease®) + cimetidine [2]	6.9 ± 0.8 [3,b]	-
				ECM (Pancrease®) + antacid [2]	11.2 ± 2.1 [3,b]	-
Lancellotti *et al.* 1996	20	17.7	(10.9 - 29.5)	ECM (Pancrease®), low strength	12.7 ± 18.6	-
				ECM (Pancrease®), high strength	16.7 ± 14.3	-
Robinson & Sly 1990	17	6.2	(0.5 - 13.8)	ECM (Pancrease®) + placebo	19 ± 15.9	(6 - 65)
				ECM (Pancrease®) + misoprostol [2]	14 ± 13.2 [a]	(3 - 58)
Heijerman *et al.* 1993	11	-	(20 - 42)	ECM (Pancrease®) + placebo	22.9	(12 - 44)
				ECM (Pancrease®) + omeprazole [2]	18.1	(4 - 45)

Table 2.5. Continued.

Reference	*n*	Age, yr		PERT [1]	Stool lipid excretion	
		Mean	(Range)		Mean ± SD	(Range)
					Coefficient of lipid absorption % [4]	
Beverley *et al.* 1987	19	-	(6 - 20)	Conventional (Pancrex V Forte®)	74 [5,c]	(52 - 93)
				ECM (Pancrease®)	87 [5]	(24 - 95)
				ECM (Creon®)	85 [5]	(56 - 94)
				ECM (Pancreatin Merck®)	81 [5,e]	(34 - 94)
George *et al.* 1990	21	8.9	(3 - 27)	ECM (Pancrease®)	80.7 ± 2.7 [3]	-
				ECM (Creon®)	81.2 ± 3.1 [3]	-
Williams *et al.* 1990	27	-	(5 - 17)	ECM (Pancrease®)	91.3 [5]	(55.1 - 95.4)
				ECM (Creon®)	92.4 [5]	(70.4 - 98.8)
Carroccio *et al.* 1992	10	12.5	(7 - 18)	ECM (Pancrease ®) + placebo	74.4 ± 13.4	-
				ECM (Pancrease ®) + famotidine [2]	87.3 ± 4.2 [a]	-

[1] Conventional PERT = powder, tablet or capsule and ECM PERT = enteric coated microspheres.
[2] Acidity regulator.
[3] Mean ± SEM.
[4] Coefficient of lipid absorption = [(Dietary lipid intake - Stool lipid losses) / Dietary lipid intake × 100].
[5] Median.
Statistically significantly different from: [a] placebo, [b] conventional PERT, [c] ECM PERT, [d] without enzymes, [e] ECM PERT (Pancrease®).
- not reported.

2.6.3 Energy metabolism and growth

A balance between intake of energy and nutrients in adults results in weight maintenance whereas a positive energy balance is required for growth [Swinburn & Ravussin 1994]. Although not well understood, the amount and the proportion of substrates (macronutrients) utilised for energy production both from diet and from endogenous sources may influence body composition and growth rate. Growth is defined as an increase in stature and mass, but also involves changes in body composition and relative size of organs and tissues [Widdowson 1994]. Achievement of a genetically determined adult height (expected by the parent's height) occurs in the presence of an adequate dietary supply of energy and nutrients, an adequate hormone production (e.g. growth hormone) and a good general health including the absence of diseases. Assuming that the hormonal requirements for growth have been fulfilled, the primary determinant of the growth rate is the availability of energy [Jackson & Wootton 1990]. However, the requirements for all the nutrients for the growth have to be satisfied and ultimately the availability of the nutrient, which is available in the least amount relative to the demands for growth, a limiting nutrient, determines the growth rate [Jackson & Wootton 1990]. The use of other nutrients is dependent on the availability of the limiting nutrient and the energy utilisation may become less efficient if the availability of any vitamin or mineral is insufficient compared to the requirements.

Cystic fibrosis patients may have difficulties in achieving an expected adult height and weight in comparison to population growth standards and also in maintaining adult weight [Corey *et al.* 1988; Morison *et al.* 1997; Lai *et al.* 1999; McNaughton *et al.* 1999]. Disturbed growth and a poor nutritional status may be related to a decreased pulmonary function [Kraemer *et al.* 1978] and may affect the overall survival of the patients. In cystic fibrosis the intake of energy from the diet may be decreased and losses of energy in stools may be increased due to altered digestion and absorption of lipids, which in addition to possibly altered energy metabolism may influence growth rate or disturb the maintenance of a good nutritional status.

Due to a common occurrence of excessive lipid losses in stools in cystic fibrosis patients, a low-lipid diet was previously recommended. However, when two cystic fibrosis centres, one in Toronto and one in Boston, were compared it was found that growth and nutritional status parameters of patients in Toronto were greater and related to a better

survival compared to patients in Boston [Corey *et al.* 1988]. In Boston, patients were advised to consume a low-lipid diet, whereas in Toronto a high-lipid and high-energy diet was introduced as part of the patient management. Comparing the two centres again after the high-lipid, high-energy diet was implemented in Boston, the differences in the growth indices between the centres had reduced [Lai *et al.* 1999] suggesting that the increase in dietary lipid and energy intake had improved growth of the cystic fibrosis patients. However, in both centres the average growth indices of the cystic fibrosis patients were below the population average ranging from –0.3 to –0.7 SD in Toronto and –0.4 to –0.8 SD in Boston. In the UK the mean height and weight SD scores of cystic fibrosis patients aged 1 to 10 years were also about -0.5 SD [Morison *et al.* 1997]. In addition, Morison and co-workers observed a decline in growth SD scores to an average of about –1 SD in cystic fibrosis children over 10 years of age.

It has been shown that weight gain and an increase in height may be achieved in cystic fibrosis patients by increasing energy intake [Parsons *et al.* 1983]. When adequate nutrition is reintroduced following a growth delay or weight loss, the growth velocity can accelerate due to catch-up growth [Jackson & Wootton 1990]. If a sufficient energy intake for growth is not achieved by increasing dietary energy intake or by additional oral supplements, an overnight nasogastric or gastrostomy feeding may be useful. It has been shown that a long-term overnight gastrostomy feeding induces growth in malnourished cystic fibrosis patients [Levy *et al.* 1985; Rosenfeld *et al.* 1999]. Although supplementary feeding induces growth, the increased energy intake may not be sufficient alone to normalise growth of cystic fibrosis patients to the level of population average [Levy *et al.* 1985]. The complete recovery in size might not be possible especially if the undernutrition occurs when the growth is rapid. This may be because cell division is hindered during undernutrition and despite adequate supply of food later on, the cell divisions that were lost may not be regained [Widdowson 1994]. It has been suggested that the weight gain due to supplementary feeding is due to an increase in both body fat mass and LBM [Levy *et al.* 1985]. However it is not know whether both fat mass and LBM mass are increased in similar proportions during refeeding. Rosenfeld *et al.* (1999) showed that supplemental feeding in cystic fibrosis patients resulted initially in weight gain, which was followed by increase in height suggesting that body composition may be affected by feeding.

In addition to an inadequate dietary intake, disturbed growth and difficulties in maintaining weight or weight loss in cystic fibrosis patients may also result from increased

energy losses in stools due to maldigestion and/or malabsorption of lipids (See 2.6.2) or due to increased energy requirements. Several studies have reported increased BMR by 10% to 30% in cystic fibrosis patients compared to control subjects or compared to predicted energy expenditure [Vaisman *et al.* 1987; Buchdahl *et al.* 1988; Spicher *et al.* 1991; Grunow *et al.* 1993]. However, also similar BMR in cystic fibrosis patients have been reported [Ward *et al.* 1999]. Reasons for the possibly increased BMR are disputed and are most likely to be multifactorial. Increased BMR may be associated with decreased pulmonary function and increased energy cost of breathing [Vaisman *et al.* 1987; Fried *et al.* 1991; Hirsch *et al.* 1989]. It has been suggested that the breathing work is increased in cystic fibrosis to deliver the required oxygen due to airflow obstruction [Hirsch *et al.* 1989]. However, it has been shown that the BMR may be elevated in cystic fibrosis patients even when the pulmonary function is normal [Vaisman *et al.* 1987] or no association has been found with increased BMR and the severity of the pulmonary disease [Tomezsko *et al.* 1994; Zemel *et al.* 1996]. It is possible also that a subclinical pulmonary inflammation could explain increased BMR [Thomson *et al.* 1996]. It has been suggested that the increased BMR in cystic fibrosis is related to a specific gene defect. A higher BMR has been associated with ΔF508 homozygous genotype compared to other genotypes [O'Rawe *et al.* 1992; Thompson *et al.* 1996]. However Fried and co-workers (1991) and Tomezsko and co-workers (1994) found increased BMR in cystic fibrosis patients, which was not related to the genotype. There may also be a basal defect in energy metabolism of cystic fibrosis patients related to alterations in calcium movement to mitochondria that could contribute to the increased energy expenditure in the cystic fibrosis [Feigal *et al.* 1982]. However, the significance of the altered calcium metabolism for energy metabolism is not understood.

Total energy expenditure determines the overall energy requirements irrespective of an increase in BMR. Both similar [Spicher *et al.* 1991] and increased [Shepherd *et al.* 1998; Tomezsko *et al.* 1994] total energy expenditures have been reported in cystic fibrosis patients compared to control subjects. Total energy expenditure may be increased in the cystic fibrosis patients due to increased BMR, but equally cystic fibrosis patients may compensate for the increased BMR by decreasing activity due to ill health. An increased energy expenditure in cystic fibrosis patients has been found during both rest and exercise but the incremental increase in the energy expenditure from resting to activity levels did not differ between the cystic fibrosis patients and the control subjects suggesting that the increase in the energy expenditure was due to increased BMR [Grunow *et al.* 1993]. Also

similar energy expenditures in the cystic fibrosis patients and control subjects during exercise have been observed [Spicher *et al.* 1991], which suggests that the total energy expenditure of the cystic fibrosis patients is similar to that of the control subjects or that the cystic fibrosis patients expend less energy in excess to their resting values compared to controls.

Contribution of substrates utilised for energy expenditure has been measured in cystic fibrosis patients only during postabsorptive period. Higher postabsorptive respiratory exchange ratio (RER) has been observed in cystic fibrosis patients compared to control subjects [Hirsch *et al.* 1989: 0.86 vs. 0.78; Spicher *et al.* 1991: 0.834 vs. 0.797; O'Rawe *et al.* 1992; 0.87 vs. 0.80, cystic fibrosis vs. controls respectively], which suggests that carbohydrate oxidation contributes a greater proportion of BMR in the cystic fibrosis patients. When expressing substrate utilisation as a proportion of the BMR, a higher carbohydrate oxidation has been observed in the cystic fibrosis patients compared to the control subjects [Spicher *et al.* 1991: 50% vs. 26% of BMR; Bowler *et al.* 1993: 44% vs. 30% of BMR, cystic fibrosis vs. controls respectively], whilst no difference was found in the lipid oxidation between the groups. However, Ward *et al.* (1999) and Hardin *et al.* (1999) found no differences in the postabsorptive carbohydrate or lipid oxidation rates amongst patients with cystic fibrosis, patients with cystic fibrosis related diabetes mellitus and control subjects. Bowler *et al.* (1993) were the only group to measure the previous dietary intake of macronutrients and found that the intake of carbohydrates was higher in the cystic fibrosis patients compared to the control subjects during 24 hours prior to the measurement, which could account for the greater contribution of carbohydrates to the BMR. Although these studies indicate that carbohydrates contribute a greater proportion of total energy expenditure in cystic fibrosis patients, further studies are required to confirm the findings especially during postprandial state and also to investigate what factors determine the substrate utilisation in cystic fibrosis.

2.7 Summary, hypothesis and aims

A co-ordinated functional capacity of the gastrointestinal tract, which results in almost complete digestion and absorption of dietary lipid (stool lipid losses less than 7 g/d), for subsequent partitioning and metabolism is important in providing the necessary conditions for growth and maintenance of good nutritional status. Cystic fibrosis patients commonly have excess stool lipid losses due to various disturbances in gastrointestinal function including alterations in pancreatic capacity, bile salt metabolism and luminal environment. Excess stool lipid losses cause gastrointestinal symptoms and may limit the energy and nutrient availability from the diet resulting in disturbed growth, weight loss and poor nutritional status. Patients who show disturbed growth or weight loss sometimes receive supplementary feeding, which usually increases weight gain. However, the nutritional status of some cystic fibrosis patients is poor despite dietary intervention and PERT. Elevated stool lipid losses in some patients indicate that PERT alone is an inadequate therapy for the normalisation of lipid digestion and absorption and it is possible that some patients have malabsorption in addition to maldigestion. The ability to distinguish between and quantify the separate processes of maldigestion and malabsorption would be of benefit for current treatment and enable the development of new therapies for management of excessive lipid losses in stools.

Persistant growth problems and a poor response to routine clinical management in some patients suggest that the metabolism of absorbed lipid may be altered. However, little is known about the metabolism of absorbed lipid in cystic fibrosis and it has generally been assumed not to differ from that of healthy individuals. The postabsorptive energy expenditure measurements suggest that BMR is elevated and carbohydrates are used for energy production to a larger extent in cystic fibrosis patients compared to healthy individuals. However, postprandial substrate metabolism is poorly understood. It is not known whether the metabolic partitioning of lipids and the capacity to oxidise lipids of cystic fibrosis patients is similar to healthy individuals. Metabolic partitioning of absorbed lipids may influence growth and body composition and therefore it is important to understand the determinants of the metabolic disposal of dietary lipid.

Poor understanding of both the gastrointestinal handling and the metabolic disposal of dietary lipid may be partially explained by lack of methods that would enable direct measurements of lipid metabolism within human body. A novel approach, stable isotope

tracer technique, offers an opportunity to quantitatively explore the digestion and absorption processes and the subsequent metabolic disposal of dietary lipid in both children and adults, although further development of the methods is required.

These observations led to the hypothesis of this thesis that:

> Poor nutritional status or disturbed growth in cystic fibrosis patients is associated with two metabolic disturbances: 1) increased stool lipid losses due to malabsorption of dietary lipid in addition to maldigestion and 2) altered metabolism of absorbed dietary lipid.

To test the hypothesis the aims of this thesis were:

1) To develop and evaluate methods, which may be used to differentiate between maldigestion and malabsorption of dietary lipid.
2) To determine amount and type of lipid losses in stools in cystic fibrosis patients compared to control subjects.
3) To investigate the metabolic disposal of absorbed lipid in cystic fibrosis patients compared to control subjects.

Patients who receive supplementary feeding are most likely to have problems with the gastrointestinal handling and metabolic disposal of dietary lipid and to be at risk of developing poor growth or nutritional status. This patient group was therefore selected for investigation in this thesis. The findings of these investigations would make it possible to develop a conceptual framework, which is required to improve the nutritional management of cystic fibrosis patients and other patients with gastrointestinal dysfunction.

Chapter 3. Development and evaluation of methods to differentiate between digestion and absorption of lipids

3.1 Introduction

A quantitative differentiation between digestion and absorption of dietary lipid may be achieved by characterising the form in which ^{13}C-label is excreted in stools following the oral administration of ^{13}C-labelled TAG and is illustrated in Figure 3.1. The appearance of the label in the stool TAG fraction indicates maldigestion and the appearance of the label in the FA or MAG fractions indicates that digestion has taken place but the products of digestion have not been absorbed. Measurement of ^{13}C enrichment in the stool TAG and FA fractions required three clearly defined processes:

1) Extraction of all lipids (total lipid) from stools.
2) Separation of TAG and FA fractions from stool total lipid in such a way that only the appropriate lipid is present in each fraction.
3) Measurement of ^{13}C enrichment in TAG and FA fractions above baseline abundance.

The development and validation of these analytical methods is reported in this chapter. An outline of the final analytical methods, which were applied to investigate maldigestion and malabsorption of dietary lipid in cystic fibrosis patients, reported in Chapter 6, is shown in Figure 3.2. Analysis of the ^{13}C enrichment in TAG and FA fractions required collection of a baseline stool specimen and all stools passed for up to five days following an oral administration of ^{13}C-labelled TAG. It has been shown previously that following the administration of ^{13}C-labelled TAG, the ^{13}C enrichment in stools usually returns to the baseline level by three days [Murphy *et al.* 1998]. The collected stools were immediately frozen and subsequently homogenised and freeze-dried. The total ^{13}C enrichment of the dry stools was measured by continuous flow isotope ratio mass spectrometry (CF-IRMS) with a gas solid liquid (GSL) interface (Protocol 3). The total lipids were extracted from dry stool samples using solvent and acid extraction (Protocol 5), the TAG and the FA fractions were separated by thin layer chromatography (TLC; Protocol 2) and the ^{13}C enrichment of TAG and FA fractions was measured by CF-IRMS. Figure 3.2 also shows the procedure for analysis of ^{13}C enrichment by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS), which was used in the development and validation of the

methods.

A detailed description of the investigations that led to the final analytical methods is summarised in this chapter. Initially the methods for lipid extraction and separation of lipid fractions used in our laboratory (Clinical Nutrition & Metabolism Unit, Institute of Human Nutrition) primarily for analysis of blood specimens were chosen for the evaluation of the analysis of stool specimens. The CF-IRMS was used to measure the total ^{13}C enrichment of the stools or lipid fractions, whilst GC-C-IRMS was used to measure the ^{13}C enrichment of specific fatty acids, such as palmitic acid. Methods for total lipid extraction from stools (See 3.2.1; Protocol 1), separation of lipid fractions from total lipid by TLC (See 3.2.2; Protocol 2) and analysis of ^{13}C enrichment by CF-IRMS (See 3.2.3.1; Protocol 3) or GC-C-IRMS (See 3.2.3.2; Protocol 4) and the evaluation of the methods to differentiate between lipid digestion and absorption (See 3.2.4 and 3.2.5) are described in section 3.2. The limitations of the initially chosen methods for the analysis of the stool TAG and FA fractions become evident when it was found by GC-C-IRMS that the TAG fraction inappropriately contained derivatised FA standard (See 3.2.5.). A series of experiments was carried out to characterise the cause of the error and to develop an analytical procedure for the separation of TAG and FA fractions so that only the appropriate lipids were present in each fraction (See 3.3.1 and 3.3.2). Finally the issues related to the stool sample storage were addressed (See 3.3.3.). All chemicals and laboratory materials used are presented in Appendix 1.

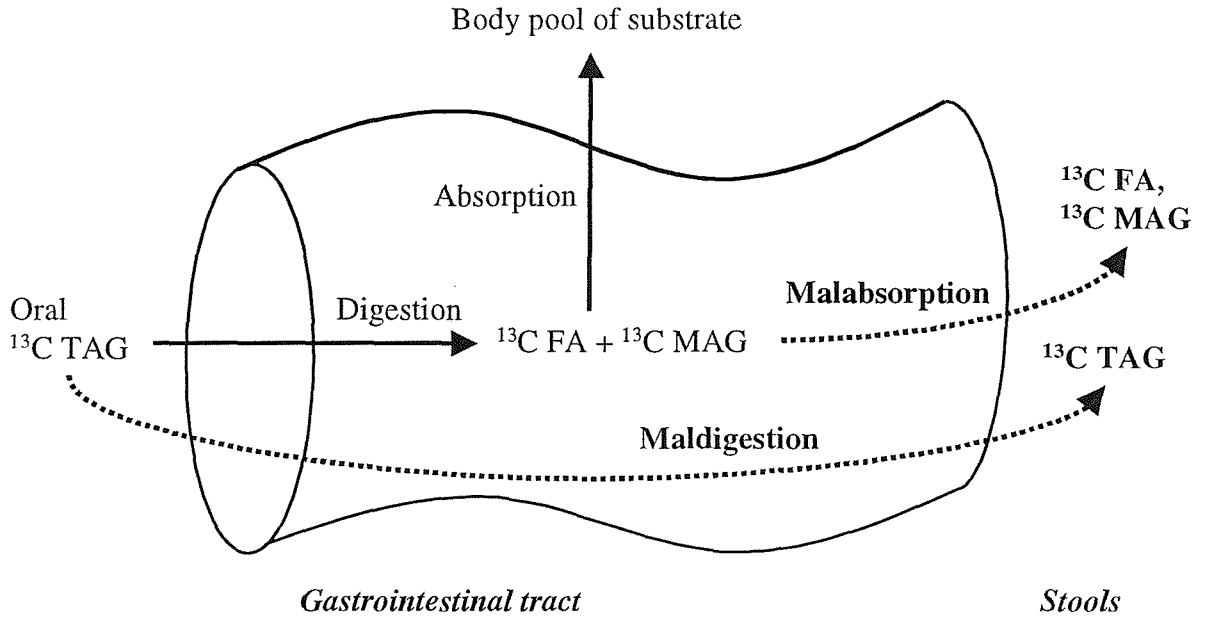


Figure 3.1. Principle of ^{13}C-labelling studies to differentiate between digestion and absorption of dietary lipid by characterising the form in which ^{13}C-label is excreted in stools following the oral administration of ^{13}C-labelled TAG. The appearance of the label in stool TAG fraction indicates maldigestion and the appearance of the label in FA or MAG fractions indicates malabsorption.

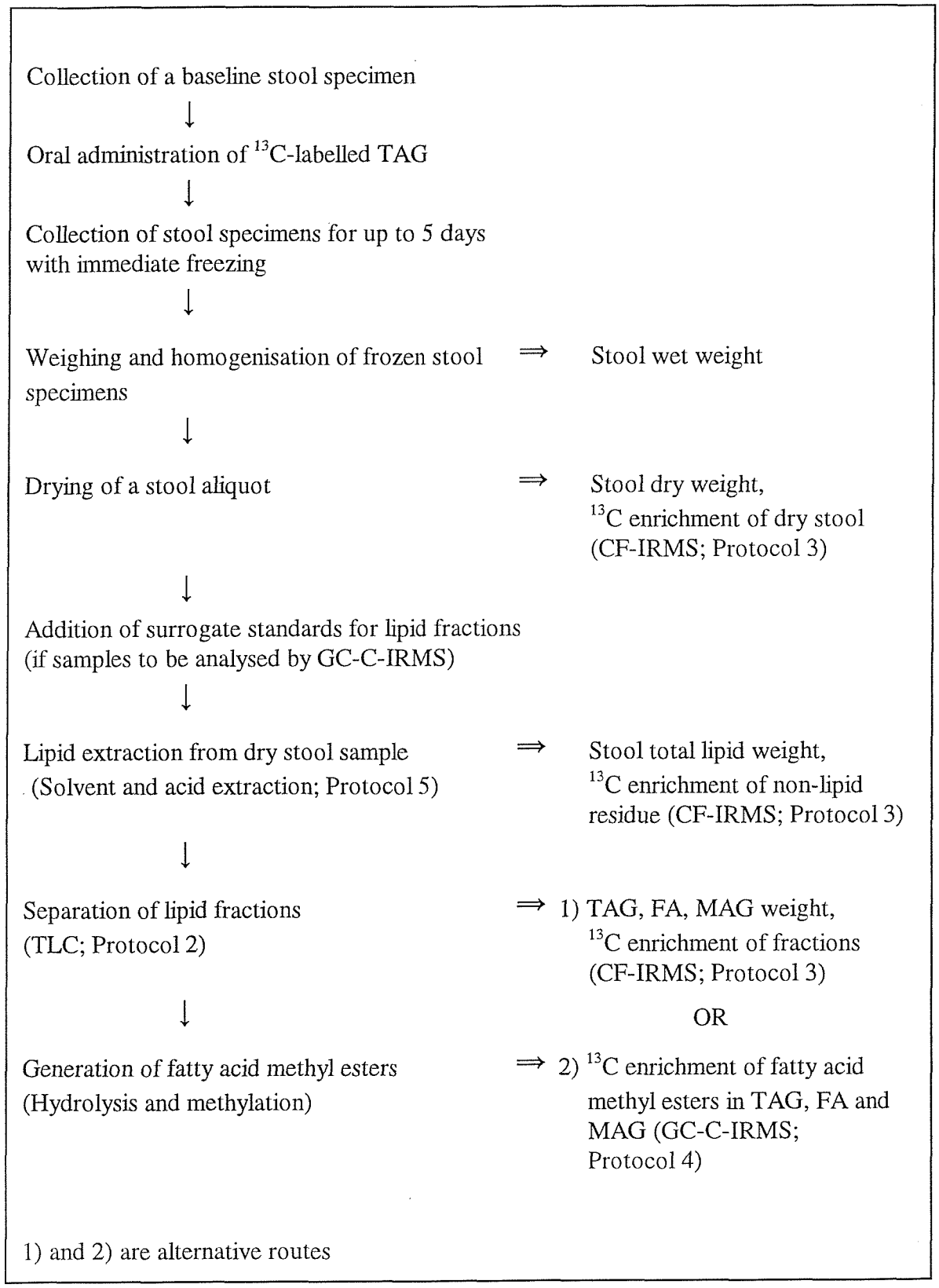


Figure 3.2. Outline of the analytical methods. Procedures are shown on the left, outcome variables on the right and the methods in brackets.

3.2 Description and evaluation of analytical methods

The initial choice of methods for total lipid extraction from stools, separation of TAG and FA fractions from total lipid and measurement of ^{13}C enrichment are described. The criteria for selection of these methods and their validation and modifications are presented. In the following description the term surrogate standard refers to triheptadecanoin as TAG, heneicosanoic acid as FA or monoerucin C22:1[cis]-13 as MAG standard of known concentration added to the stool sample before lipid extraction, which will be used to calculate sample concentration with correction for losses during processing. For this purpose standards which do not naturally occur in the samples being analysed were selected.

Archived stool specimens collected in a previous study [Murphy *et al.* 1998] from cystic fibrosis patients and healthy control subjects were used for the validation of the methods. The stool specimens were obtained following an oral administration of [1,1,1-^{13}C] tripalmitin as a glucose-sucrose-casein emulsion drink and were immediately frozen, homogenised with distilled water and stored as homogenates at -20°C. The frozen homogenates were thawed and dried and all subsequent analysis was conducted with dry stool. Stool samples known to be enriched with ^{13}C compared to respective baseline sample as analysed by CF-IRMS (See 3.2.3.1) were chosen for the validation studies.

3.2.1 Total lipid extraction from stools

For the extraction of lipids, Protocol 1 was adopted as a basis for the examination in detail of the factors reported in this section.

Protocol 1: Method for total lipid extraction from stools (modified from Folch et al. 1957)

1) Weigh 50 mg dry stool into screw topped tubes (10 ml).
2) Add 1.0 ml chloroform:methanol (2:1; v:v including 50 mg/litre of butylated hydroxytoluene). To samples for analysis by GC-C-IRMS use chloroform:methanol (2:1, v:v) mixture containing 0.3 mg surrogate TAG standard (triheptadecanoin) and 0.3 mg surrogate FA standard (heneicosanoic acid) and 0.15 mg surrogate MAG standard (monoerucin).
3) Acidify samples by adding 10 μl HCl (12 mol/l).
4) Add 1 ml chloroform:methanol (2:1; v:v).
5) Replace caps firmly and seal tubes with parafilm and store tubes at 4°C overnight.
6) Centrifuge for 10 min at 1600 g.
7) Remove solvent layer into weighed screw topped tubes.
8) Add 1 ml chloroform:methanol (2:1; v:v), 10 μl HCl and another 1 ml chloroform:methanol (2:1; v:v) to residue.
9) Leave for 30 min and then centrifuge for 10 min at 1600 g.
10) Remove solvent and combine with previous extract.
11) Wash lipid extracts by adding 0.88 ml of 0.04% $CaCl_2$, mix using vortex and centrifuge for 10 min at 1600 g.
12) Discard the upper layer.
13) Wash lipid extracts by adding 0.88 ml of 0.04% $CaCl_2$:chloroform:methanol in the ratio of 47:3:48 (v:v:v), vortex mix samples and centrifuge for 10 min at 1600 g.
14) Discard the upper layer.
15) Add 200 μl ethanol and dry samples under nitrogen flow.
16) Weigh tubes when samples are dry and calculate the mass of lipid and the % lipid dry stool weight.
17) Add 500 μl chloroform:methanol (2:1, v:v), store under nitrogen in screw capped tubes secured with parafilm at -20°C for further analysis.

Total lipids were extracted from dry stools by the modified method of Folch *et al.* (1957). Samples were adjusted to pH less than 3 by the addition of HCl [Stolinski *et al.* 1997] to extract fatty acid salts. Acidification of the stools hydrolysed the fatty acid salts and released the fatty acids. Surrogate standards for TAG, FA and MAG containing 0.15 mg to 0.3 mg standard per 50 mg dry stool were added to samples prior to total lipid extraction if the samples were to be later analysed by GC-C-IRMS. The lipid extracts were washed with salt solutions to remove water-soluble impurities. The modified method of Folch *et al.* (1957) was originally chosen for extracting total lipids from stools, however limitations to this procedure later became evident and the development of suitable modifications is described in 3.3.2.

3.2.2 Separation of lipid fractions from total lipid

Protocol 2 was used to separate TAG, FA and MAG fractions from total lipid by TLC. It was necessary to run two plates with different solvent systems to obtain clear separation of all three fractions.

Protocol 2: Method for separation of lipid fractions from total lipid by TLC

Separation of TAG and FA fractions:

1) Prepare silica gel TLC plate by drawing a line 2 cm from the bottom of the plate and another line 15 cm from the first line. Prepare TLC tank by covering the walls of the tank with filter paper and loading the tank with hexane, diethyl ether and acetic acid (70:30:1.8, v:v:v).
2) Pipette a maximum of 5 mg lipid in chloroform:methanol (2:1, v:v) onto the bottom line of the TLC plate.
3) Add standards for TAG (triheptadecanoin) and for FA (heneicosanoic acid) in chloroform onto the first line next to the lipid samples.
4) Place the plate into the TLC tank containing the solvent system until the solvent has reached the upper line (approximately 30 min), remove from the tank and allow the solvent to evaporate in the fume cupboard.
5) Stain the plate with Rhodamine B (0.25% in ethanol) or fluorescein (0.25% in ethanol).
6) Detect bands under UV-light and mark with pencil.
7) Scrape off bands and place into screw topped tubes for further processing.

Separation of MAG fraction:

1) Prepare TLC plates as described for separation of TAG and FA fractions and place the TLC plate into a tank containing the solvent system toluene, propan-2-ol and water (100:10:0.25, v:v:v). Use MAG standard (monoerucin) in chloroform to visualise the migration of the MAG.

3.2.3 Analysis of ^{13}C enrichment

The ^{13}C enrichment was measured either by CF-IRMS or by GC-C-IRMS. In both systems IRMS (20-20 stable isotope analyser, PDZ Europa Ltd., Crewe, UK) was used to analyse the ^{13}C to ^{12}C ratio of the sample with reference to working standards (beet sugar for solid samples and CO_2 for gaseous samples in CF-IRMS; tricosanoic acid methyl ester in GC-C-IRMS). The CF-IRMS was calibrated to a certified reference sucrose standard (-10.4 δ units; International Atomic Energy Agency, Vienna, Austria) and certified reference CO_2 standards (-23.52 δ units, 6.55 δ units and 16.1 δ units; Cambridge Isotope Laboratories Inc., Andover, MA, USA), which have been adjusted against PDB. Repeatability of the abundance measurements by GC-C-IRMS was monitored by analysis of fatty acid standards (palmitic acid -31.2 δ units, triheptadecanoin -33.9 δ units, stearic acid -22.1 δ units, oleic acid -31.1 δ units; Sigma-Aldrich Company Ltd., Poole, UK).

The IRMS is equipped with triple collectors for the simultaneous recording of CO_2^+ ions. The instrument is set to measure the major CO_2^+ ions: m/z = 44[$^{12}C^{16}O^{16}O$], 45[$^{13}C^{16}O^{16}O$] or 45[$^{12}C^{17}O^{16}O$] and 46[$^{12}C^{18}O^{16}O$]. The ^{13}C enrichment of dry stool and non-lipid residue samples and total TAG and total FA fractions were analysed by CF-IRMS and the ^{13}C enrichment of individual fatty acids by GC-C-IRMS. The sample preparation and the analysis of the ^{13}C enrichment by CF-IRMS and GC-C-IRMS and the calculations of the sample ^{13}C enrichment are described below.

3.2.3.1 CF-IRMS

The ^{13}C enrichment and carbon content of dry stool and non-lipid residue samples and TAG and FA fractions were analysed by CF-IRMS with an automated GSL system (PDZ Europa Ltd., Crewe, UK) according to Barrie *et al.* (1989). Protocol 3 was used for the preparation of samples for CF-IRMS analysis.

Protocol 3: Preparation of samples for CF-IRMS analysis

Aliquots of dry stool or non-lipid residue (1.8 mg) were weighed into tin capsules and analysed in duplicate by CF-IRMS. For analysis of the ^{13}C enrichment of TAG and FA fractions lipids were extracted from TLC scrapings as follows:

1) Add 2 ml chloroform:methanol (2:1, v:v) to TAG and FA scrapings from TLC in 10 ml screw topped tubes.
2) Vortex mix for 15 min.
3) Centrifuge 10 min at 1600 g.
4) Remove lipid extract to weighed 10 ml screw topped tubes.
5) Repeat steps 1 to 3 and remove lipid extract to weighed tubes combining the solvents from step 4.
6) Dry lipid extracts under nitrogen flow.
7) Weigh tubes when the samples are dry and calculate the mass of TAG or FA in the sample.
8) Resuspend TAG or FA in 200 μl chloroform:methanol (2:1, v:v).
9) Pipette TAG or FA into weighed tin capsules containing non-carbon absorbent (chromosorb) and weigh capsules again to determine the amount of TAG or FA.

Analysis of ^{13}C enrichment by CF-IRMS

The process of CF-IRMS is illustrated in Figure 3.3 according to Barrie *et al.* (1989). Samples of unknown enrichment in tin capsules were loaded into the autosampler. Two reference standard samples (sugar beet) were placed after each set of 6 samples. The CF-IRMS was set to measure carbon content of a sample with a range of 50 μg to 1000 μg. The combustion of 1.8 mg of sugar beet generated 750 μg of carbon and a similar amount of carbon is generated from 1.8 mg dry stool and non-lipid residue and 1.0 mg lipid extracted from stools, which were the target sample weights used in all studies.

In this continuous flow system, the sample in the tin capsule is combusted at 1000°C to generate CO_2. Complete oxidation is ensured by passing the combustion products

through a bed of chromium trioxide using a helium carrier gas. A layer of copper oxide and silver wool completes the oxidation and removes any sulphur. Water is removed in a trap containing anhydrous magnesium perchlorate. A gas chromatography (GC) capillary column separates CO_2 from nitrogen and interfacing impurities. Approximately 1% of the GC effluent is admitted to the IRMS analyser to prevent excess pressure. The ^{13}C enrichment is expressed either as atom % or as δ value (See 2.4.2) by the software provided by the manufacturer (PDZ Europa Ltd., Crewe, UK). The analytical precision of CF-IRMS in the laboratory has been shown to be (SD) 0.06 δ units and 0.03 δ units for reference gas and sugar beet respectively for 10 samples [Murphy *et al.* 1995].

Calculation of ^{13}C enrichment in total stools, TAG, FA and MAG fractions

The ^{13}C enrichment above baseline abundance in total stools, TAG, FA and MAG fractions was calculated as mmol ^{13}C or as a proportion of the administered dose using the following equations:

^{13}C (above baseline abundance) in the sample as a proportion of the administered dose =

$$\frac{^{13}C \text{ in sample above baseline abundance (mmol)}}{^{13}C \text{ administered (mmol)}} \times 100$$

Where:

1) ^{13}C in sample above baseline (mmol) =

$$\frac{(\text{atom\%}^1 \text{ of sample} - \text{atom\%}^1 \text{ of baseline sample})}{100} \times \text{carbon in sample (mmol)}^2$$

[1] From CF-IRMS analysis.

[2] From CF-IRMS analysis (mg carbon in the sample ÷ mol weight of carbon, 12.01 g/mol) extrapolated to the total stool or TAG, FA or MAG fraction

2) 1 mol of [1,1,1-^{13}C] tripalmitin contains 3 mol of ^{13}C, therefore mmol ^{13}C administered =

$$\left(\frac{\text{mg [1,1,1-}^{13}\text{C] tripalmitin administered}}{\text{mol wt of [1,1,1-}^{13}\text{C] tripalmitin}^4}\right) \times 3 \times \left(\frac{\text{isotopic purity of [1,1,1-}^{13}\text{C] tripalmitin}^3}{100}\right)$$

[3] 99%,

[4] 810.7 g/mol

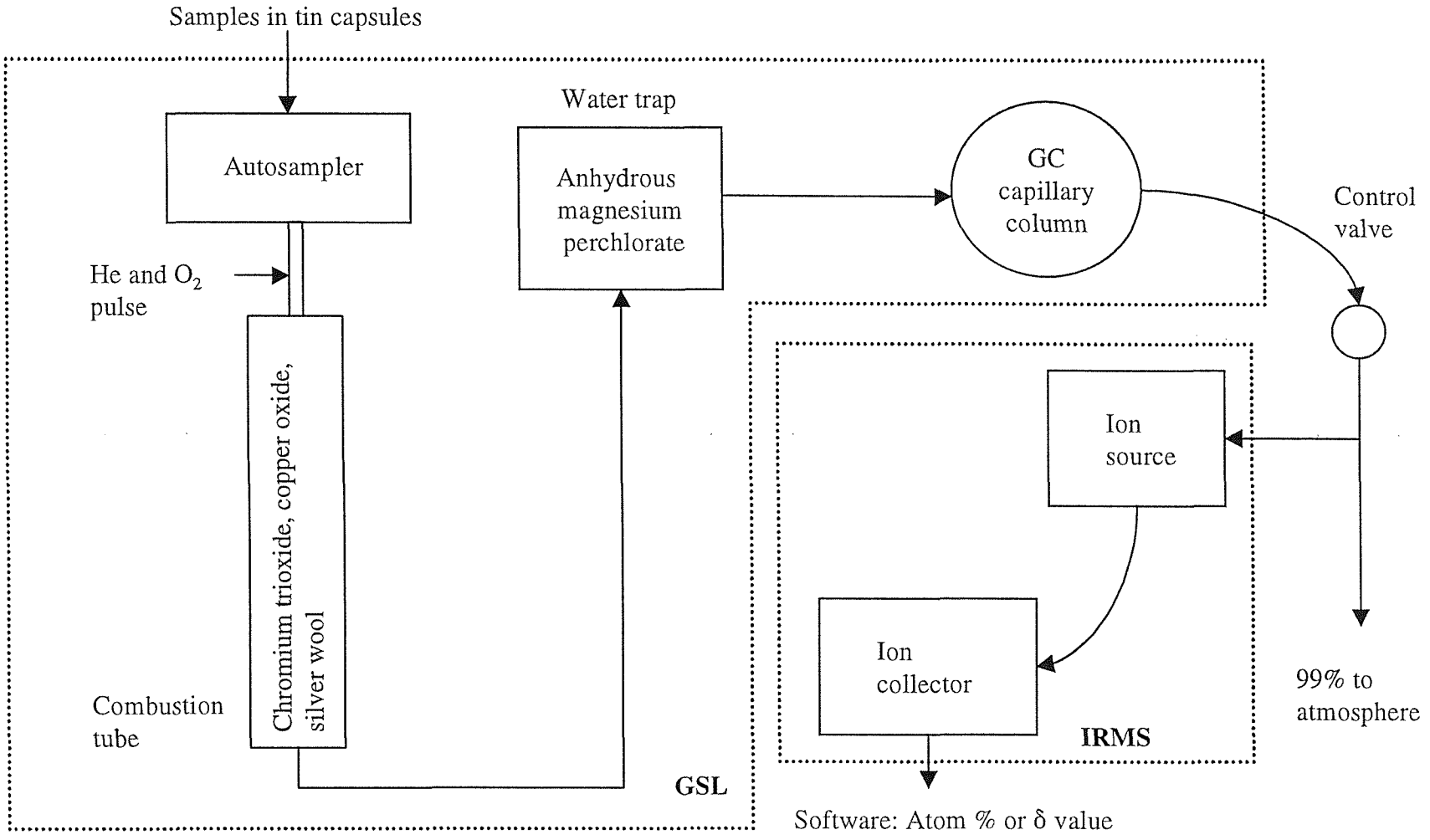


Figure 3.3. The CF-IRMS system for the analysis of ^{13}C enrichment.

3.2.3.2 GC-C-IRMS

The ^{13}C enrichment of specific fatty acids was analysed by GC-C-IRMS. Protocol 4 was used to prepare samples of total lipid and lipid fractions separated by TLC for GC-C-IRMS analysis. The protocol involves hydrolysis and methylation of lipid to generate fatty acid methyl esters (FAME) for the analysis of the ^{13}C enrichment by GC-C-IRMS.

Protocol 4: Preparation of FAME for GC-C-IRMS

1) Extract lipids from dry stools and separate lipid fractions by TLC. Scrape off the identified TAG and FA bands from the TLC plates and place scrapings in screw topped tubes (10 ml).
2) Add 1 ml toluene and 2 ml 2% H_2SO_4 in methanol into tubes containing total lipid or TLC scrapings.
3) Seal tubes with tape over screw tops and place samples into a heating block (50°C) overnight.
4) Cool samples to room temperature.
5) Add 2 ml neutralising reagent (25 g $KHCO_3$ and 34.55 g K_2CO_3 in 500 ml distilled water) and 2 ml hexane.
6) Vortex mix for 15 min.
7) Centrifuge for 10 min at 700 g.
8) Remove upper layer containing FAME into mini vials with inserts.
9) Add another 2 ml hexane, vortex mix for 15 min and centrifuge for 10 min at 700 g.
10) Remove upper layer into mini vials with inserts combining with the solvents from step 8.
11) Dry samples under nitrogen.
12) Analyse ^{13}C enrichment of FAME by GC-C-IRMS following addition of hexane and internal standard (tricosanoic acid methyl ester).

Analysis of ^{13}C enrichment of FAME by GC-C-IRMS

Figure 3.4 shows the GC-C-IRMS system for the analysis of the ^{13}C enrichment of FAME. The ^{13}C enrichment of the FAME was analysed by GC (Model 5890, Hewlett Packard, Bracknell, UK) interfaced to an IRMS (Orchid GC interface; PDZ Europa Ltd., Crewe, UK). The FAME were separated by GC on a BPX-70 column (0.25 mm i.d., 50 m; SGE Ltd., Milton Keynes, UK). Helium was used as the carrier gas at a column head pressure of 120 kPa. The injector port temperature was set at 270°C and the detector set at 295°C. The GC oven temperature programme is presented in Table 3.1. The FAME were identified by comparison of retention times with known standards. For isotopic mass spectrometric analysis following GC separation, the sample was diverted to a combustion tube containing platinised copper oxide powder (Elemental Microanalysis, Okehampton, UK) maintained at 800°C. Water was removed by the use of a hygroscopic ion exchange membrane (Perma Pure Inc, New York, USA) in a continuous drying treatment. This process resulted in discrete samples of CO_2 flowing directly into the source of the IRMS. Data processing and enrichment measurements (δ value) were determined using the software provided by the manufacturer (VANCA-GC; PDZ Europa Ltd., Crewe, UK). Enrichments of the FAME were calculated against internal standard (tricosanoic acid methyl ester, δ value: -32.00) added prior to the injection of the sample. All samples were injected in amounts calculated to give peak areas of comparable beam strengths to those used with standards [Stolinski *et al.* 1997]. The analytical precision of GC-C-IRMS in this laboratory has been determined from 10 consecutive injections and the SD was found to be 0.60 δ units for palmitic acid [Stolinski *et al.* 1997].

Table 3.1. The GC oven temperature programme.

Oven ramp	°C / min	Next °C	Temperature hold (min)
Starting temperature		140	4
Ramp 1	6	175	3
Ramp 2	20	240	2
Ramp 3	60	250	7

Calculation of ^{13}C enrichment in fatty acids (^{13}C palmitic acid)

Preliminary studies of FAME in TAG and FA fractions of stools by GC-C-IRMS showed that the ^{13}C-label was confined to palmitic acid following an oral administration of [1,1,1^{13}C] tripalmitin. Therefore, the ^{13}C enrichment was expressed as mass of ^{13}C palmitic acid calculated using the following formula.

μg ^{13}C palmitic acid/sample =

$$\frac{\%\ ^{13}C \text{ palmitic acid/sample} \times\ \mu g \text{ palmitic acid/sample}}{100}$$

Where,

1) Proportion of ^{13}C palmitic acid of total palmitic acid in the sample was calculated using a standard curve (Stolinski *et al.* 1997; Appendix 2) which correlates the proportion of ^{13}C of total carbon in palmitic acid with ^{13}C enrichment (analysed by GC-C-IRMS).

Proportion of ^{13}C palmitic acid =

$$\frac{(\delta \text{ value of palmitic acid} + 31.57)}{53.80}$$

2) The mass of palmitic acid, or any other fatty acid, in the sample was calculated from the ratio of sample peak area to surrogate standard peak area (triheptadecanoin for TAG, heneicosanoic acid for FA), the concentration of which was known.

μg palmitic acid/sample =

$$\frac{\text{Beam area of palmitic acid} \times \mu g \text{ of surrogate standard added/sample}}{\text{Beam area of surrogate standard}}$$

3) The recovery of the surrogate standard (triheptadecanoin for TAG, heneicosanoic acid for FA) was calculated as:

Recovery of surrogate standard =

$$\frac{\mu g \text{ of surrogate standard (measured)/sample}}{\mu g \text{ surrogate standard (added)/sample}} \times 100$$

Where,

μg of surrogate standard (measured)/sample =

$$\frac{\text{Beam area of surrogate standard} \times \mu g \text{ of internal standard added/sample}}{\text{Beam area of internal standard}}$$

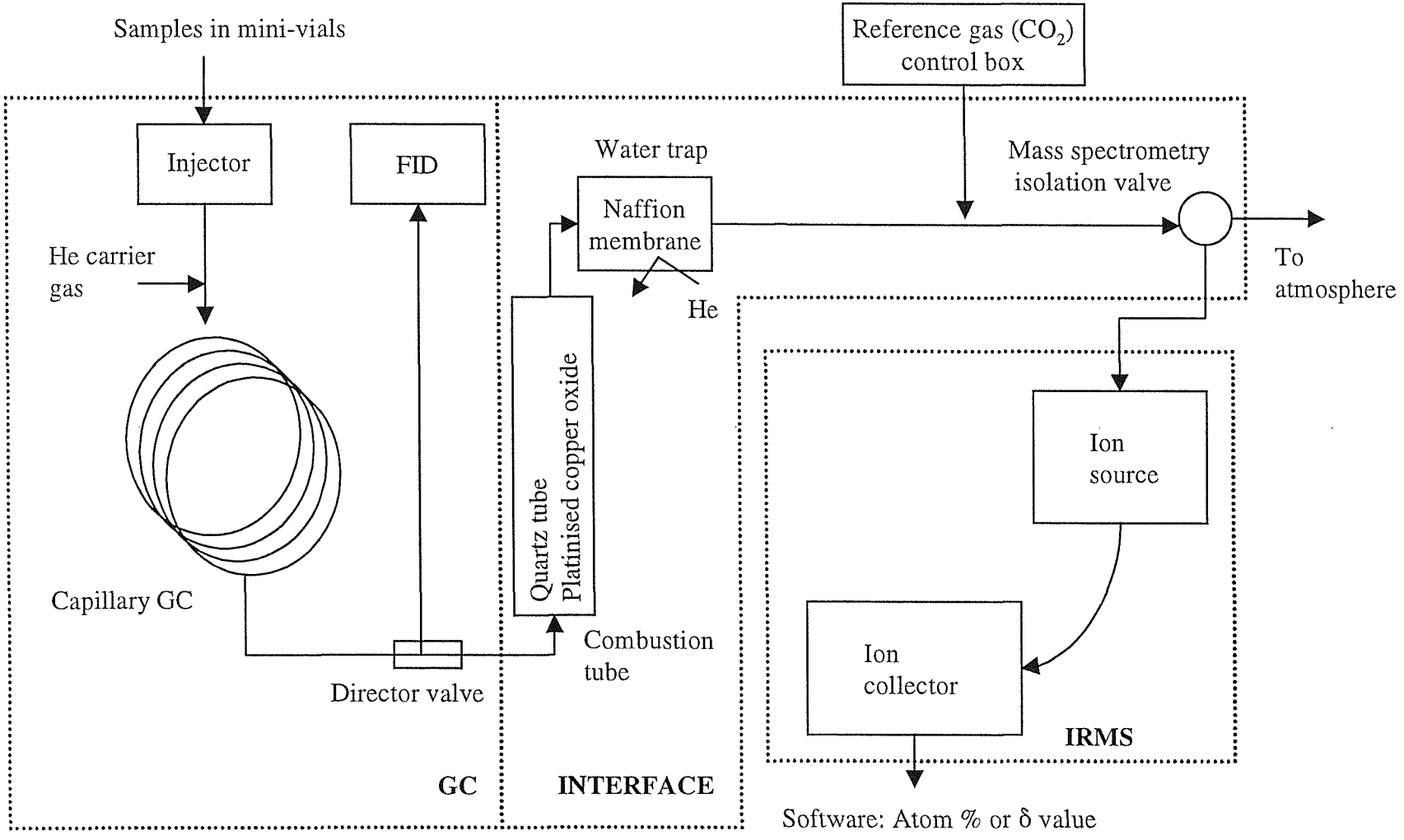


Figure 3.4. The GC-C-IRMS system for the analysis of ^{13}C enrichment of FAME.

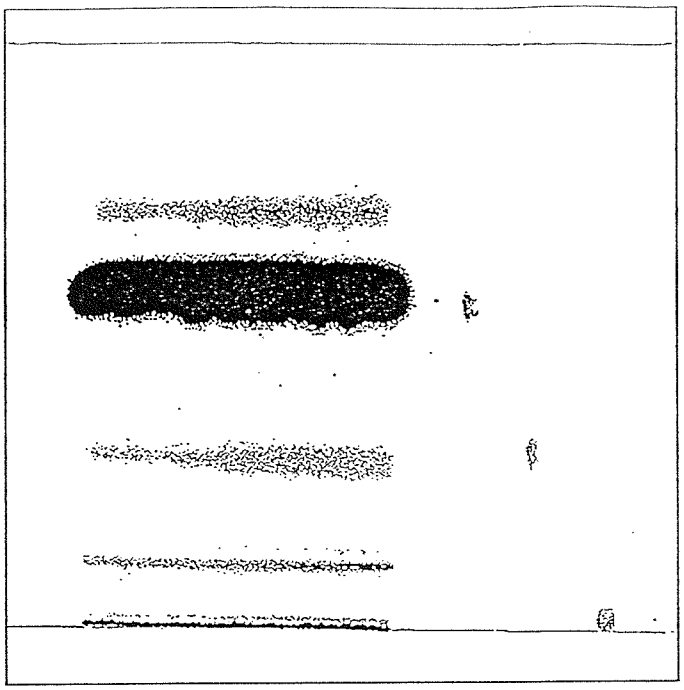
3.2.4 Evaluation and modification of analytical methods

Total lipid extraction from stools

The recovery of total lipid by extraction protocol 1 was evaluated by extracting lipids from a commercial infant formula (SMA Wysoy: soy protein infant formula). The % lipid dry weight in 9 replicate extractions was 24.4% ± 1.3% (Mean ± SD), which was similar to that reported by the manufacturer (24.0% lipid dry infant formula weight). The extraction of ^{13}C-labelled lipids from stools was examined by measuring the ^{13}C enrichment of the non-lipid residue. The ^{13}C enrichment of the non-lipid residues of an enriched stool sample and a baseline stool sample were analysed in triplicate and were found to be -23.85 δ units and -23.51 δ units. The close agreement suggested that there was not a significant amount ^{13}C-labelled lipid left in the non-lipid residue that could affect the interpretation of the results. The repeatability of the lipid extraction from stools was investigated by processing 10 replicates of a single stool sample. The % lipid dry stool weight was 38.9% ± 1.0% (Mean ± SD). Sources of variation were likely to be due to sample inhomogeneity and errors in determining the weight of lipid. For a representative 100 g stool sample (assuming 70% hydration) this variability would be equivalent to 95% confidence interval for the lipid content of 11.1 g to 12.3 g and for a 300 g stool sample 33.2 g to 36.8 g.

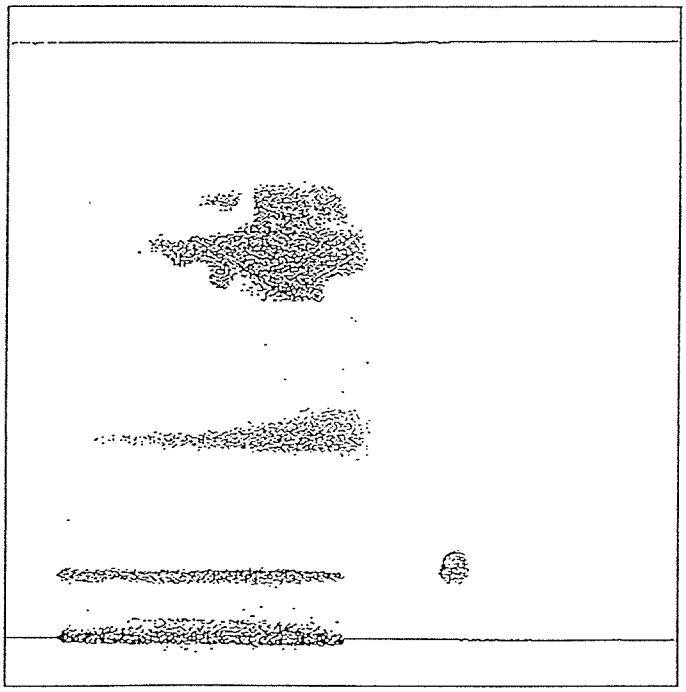
Separation of lipid fractions by TLC

The optimal mass range of lipid to be dispensed onto TLC plate was determined by a load test, where the aim was to achieve clearly separable but not overlapping lipid bands. Visually inspecting the plates the lipid bands were clearly separated from each other and the upper mass limit for clear separation of bands was found to be 5 mg of total lipid. Examples of TLC plates where TAG, FA and MAG fractions can be detected are depicted in Figure 3.5 and Figure 3.6. For visualisation of the lipid fractions for the figures, TLC plates were sprayed with 0.25% H_2SO_4 in ethanol and heated (>100°C), which made the lipid bands visible. Lipid standards for each fraction are also shown. Figure 3.5 shows that it was not possible to separate MAG fraction in the same solvent system as TAG and FA fractions. In the hexane:diethyl ether:acetic acid system MAG do not migrate from the dispensing line, whereas in toluene:propan-2-ol:water system MAG are clearly separable from other lipid fractions (Figure 3.6). Both TLC systems were used to separate the desired lipid fractions.



Lipid extract TAG FA MAG
(standards)

Figure 3.5. Example of the separation of TAG and FA fractions in a solvent system containing hexane, diethyl ether and acetic acid (70:30:1.8; v:v:v).



Lipid extract MAG
(standard)

Figure 3.6. Example of the separation of MAG fraction in a solvent system containing toluene, propan-2-ol and water (100:10:0.25; v:v:v).

Sample preparation and analysis of ^{13}C enrichment

In the studies reported here, stool homogenates were dried using a rotary evaporator (Genevac, Ipswich, UK), which allowed drying samples with or without heat. Using heat accelerates drying of the samples but may also change the physical properties of stools. Three aliquots of frozen homogenised stools from a cystic fibrosis patient and from a control subject were thawed and dried with and without heat in the rotary evaporator. The loss of the sample weight (%) was measured after 3 hours, 6 hours and 24 hours of drying. Differences in the final weight as % of the stool homogenate weight between ambient and the increased drying temperature were negligible (9.5%, 9.7% and 10.5% with heat and 10.1%, 10.2% and 10.3% without heat) in the cystic fibrosis sample. Similarly for the stools from the healthy subject, dry stool weight (%) was not affected by the use of heat during drying (8.2%, 8.6% and 8.9% with heat and 9.3%, 9.4% and 10.8% without heat). However, the samples dried with heat lost weight at a faster rate than those dried without heat. Using heat in the drying of homogenised stool samples had no advantages over drying without heat but may change the physical properties of the sample so it was decided to dry samples without heat.

The preservation of TAG during sample processing was investigated by adding [1,1,1-^{13}C] tripalmitin into a homogenised stool sample with natural ^{13}C abundance. The stool sample was dried in a rotary evaporator, total lipids were extracted, TAG and FA fractions separated by TLC and prepared for and analysed by GC-C-IRMS. The GC-C-IRMS analysis detected no ^{13}C enrichment above baseline abundance in the FA fraction indicating that no significant hydrolysis of TAG had occurred during processing of the homogenised stool samples.

The repeatability of the ^{13}C enrichment analysis was evaluated at two levels of enrichment by CF-IRMS of 10 replicates of each sample. At a mean enrichment of 1.82 δ units the SD was 0.57 and at a mean enrichment level of -22.44 δ units the SD was 0.70. For a representative stool sample this variability would be equivalent to 95% confidence intervals for the excretion of ^{13}C-label in stool as a proportion of the administered dose of 29.9% to 32.5% and 1.4% to 4.6% respectively. The major component of the variation arises from the processing of the sample because the instrumental variation (CF-IRMS) was 0.03 δ units [Murphy *et al.* 1995].

For evaluation of the repeatability of the ^{13}C enrichment analysis by GC-C-IRMS, an aliquot of a total lipid extracted from stools was separated by TLC and TAG and FA fractions were prepared for analysis by GC-C-IRMS in 10 replicate injections of FAME. The δ value of palmitic acid in TAG fraction was 197.23 ± 15.52 (Mean ± SD) and in FA fraction was 197.51 ± 10.05 (Mean ± SD). For a representative sample this variability would be equivalent to 95% confidence intervals for the excretion of ^{13}C label in fraction as a proportion of the administered dose of 5.4% to 7.1% and 5.7% to 6.8% respectively. The components of this variability were those due to homogeneity of the sample, injection technique, properties of the GC column, combustion of fatty acids and recording of CO_2 ions in IRMS.

3.2.5 Pilot study: Analysis of ^{13}C enrichment in stool TAG, FA and MAG fractions

The methods for total lipid extraction from stools, separation of TAG, FA and MAG fractions from total lipids and analysis of ^{13}C enrichment by GC-C-IRMS were applied to stool samples collected from a cystic fibrosis patient and a control subject. The aim of the pilot study was to evaluate the analytical methods for measuring the ^{13}C enrichment in TAG, FA and MAG fractions following an oral administration of [1,1,1-^{13}C] tripalmitin to differentiate between digestion and absorption of lipids. The outcome variables investigated were the stool total lipid excretion (g/d), the extent to which ^{13}C-label was extracted from stools (δ value of the non-lipid residue) and the excretion of ^{13}C-label in specific fatty acids in TAG, FA and MAG fractions.

Samples and methods

The analysed stool specimens were collected previously from a cystic fibrosis patient and a control subject over a 3 day study period following an oral administration of [1,1,1-^{13}C] tripalmitin [Murphy *et al.* 1998]. All stools collected by the two subjects (four specimens each) were analysed separately in triplicate. The frozen stool samples were thawed and dried in a rotary evaporator. Total lipids were extracted from 3 aliquots of dried stool with chloroform:methanol (2:1, v:v) and HCl (See 3.2.1; Protocol 1), after addition of surrogate standards for TAG, FA and MAG. Lipid fractions were separated by TLC (See 3.2.2; Protocol 2), hydrolysed, methylated and analysed by GC-C-IRMS (See 3.2.3.2; Protocol 3). The GC-C-IRMS was used to locate the enrichment to a specific fatty acid. The ^{13}C

enrichment of total stools and non-lipid residues were determined by CF-IRMS (See 3.2.3.1).

Results

The total lipid excretion in stools of the cystic fibrosis patient was 14.2 g/d and of the control subject 2.2 g/d. Excretion of the ^{13}C-label in stools was 64.4% of the administered dose in the cystic fibrosis patient and 9.5% of the administered dose in the control subject. Both the total lipid and the ^{13}C-label excretion in stools of the cystic fibrosis patient exceeded that of the control subject and was higher compared to the total lipid and the ^{13}C-label excretion in stools of healthy individuals in the previous studies [Wollaeger *et al.* 1947; Murphy *et al.* 1998].

In the cystic fibrosis samples the δ values of the non-lipid residues from the enriched samples ranged from -24.96 to -23.50, which were similar to the δ value of the unenriched non-lipid residue (-23.51). In the samples of the control subject, the δ values of the non-lipid residues of the enriched samples ranged from -26.45 to -24.57, which were similar to the δ value of the unenriched non-lipid residue (-26.28). Therefore, there were no significant amounts of ^{13}C-label left in the non-lipid residues suggesting that the ^{13}C-labelled lipids were completely extracted from stools.

The GC-C-IRMS analysis of lipid fractions revealed that in addition to the TAG standard, 40% to 50% of the FA standard was present in the TAG fraction, whereas the TAG standard and the MAG standard were detected only in their appropriate fractions. This implies that some of the fatty acids from the FA fraction were inappropriately recovered in the TAG fraction. It has been previously mentioned that the surrogate lipid standards are not naturally occurring and so any surrogate FA standard cannot be the product of TAG hydrolysis. If the effect of fatty acids from the FA fraction translocating to the TAG fraction is not taken into account, this effect would lead to erroneously high TAG and low FA concentrations. The GC-C-IRMS detected ^{13}C enrichment in both the TAG and the FA fraction. Only palmitic acid was found to be enriched with ^{13}C. However, the ^{13}C enrichment of fatty acids within the TAG fraction may be altered by the label derived from the FA fraction. In order to calculate the mass of recovered ^{13}C enriched fatty acids, both the mass of the fatty acid and the δ value must be known. As the surrogate FA standard had translocated inappropriately to TAG fraction by virtue of the method one can assume that

some of the sample will also have translocated resulting in both erroneous mass of fatty acids and ^{13}C enrichment of fatty acids. Therefore, it was not possible to calculate the ^{13}C-label excretion in the TAG and FA fractions in this pilot study.

The appropriate MAG standard was only found within the MAG fraction and so the fatty acid concentration and the ^{13}C enrichment for the MAG fraction appear correct. Table 3.2 shows total stool lipid and total fatty acids in MAG fraction and ^{13}C palmitic acid excretion in the MAG fraction in individual stool samples of the cystic fibrosis patient and the control subject. The mean total fatty acid content of the MAG fraction in the cystic fibrosis patient was 0.2 g/d and in the control subject was 0.1 g/d, which are 1.6% and 6.5% of the gravimetrically determined total stool lipid per day respectively. Palmitic acid was the only enriched fatty acid within the MAG fraction. The cystic fibrosis patient excreted 0.18% of the administered ^{13}C dose and the control subject excreted 0.05% of the administered ^{13}C dose within the MAG fraction over the study period.

Table 3.2. Total stool lipid and total fatty acids and ^{13}C palmitic acid excretion in the MAG fraction in individual stool samples of a cystic fibrosis patient and a control subject.

Stool sample	Total stool lipid (g/d) [1]	Total fatty acids in MAG (g/d) [2]	^{13}C palmitic acid in MAG (μg/d) [2]	^{13}C in MAG % administered dose
Cystic fibrosis patient				
Baseline	8.3	0.23	0	0
Day 1	17.6	0.25	107	0.04
Day 2	9.4	0.22	274	0.11
Day 3	21.4	0.19	62	0.03
Control subject				
Baseline	3.1	0.18	0	0
Day 1	1.6	0.11	26	0.02
Day 2	1.8	0.12	21	0.02
Day 3	2.5	0.16	8	0.01

[1] Analysed gravimetrically.
[2] Analysed by GC-C-IRMS.

Conclusions

Established methods for lipid extraction, separation of TAG and FA fractions and analysis of ^{13}C enrichment by GC-C-IRMS were used in this study to evaluate their applicability to differentiate between lipid maldigestion and malabsorption. It was found that a portion of the fatty acids from the FA fraction was recovered in the TAG fraction resulting in erroneous TAG and FA concentrations and ^{13}C enrichments. It was therefore necessary to develop methods that avoid such errors.

The concentration and ^{13}C enrichment of fatty acids within the MAG fraction were detectable by the GC-C-IRMS analysis. However the mass of MAG in stool lipid was small, and less than 1% of the administered ^{13}C dose was excreted in the MAG fraction. The proportion of the ^{13}C-label excreted in the MAG fraction was negligible compared to the excretion of the label in total stools and did not differ between the cystic fibrosis patient and the control subject despite the large difference in the total label excretion in the stools. Therefore, it is unlikely that the analysis of ^{13}C enrichment of the MAG fraction is required to differentiate between the impairment of digestion and absorption of lipids. Only the ^{13}C enrichment in TAG and FA fractions was measured in the future studies. However, the products of TAG digestion are FA and MAG and omission of the MAG fraction could result in a slight underestimation of the malabsorbed lipid.

3.2.6 Summary

The methods for total lipid extraction from stools, separation of TAG, FA and MAG fractions from total lipid and analysis of the ^{13}C enrichment were described in this section. The results from these experiments can be summarised as:

1) Lipid extraction from stools using the modified method of Folch *et al.* (1957) with acidification (HCl) resulted in complete extraction of the ^{13}C-labelled lipids from dry stools as indicated by the similar δ values of non-lipid residues from enriched and unenriched stool samples.
2) The TLC method separated TAG, FA and MAG fractions from total lipid as evaluated visually under UV-light. Two different solvent systems were required to separate TAG and FA and MAG fractions.

3) Processing (drying, lipid extraction, TLC, GC-C-IRMS) of the stool homogenates did not hydrolyse the added TAG in the samples.
4) The analysis of the ^{13}C enrichment in stools by CF-IRMS was found to be sufficient when total ^{13}C enrichment of a sample was of interest, although GC-C-IRMS enabled detection of the ^{13}C enrichment in a specific fatty acid.
5) A portion of fatty acids from the FA fraction was recovered in the TAG fraction and it was therefore necessary to develop methods that avoided this effect, and this is addressed in 3.3.
6) Both the fatty acid concentrations and the ^{13}C enrichment of the fatty acids within the MAG fraction were detectable by the GC-C-IRMS analysis. The mass of MAG and the ^{13}C enrichment of the MAG fraction was negligible and unlikely to contribute to the differentiation between maldigestion and malabsorption of lipids. Consequently only ^{13}C enrichment in TAG and FA fractions was measured.

3.3 Development and evaluation of methods for measuring stool TAG and FA fractions

The previously described study (See 3.2.5) evaluated the methods for measuring the ^{13}C-label excretion in stool TAG, FA and MAG fractions following the administration of ^{13}C-labelled tripalmitin. The following experiments were designed to characterise the cause of the inappropriate recovery of the FA standard in the TAG fraction and to develop an analytical procedure for the separation of TAG and FA fractions so that only the appropriate lipids are present in each fraction. Experiments were designed to determine whether the inappropriate recovery of fatty acids in the TAG fraction was 1) due to the procedure of TLC for separation of TAG and FA fractions or 2) due to the procedure of total lipid extraction from stools. Having identified the cause of the error, a procedure for separation of TAG and FA fractions containing only their appropriate lipids was developed and evaluated.

3.3.1 Separation of TAG and FA fractions from total lipid by TLC

Overloading a TLC plate with sample is a recognised cause of poor separation of components and could cause fatty acids from the FA fraction to appear in the TAG fraction. Previously the lipid load, which gave satisfactory separation of lipid fractions in TLC had been evaluated by visual inspection of the plates under UV-light. This experiment was carried out to exclude the possibility that the visual inspection was not sufficiently sensitive to fully characterise the separation. Total lipids were extracted from a cystic fibrosis stool sample following addition of surrogate TAG and FA standards (See 3.2.1; Protocol 1) and TAG and FA fractions separated by TLC (See 3.2.2; Protocol 2) and the sections between and around the visually identified TAG and FA fractions were removed from the plate and analysed by GC-C-IRMS (See 3.2.3.2). The plates were loaded with three different masses of lipid extract (0.5 mg, 1.0 mg and 6.0 mg lipid/TLC plate).

Visually separate TAG and FA fractions were obtained from all the three lipid masses. No fatty acids were detected in the sections above the TAG band, between the TAG and the FA band or below the FA band as analysed by GC-C-IRMS. Despite separation of the apparent TAG and FA fractions, the FA standard was found in the TAG fraction at all the 3 lipid masses. Therefore, it was demonstrated that 6 mg of total lipid can be dispensed onto the TLC plate without overloading and that complete separation of TAG and FA fractions by TLC does not cause the inappropriate recovery of the fatty acids from the FA fraction to the TAG fraction.

To confirm that TLC could satisfactorily separate the TAG and FA surrogate standards, lipids were extracted from stools by the standard procedure, but the surrogate standards were not added until immediately before the application to the TLC plate. The GC-C-IRMS analysis showed that standards were only present in the appropriate fractions.

Conclusion

It was shown that up to 6 mg of total lipid extract can be satisfactorily applied to the TLC plate. The inappropriate recovery of the FA standard in the TAG fraction was independent of the TLC and it was therefore concluded that the analytical error occurred earlier during the extraction stage.

3.3.2 Extraction of total lipid from stools

Extraction of total lipid from stools may change the properties of the lipid causing the inappropriate recovery of fatty acids from the FA fraction in the TAG fraction. This section describes investigations of mechanisms that could cause the inappropriate recovery of the FA standard in the TAG fraction and the development of a reliable total lipid extraction method.

3.3.2.1 Acidification

In the previous experiments total lipids were extracted from stools using the modified method of Folch *et al.* (1957, See 3.2.1; Protocol 1). In the modified lipid extraction method stool samples were acidified by HCl to increase the recovery of the extracted lipid [Stolinski *et al.* 1997]. A portion of the fatty acids released by digestion of TAG form salts in the gastrointestinal tract with ions such as calcium and magnesium [Sammons & Wiggs 1960]. Acidification of stool samples released fatty acids that were bound as salts. However, neither the extent of acidification needed for complete extraction of lipids from stools nor the effect of acidification on the properties of the extracted lipids was known. It has also been suggested that ethanol used with solvents disrupts hydrogen bonding and the ionic forces between lipids and proteins [Gunstone *et al.* 1994] and may result in a more efficient lipid extraction. The effect of HCl or ethanol on the recovery of ^{13}C-labelled lipids extracted from stools as assessed by the ^{13}C enrichment of the non-lipid residues and by the presence of surrogate standards in TAG and FA fractions was investigated.

A stool specimen obtained in a previous study in which [1,1,1-^{13}C] tripalmitin was administered to cystic fibrosis patients was used in the experiments [Murphy *et al.* 1998]. A stool specimen known to be enriched with ^{13}C compared to the respective baseline specimen from the same individual was thawed and dried. Total lipids were extracted (See 3.2.1; Protocol 1) from dry stools (50 mg) and a mixture of fatty acid standards, palmitic acid (C16:0), oleic acid (C18:1) and heneicosanoic acid (C21:0) (1.0 mg) using varied amounts of HCl or ethanol. The TAG and FA fractions were separated by TLC, prepared for and analysed by GC-C-IRMS. The δ values of non-lipid residues analysed by CF-IRMS from the enriched and unenriched samples were evaluated for completeness of the lipid extraction.

Acidification of the stool samples was required for complete extraction of ^{13}C-labelled

lipids from stools. Without HCl the δ value of the non-lipid residue of the enriched sample was -16.98, whilst the non-lipid residue from the unenriched sample had a δ value of -23.51. With addition of 1 μl, 10 μl and 20 μl of HCl the δ values were -23.01, -23.48 and -23.80, respectively. The minimum amount of HCl needed for the extraction of the ^{13}C-labelled lipids from dry stools was 1 μl per 50 mg stool sample. Adding ethanol in lipid extraction (method as described in 3.2.1; Protocol 1, except ethanol added instead of HCl) did not extract all the ^{13}C-labelled lipids from dry stools. The δ values of the non-lipid residues were -15.73, -16.12 and -13.61 for 50 μl, 100 μl and 500 μl of ethanol, respectively and the δ value of the unenriched non-lipid residue was -23.51.

The TAG and the FA standards were found in their respective fractions when total lipids were extracted from dry stools without acidification. However, when HCl was added the FA standard appeared in the TAG fraction. Without acid, fatty acids from the fatty acid standard mixture migrated to FA band on TLC plate, but with addition of HCl, a 'TAG' band was detected on TLC plate. The GC-C-IRMS analysis of the 'TAG' band showed the presence of all fatty acids from the fatty acid standard mixture (C16:0, C18:1 and C21:0). It is likely that fatty acids and methanol form FAME in the presence of acid during lipid extraction. A series of FAME standards was applied to a TLC plate and were found to have similar retention factors (Distance of the component migrated on TLC plate divided by the maximum migration distance) as TAG standards. It was therefore necessary to develop methods for the complete extraction of lipid without the formation of FAME, which accompanies acidification with HCl in methanol. Acidification of the stool sample during lipid extraction dissociates fatty acid salts, but the released fatty acids simultaneously form FAME with the methyl group from methanol, which is used to extract the lipids together with chloroform. Neutralisation of lipid extracts was examined, but did not resolve the problem.

Conclusion

The acidification of stools with HCl in total lipid extraction caused the inappropriate recovery of the FA standard in the TAG fraction. This was due to the formation of the FAME from fatty acids and methanol in the presence of HCl and these migrated the same distance on the TLC plate as TAG. However, the acidification of the samples was required for the complete extraction of the ^{13}C-labelled lipids from dry stools. A minimum amount of

HCl needed for the extraction of the ^{13}C-labelled lipids from dry stools was determined, but this still caused the formation of FAME. It was therefore necessary to develop an extraction procedure that results in complete extraction of lipids without the use of methanol and HCl together.

3.3.2.2 Extraction of total lipid from stools using various solvent and acid combinations

The most widely used method of analysis of total lipid in stools has been that of Van de Kamer *et al.* (1949) which involves the initial hydrolysis of all lipids to fatty acids. Since that method does not allow separation and quantification of individual lipid fractions in stools, little information has been available on a suitable extraction method for separation of TAG and FA from stools. The lipid extraction method with chloroform and methanol without acidification [Folch *et al.* 1957; Bligh & Dyer 1959] has been used for stools [Morgan *et al.* 1998; Kalivianakis *et al.* 1999] and these results must be questioned because the total lipid will not include fatty acid salts. Lipid extraction methods that exclude fatty acid salts will underestimate stool lipid losses, especially in cases of malabsorption of lipid. It has been shown above (See 3.3.2.1) that acidification of the standard chloroform and methanol lipid extraction system gave correct total lipid but erroneous composition of lipid fractions. Therefore an alternative lipid extraction procedure needed to be developed. The studies examining solvent and acid combinations other than chloroform:methanol (2:1, v:v) and HCl (Protocol 1) for the complete extraction of lipid from stools without formation of FAME from fatty acids are described in this section.

Toluene

Toluene has been used to extract lipid from stools [Henry *et al.* 1974] but its use for subsequent separation of TAG and FA fractions has not been investigated. An initial experiment showed that acidification was also needed when total lipids were extracted from stools using toluene. Acetic acid was used because it is more soluble than HCl in toluene. For acetic acid volumes of 0 μl, 50 μl, 100 μl and 250 μl per 50 mg dry stool, the δ values of the non-lipid residue were -1.17, -23.42, -23.27 and -23.67, respectively. With toluene and acetic acid extraction, the δ values were similar to the δ values of the non-lipid residues

extracted with chloroform:methanol (2:1, v:v) and HCl (-23.51). Importantly, the surrogate TAG and the FA standards were found in their appropriate fractions as analysed by GC-C-IRMS, following lipid extraction with toluene and acetic acid. The toluene and acetic acid extraction was further studied by comparing with chloroform:methanol (2:1, v:v) and HCl extraction. In the toluene and acetic acid system, the stools were extracted with 2 ml toluene and 0.5 ml acetic acid, and the aqueous washes of the extract were omitted.

Total lipids were extracted from a commercial infant formula (SMA Wysoy: soy protein infant formula) and from a stool sample using both methods. Lipid extraction with chloroform:methanol (2:1, v:v) and HCl resulted in a similar proportion of total lipid in dry infant formula (24.4% ± 1.3% lipid dry formula weight, Mean ± SD of 9 replicates) as reported by the manufacturer (24% lipid dry formula weight). Recovery of lipid from the infant formula with the toluene and acetic acid (18.9% ± 2.9% lipid dry weight, Mean ± SD of 10 replicates) was substantially less than with chloroform:methanol (2:1, v:v) and HCl extraction ($P < 0.001$, Independent samples t-test). The proportion of lipid extracted with toluene and acetic acid from the stool sample was 31.2% ± 3.2% dry stool weight (Mean ± SD of 10 replicates) and with chloroform:methanol (2:1, v:v) and HCl 39.1% ± 2.1% dry stool weight (Mean ± SD of 5 replicates, $P < 0.001$, Independent samples t-test). The δ values of the non-lipid residues were similar following both extractions (-23.98 ± 0.1 and -24.98 ± 0.1, chloroform:methanol (2:1, v:v) with HCl and toluene with acetic acid, respectively, Mean ± SD), and equal to unenriched non-lipid residue δ value (-23.51), suggesting that the ^{13}C-labelled lipids were completely extracted from stools with both methods.

The fatty acid compositions of the lipids extracted by the two methods were compared. Total lipids were extracted by each method from 5 replicates of a stool sample, hydrolysed, methylated and analysed by GC-C-IRMS. Table 3.3 shows the concentration of fatty acids and ^{13}C-labelled palmitic acid in total lipids extracted with chloroform:methanol (2:1, v:v) and HCl or toluene and acetic acid. The sum of fatty acids within total stool lipid was similar between the methods. Also the individual fatty acid concentrations (μg/mg stools) were similar, but C18:1 was lower in total lipid extracted with toluene and acetic acid than with chloroform:methanol (2:1, v:v) and HCl. The concentration of the ^{13}C palmitic acid (μg/mg stools) was similar between the methods.

The fatty acid and ^{13}C palmitic acid concentrations (μg/mg stools) in total lipid were compared to the fatty acid and ^{13}C palmitic acid concentrations in TAG and FA fractions

from toluene and acetic acid extraction shown in Table 3.4. Total lipids were extracted from 5 replicates of a stool sample with toluene and acetic acid, TAG and FA fractions were separated by TLC, prepared for and analysed by GC-C-IRMS. Unexpectedly, no fatty acids, except TAG standard, were detected in the TAG fraction. Fatty acid concentrations and ^{13}C palmitic acid concentration in the FA fraction were similar to those in the total lipid extract, except C18:1 and C18:2 which were lower in the FA fraction compared to the total lipid.

Low stool total lipid by the toluene with acetic acid extraction compared to the chloroform:methanol (2:1, v:v) with HCl extraction and the low recovery of the TAG standard (27% to 34%) shown by the GC-C-IRMS analysis suggested that toluene with acetic acid extraction was not suitable for stools. However, the lack of fatty acids in the TAG fraction may have been due to the hydrolysis of TAG during storage of the stool specimens. To investigate further the toluene and acetic acid extraction, a fresh stool specimen was obtained from a patient with cystic fibrosis and immediately processed. Although, fatty acids were detected in the stool TAG fraction, a low recovery of 24% was obtained.

These experiments indicated that although the appropriate surrogate standards were recovered in the TAG and FA fractions, the toluene and acetic acid extraction resulted in a low recovery of the surrogate standards. Therefore, this extraction system resulted in an artificially low lipid content of the stools. The lack of TAG fraction in stools may have been due to hydrolysis of the TAG during storage and the effect of the storage conditions of the stool specimens on the stool lipid composition required evaluation (See 3.3.3).

Table 3.3. Fatty acid and ^{13}C palmitic acid concentration in total lipid extracted from stools either with chloroform:methanol (2:1, v:v) and HCl or toluene and acetic acid (n = 5 replicates).

Fatty acid (μg/mg stools)	Chloroform:methanol (2:1, v:v) and HCl	Toluene and acetic acid	
	Mean ± SD	Mean ± SD	P value [1]
C14:0	7.0 ± 2.1	8.7 ± 0.5	0.114
C16:0 [2]	83.9 ± 3.9	81.3 ± 3.5	0.293
C18:0	33.5 ± 1.3	33.4 ± 1.1	0.860
C18:1	43.9 ± 1.7	40.1 ± 2.0	0.010
C18:2	14.4 ± 0.4	14.2 ± 0.6	0.458
Sum of fatty acids [3]	182.8 ± 7.5	177.8 ± 6.2	0.269
^{13}C palmitic acid	3.4 ± 0.2	3.3 ± 0.1	0.164

[1] Independent samples t test.
[2] Total (^{13}C + ^{12}C) palmitic acid.
[3] Sum of C14:0, C16:0, C18:0, C18:1 and C18:2.

Table 3.4. Fatty acid and ^{13}C palmitic acid concentration in TAG and FA fractions and in total lipid extracted from stools with toluene and acetic acid (n = 5 replicates).

Fatty acid (μg/mg stools)	TAG	FA	Total lipid	
	Mean	Mean ± SD	Mean ± SD	P value [1]
C14:0	0	8.1 ± 0.7	8.7 ± 0.5	0.132
C16:0 [2]	0	82.0 ± 3.9	81.3 ± 3.5	0.813
C18:0	0	32.8 ± 1.7	33.4 ± 1.1	0.512
C18:1	0	36.3 ± 2.1	40.1 ± 2.0	0.018
C18:2	0	12.7 ± 0.7	14.2 ± 0.6	0.008
Sum of fatty acids [3]	0	171.8 ± 11.0	177.8 ± 6.2	0.335
^{13}C palmitic acid	0	3.4 ± 0.2	3.3 ± 0.1	0.530

[1] Independent samples t test, FA vs. total lipid.
[2] Total (^{13}C + ^{12}C) palmitic acid.
[3] Sum of C14:0, C16:0, C18:0, C18:1, C18:2.

Various solvent and acid combinations

In the previous experiments fatty acids were not detected in the TAG fraction extracted from stool samples that had been stored at -20°C as a homogenate for 1 to 2 years. Moreover, the surrogate TAG standard was extracted so that it is possible that the stool TAG had been hydrolysed during sample handling and storage. In the previous experiments neither chloroform:methanol (2:1, v:v) and HCl nor toluene and acetic acid resulted in a desired extraction of total lipids from stools. The chloroform:methanol (2:1, v:v) and HCl extraction caused an inappropriate recovery of the FA standard in the TAG fraction and the recoveries of the surrogate standards were poor in the toluene and acetic acid extraction. Therefore, various other solvent and acid combinations were investigated.

The conditions used in a series of extraction studies are presented in Table 3.5. Rapidly processed freeze-dried stools were utilised, since the use of wet stools is not practical when many samples are to be processed in the course of a clinical study. Single total lipid extracts using each of the detailed methods were subsequently separated by the standard TLC procedure (See 3.2.2, Protocol 2) to derive TAG and FA fractions and the fatty acid concentration of each fraction was measured by GC-C-IRMS (See 3.2.3.2). The results are presented in Table 3.5. With all systems, except chloroform:methanol (2:1, v:v) and HCl, the surrogate standards occurred in the appropriate fractions. Artefactual recovery of methylated fatty acids in the TAG fraction with chloroform:methanol (2:1, v:v) and HCl was responsible for the grossly elevated concentration shown in Table 3.5.

Chloroform:methanol (2:1, v:v) with acetic acid appeared to be the most suitable method for extracting total lipids from stools. Surrogate standards were in the appropriate fractions and were efficiently recovered. This method required only a small modification to the existing method (Protocol 1) and was therefore further evaluated (See 3.3.2.3).

Table 3.5. Proportion of total lipid in stools and fatty acid concentrations in TAG and FA fractions extracted from dry stools with various solvent and acid combinations.

	Total lipid (% dry wt)	TAG fraction (μg/mg stools)						FA fraction (μg/mg stools)					
		C16:0	C18:0	C18:1	C18:2	Sum of fatty acids	Standard recovery (%) [2]	C16:0	C18:0	C18:1	C18:2	Sum of fatty acids	Standard recovery (%) [2]
Chloroform:methanol[1] (4 ml)	8.3	0.75	0.21	1.67	1.66	4.29	37	0.97	1.13	0.79	0.72	3.61	52
Chloroform:methanol[1] (4 ml), HCl (20 μl)	18.5	6.00	5.04	6.08	4.97	22.09	58	6.49	7.92	2.59	1.29	18.42	57
Chloroform:methanol[1] (4 ml), Acetic acid (200 μl)	16.8	0.76	0	2.64	2.03	5.44	71	6.46	7.57	2.41	1.20	17.65	71
Toluene (4 ml), Acetic acid (200 μl)	12.5	0.51	0	1.88	1.52	2.84	42	6.76	6.65	2.33	1.13	15.87	63
Toluene (2 ml), Chloroform (2 ml), Acetic acid (200 μl)	10.6	1.01	0.36	2.44	1.96	5.77	33	7.52	11.25	4.67	3.78	27.22	15
Toluene (2 ml), Chloroform:methanol[1] (2 ml), Acetic acid (200 μl)	24.6	0.86	0	2.83	2.20	5.89	76	5.40	6.46	2.31	1.19	15.36	71

[1] Chloroform:methanol (2:1, v:v).
[2] Recovery of surrogate TAG or surrogate FA standard.

3.3.2.3 Evaluation of lipid extraction from stools with chloroform:methanol and acetic acid and analysis of ^{13}C enrichment of TAG and FA fractions

The extraction of total lipid from stools using chloroform:methanol (2:1, v:v) and acetic acid was evaluated using protocol 5.

Protocol 5: Method for total lipid extraction from stools with chloroform:methanol and acetic acid

1) Weigh 50 mg dry stool into screw topped tubes (10 ml).
2) Add 2 ml chloroform:methanol (2:1; v:v including 50 mg/litre of hydroxybutyrate). To samples for analysis by GC-C-IRMS use chloroform:methanol (2:1, v:v) mixture containing 0.3 mg surrogate TAG standard (triheptadecanoin) and 0.3 mg surrogate FA standard (heneicosanoic acid).
3) Acidify samples by adding 500 μl acetic acid.
4) Add 2 ml chloroform:methanol (2:1; v:v) and mix samples using vortex.
5) Replace caps firmly and seal tubes with parafilm and store tubes at 4°C overnight.
6) Centrifuge for 10 min at 1600 g.
7) Remove solvent layer into weighed screw topped tubes.
8) Add 2 ml chloroform:methanol (2:1; v:v), 500 μl acetic acid and another 2 ml chloroform:methanol (2:1; v:v) to residue and mix using vortex.
9) Leave for 30 min and then centrifuge for 10 min at 1600 g.
10) Remove solvent and combine with previous extract.
11) Wash lipid extracts by adding 1.76 ml of 0.04% $CaCl_2$, mix using vortex and centrifuge for 10 min at 1600 g.
12) Discard the upper layer.
13) Wash lipid extracts by adding 1.76 ml of 0.04% $CaCl_2$:chloroform:methanol in the ratio of 47:3:48 (v:v:v), vortex mix samples and centrifuge for 10 min at 1600 g.
14) Discard the upper layer.
15) Add 200 μl ethanol and dry samples under nitrogen flow.
16) Weigh tubes when samples are dry and calculate the mass of lipid and the % lipid dry stool weight.
17) Add 500 μl chloroform:methanol (2:1, v:v), store under nitrogen in screw capped tubes secured with parafilm at -20°C for further analysis.

To investigate the completeness of extraction of ^{13}C-labelled lipid from stools using Protocol 5, samples of ^{13}C enriched and unenriched stool specimen from the same subject were extracted and the ^{13}C enrichment (δ value) of the non-lipid residues were compared. The δ values of the non-lipid residues were -24.46 ± 0.35 (Mean ± SD, 5 replicates) and -25.78 ± 0.10 for the enriched and unenriched samples respectively. Samples from the same stool specimen were also extracted with Protocol 1 using chloroform:methanol (2:1, v:v) and HCl. The δ values of the non-lipid residues were -24.89 ± 0.38 (Mean ± SD, 5 replicates). The total lipid extracted was 11.8% ± 3.8% stool dry weight (Mean ± SD, 5 replicates) for protocol 5 and 12.8% ± 3.0% lipid dry stool weight (Mean ± SD, 5 replicates) for Protocol 1. Thus, the total lipid extracted with chloroform:methanol (2:1, v:v) and acetic acid was comparable to that with chloroform:methanol (2:1, v:v) and HCl.

Fatty acid and ^{13}C palmitic acid concentrations (μg/mg stools) in total lipid and TAG and FA fractions were analysed by GC-C-IRMS. The extracted total lipids from three replicates of a stool sample were divided into two aliquots. Total lipids in the first aliquot were hydrolysed and methylated to form FAME by protocol 4 (See 3.2.3.2). From the second aliquot the TAG and FA fractions were separated by TLC (See 3.2.2), hydrolysed and methylated. The fatty acids and ^{13}C palmitic acid concentrations in the total lipid, TAG and FA fractions are shown in Table 3.6. The GC-C-IRMS analysis showed that the surrogate TAG and FA standards were found only in their appropriate fractions. Figures 3.7a and 3.7b depict examples of the m/z = 44 and m/z = 45 GC-C-IRMS chromatograms of FAME from TAG fraction derived from chloroform:methanol (2:1, v:v) and acetic acid extraction. Similarly Figures 3.8a and 3.8b depict examples of the m/z = 44 and m/z = 45 GC-C-IRMS chromatograms of FAME from FA fraction. The chromatograms show that surrogate standards were only found in their appropriate fractions and illustrate the relative isotope abundance of each component FAME. When a standard solution of internal standard (tricosanoic acid methyl ester) was analysed under conditions that loaded the column with 100 ng, the signals from both m/z 44 and m/z 45 had a signal to noise (s/n) ratio of 20. Signal to noise ratio was calculated as: (peak height / (6 × SD of baseline signal).

Table 3.6. Fatty acids and ^{13}C palmitic acid concentration in stool total lipid, TAG and FA fractions analysed by GC-C-IRMS. Mean of 3 replicates of a stool sample.

Fatty acid (μg/mg stools)	Total lipid	TAG	FA	Sum of TAG and FA
C16:0 [1]	5.68	0.46	4.21	4.67
C18:0	6.45	0.23	5.32	5.55
C18:1	4.88	1.33	3.26	4.59
C18:2	1.58	0.40	0.45	0.85
Sum of fatty acids [2]	18.59	2.41	13.24	15.65
^{13}C palmitic acid	0.15	0.06	0.07	0.13

[1] Total (^{13}C + ^{12}C) palmitic acid.
[2] Sum of C16:0, C18:0, C18:1 and C18:2.

The technique of GC-C-IRMS was required for the resolution of the errors which were found to occur when a standard lipid extraction procedure was applied to the extraction of lipids from stools. The experiments performed for resolving the inappropriate recovery of the FA standard in the TAG fraction illustrated the effects of different solvent and acid combinations for the completeness of the lipid extraction and for the extraction of the desired lipid fractions containing only their appropriate lipids.

Having resolved these errors, the requirement of the finalised method was to measure the ^{13}C enrichment in each of the properly separated TAG and FA fractions. The CF-IRMS technique was suitable for this purpose and could be applied to the whole lipid fraction. Although the GC-C-IRMS technique located the ^{13}C enrichment in a specific fatty acid and could therefore increase the understanding of the possible alterations of the form of the administered labelled lipid but the technique had no further advantages over the CF-IRMS in the differentiation between lipid maldigestion and malabsorption. The sample analysis time by CF-IRMS (6 min) was shorter than by GC-C-IRMS (25 min) and it was not necessary to hydrolyse and methylate the samples for CF-IRMS analysis. Consequently, the ^{13}C enrichment as analysed by CF-IRMS technique was used in the analysis of samples generated in the clinical studies to be reported in Chapter 6.

The reproducibility of determination of ^{13}C enrichment in TAG and FA fractions by CF-IRMS was evaluated from duplicate aliquots of 11 stool samples using the lipid extraction described in Protocol 5 followed by TLC separation as described in Protocol 2.

The measurement error of stool ^{13}C excretion in FA fraction expressed as a proportion of the administered dose was estimated from samples containing between 2.6% to 28.0% of dose as ^{13}C FA (Mean 13.2%, $n = 11$). Within-sample SD calculated by analysis of variance [Bland 1997] was found to be ± 2.3% of the administered dose. Similarly for stool TAG fraction in the range 0% to 23.3% of dose as ^{13}C TAG (Mean 7.3%, $n = 11$) the within sample SD was found to be ± 2.4% of the administered dose.

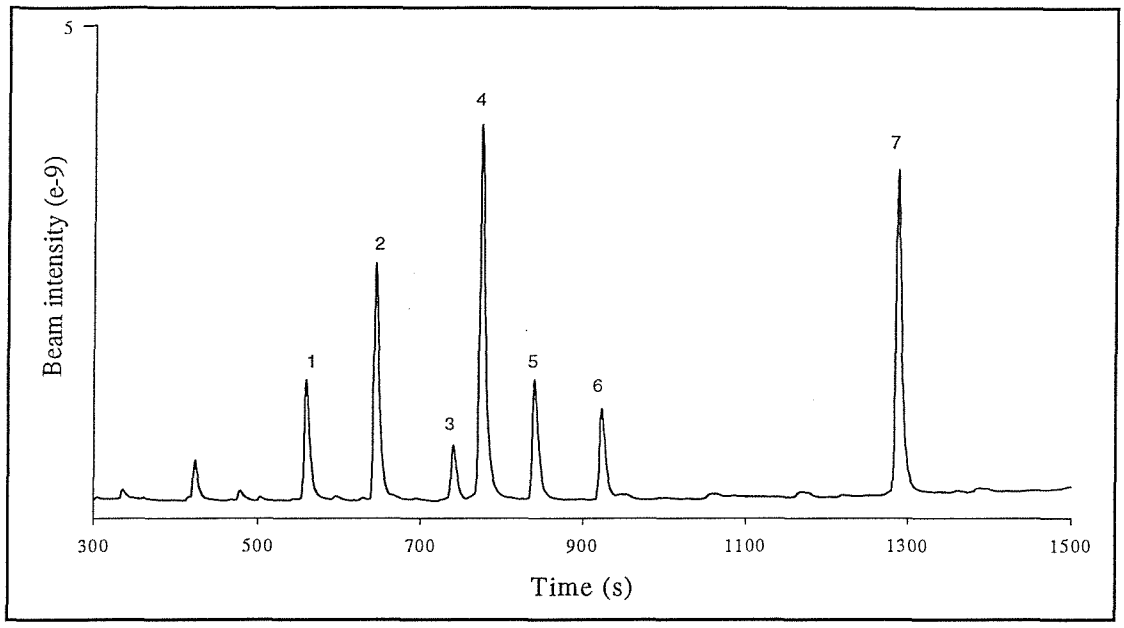


Figure 3.7a. The GC-C-IRMS detection of FAME from stool TAG as separated by TLC. The chromatogram shows CO_2 peaks obtained from m/z = 44 detector following fatty acid combustion after separation by gas chromatography. Peak 1, C16:0; peak 2, C17:0 (TAG standard); peak 3, C18:0; peak 4, C18:1; peak 5, C18:2; peak 6, C18:3; peak 7, C23:0 (internal standard).

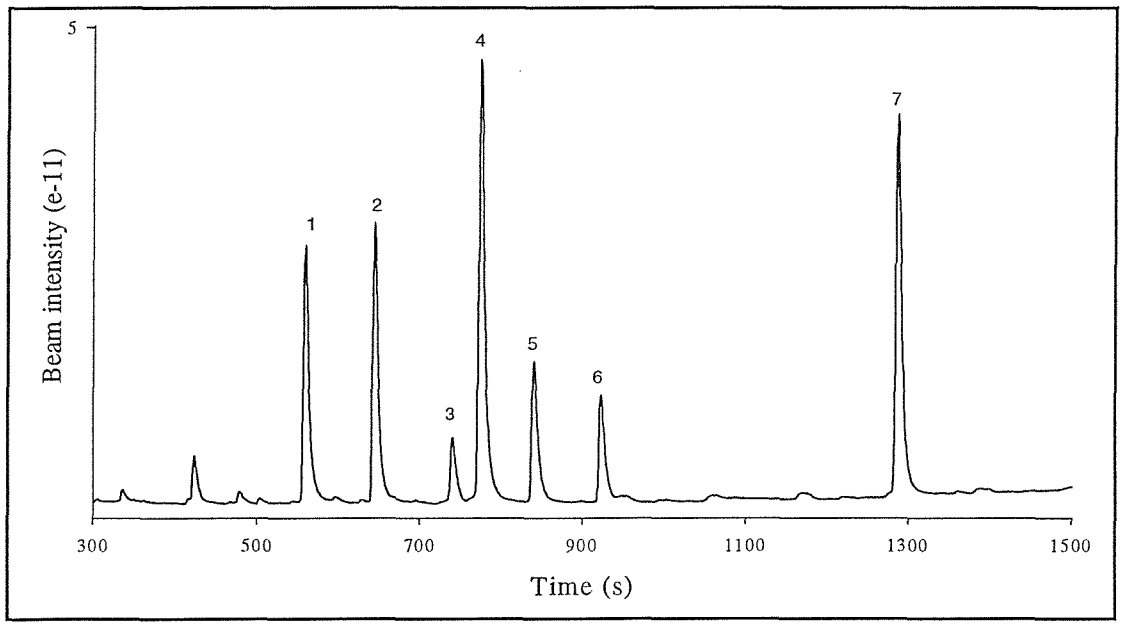


Figure 3.7b. The GC-C-IRMS detection of FAME from stool TAG as separated by TLC. The chromatogram shows CO_2 peaks obtained from m/z = 45 detector following fatty acid combustion after separation by gas chromatography. Peak 1, C16:0; peak 2, C17:0 (TAG standard); peak 3, C18:0; peak 4, C18:1; peak 5, C18:2; peak 6, C18:3; peak 7, C23:0 (internal standard).

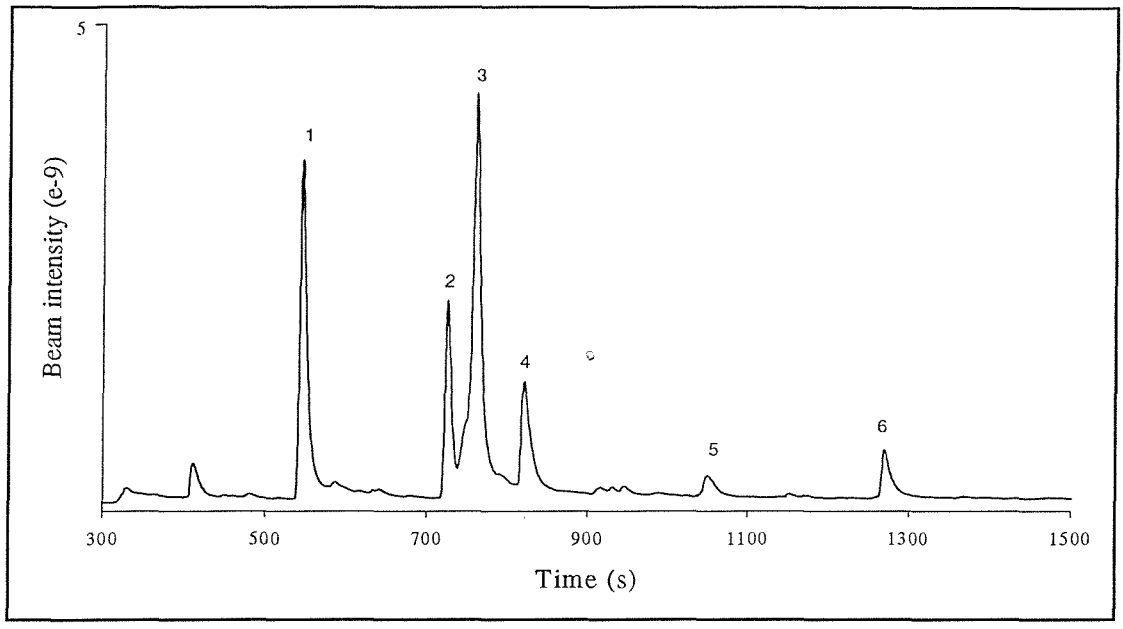


Figure 3.8a. The GC-C-IRMS detection of FAME from stool FA as separated by TLC. The chromatogram shows CO_2 peaks obtained from m/z = 44 detector following fatty acid combustion after separation by gas chromatography. Peak 1, C16:0; peak 2, C18:0; peak 3, C18:1; peak 4, C18:2; peak 5, C21:0 (FA standard); peak 6, C23:0 (internal standard).

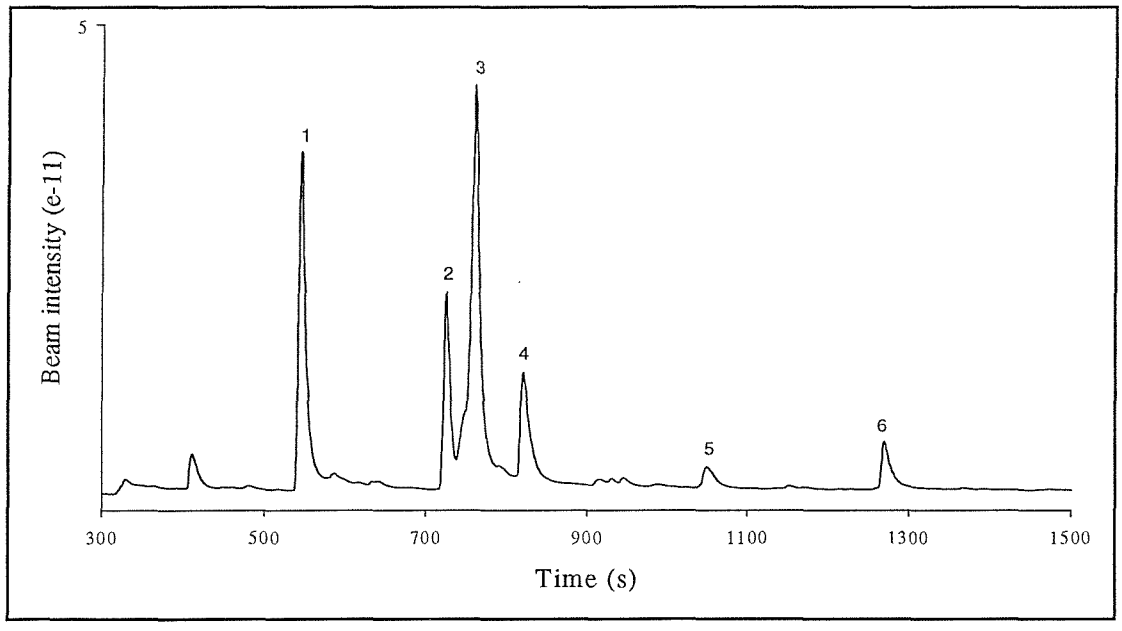


Figure 3.8b. The GC-C-IRMS detection of FAME from stool FA as separated by TLC. The chromatogram shows CO_2 peaks obtained from m/z = 45 detector following fatty acid combustion after separation by gas chromatography. Peak 1, C16:0; peak 2, C18:0; peak 3, C18:1; peak 4, C18:2; peak 5, C21:0 (FA standard); peak 6, C23:0 (internal standard).

3.3.3 Sample storage

The protocol for measuring stool losses of label following an administration of ^{13}C-labelled lipid requires stool collection for three days. It was not practicable to process stool samples immediately following defecation. Therefore, subjects placed stool samples in cool boxes containing dry ice, which are delivered to the laboratory and placed into a -80°C freezer. Stools were subsequently homogenised and freeze-dried. Dry stools were easier to handle in laboratory conditions than wet stools. However, the drying process, the type of the stool sample during storage (wet, frozen, dry) and the length of storage may have an effect on stool lipid composition. Importantly stool TAG, which is required to determine the extent of lipid maldigestion may be subjected to hydrolysis. Therefore, ^{13}C-labelled tripalmitin was added to stools and the concentration of ^{13}C palmitic acid in TAG fraction was compared amongst samples derived from wet stools and dry stools stored for different time periods.

Sample and methods

A stool sample was obtained from a healthy subject. The sample was immediately homogenised with distilled water and [1,1,1-^{13}C] tripalmitin was added as an emulsion, prepared as in Appendix 4. The homogenate was divided into aliquots, which were processed and stored in different ways. Surrogate TAG standard was added to stools prior to lipid extraction. Total lipids were extracted in triplicate using Protocol 5 (See 3.3.2.3) and prepared for GC-C-IRMS. An aliquot was processed immediately and total lipids were extracted from wet stools (Wet 0). An aliquot was stored at 4°C for 3 days before extraction of total lipids from wet stools (Wet 3 d). Three aliquots of wet stool were frozen immediately and were stored for 2 hours, 1 month or 3 months at -80°C. At the end of each storage period samples were freeze-dried and total lipids extracted from dry stools (Frozen 2 h, Frozen 1 mo, Frozen 3 mo). Dry stools from the sample frozen for 2 hours were stored at -20°C for 1 month or 3 months before extraction of total lipid (Dry 1 mo, Dry 3 mo). Additionally, the extracted total lipids from the sample which was frozen for 2 hours and then freeze-dried, were stored at -20°C for 1 month or 3 months prior to further processing (Lipid 1 mo, Lipid 3 mo). Codes in the brackets refer to Figure 3.9.

Results

Figure 3.9 shows the effects of the type of sample stored and the duration of storage on ^{13}C palmitic acid concentration in TAG fraction. There appeared to be decrease in recovery of ^{13}C-label in TAG fraction only in those samples stored frozen at -20°C for 3 months. The slight increase in the enrichment of samples stored at -20°C as dry stools or as lipid extracts for 3 months is likely to be due to analytical imprecision rather than due to true increase in the enrichment.

Conclusion

Although it was recognised that the study was carried out with only 1 stool sample, it was decided that samples should not be stored as frozen homogenates for more than 1 month before freeze-drying. Stored wet stools are a greater health and safety risk than dry stools and the drying of stool samples did not reduce the concentration of the ^{13}C palmitic acid in the TAG fraction. Immediate freezing of the sample is preferable and storing of stool samples either as dry stool or as lipid extract for up to 3 months did not decrease the ^{13}C palmitic acid concentration within TAG fraction.

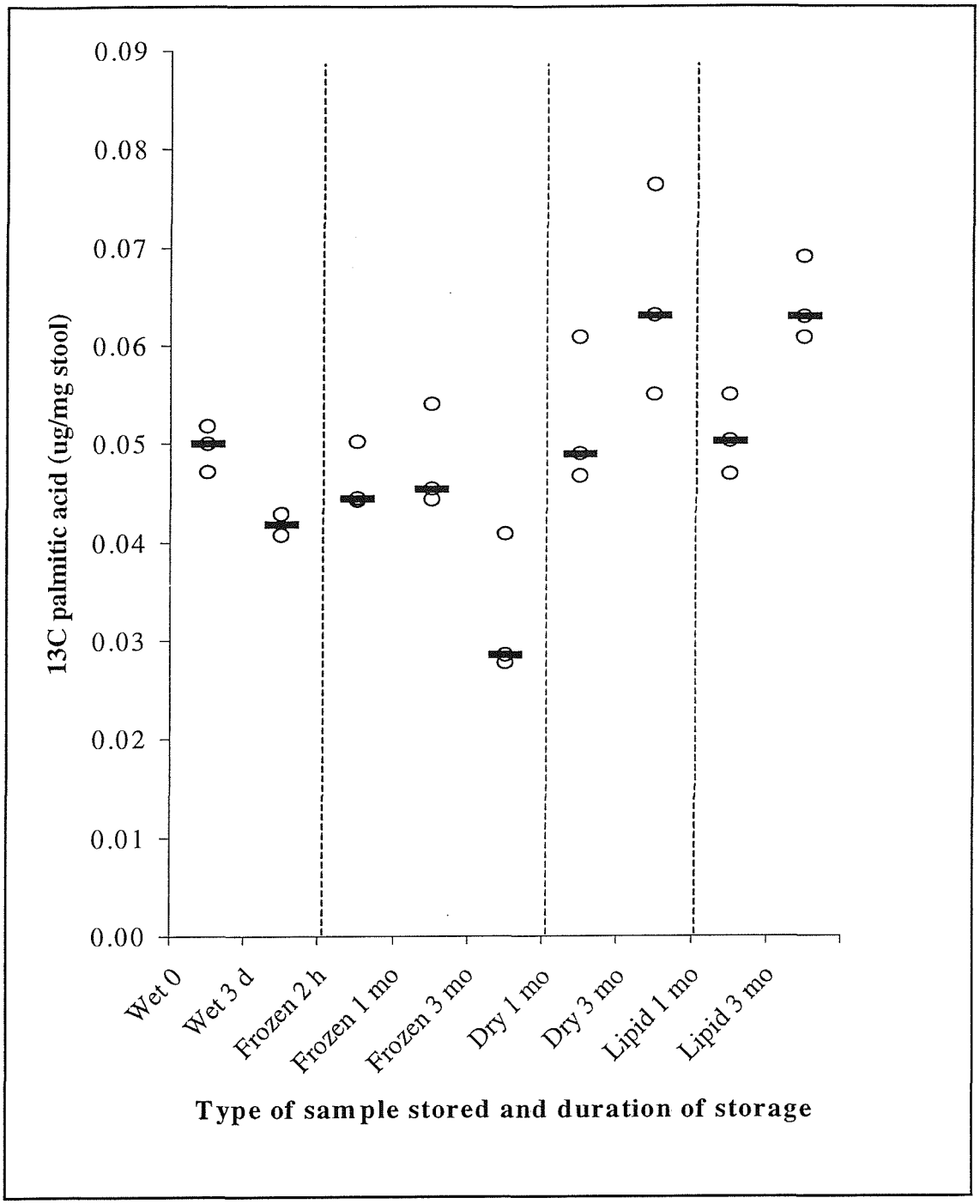


Figure 3.9. Effect of type of sample stored and duration of storage on ^{13}C palmitic acid concentration in stool TAG fraction. Median and individual values of 3 replicates. Codes are as follows: Lipids were extracted from 1) wet stools immediately = Wet 0, 2) wet stools after 3 days storage at 4°C = Wet 3 d, 3), 4) and 5) from freeze-dried stools after 2 hours, 1 month or 3 months storage as homogenate at -80°C = Frozen 2 h, Frozen 1 mo and Frozen 3 mo, 6) and 7) freeze-dried stools after 1 month or 3 month storage as dry stool at -20°C = Dry 1 mo and Dry 3 mo, 8) and 9) further processing after 1 month or 3 month storage as lipid at -20°C = Lipid 1 mo and Lipid 3 mo.

3.3.4 Summary

The cause of the inappropriate recovery of FA standard in the TAG fraction during lipid extraction and separation of lipid fractions from stools was characterised. An analytical procedure for lipid extraction, which would result in separation of TAG and FA fractions so that only the appropriate lipids are present in each fraction was developed.

The results can be summarised as:

1) The inappropriate recovery of the FA standard in the TAG fraction was not due to the TLC.
2) The inappropriate recovery of the FA standard in the TAG fraction was due to the formation of FAME from fatty acids and methanol in the presence of HCl.
3) Acidification of stools was required for the complete extraction of the ^{13}C-labelled lipids from stool as indicated by similar ^{13}C enrichments of the non-lipid residues of the unenriched and enriched stool samples.
4) Various solvent and acid combinations were investigated for extraction of total lipids from stools. Chloroform:methanol (2:1, v:v) and acetic acid extraction procedure resulted in efficient extraction of ^{13}C-labelled lipids and the surrogate TAG and FA standards were found only in their appropriate fractions. Therefore the chloroform:methanol (2:1, v:v) and acetic acid procedure was chosen for total lipid extraction from stools in the subsequent clinical studies.
5) Storing stool samples either as dry stools or as lipid extracts for up to three months did not affect the ^{13}C palmitic acid concentration in TAG fraction.

Chapter 4: General methods

The digestion, absorption and metabolic disposal of [1,1,1-^{13}C] tripalmitin were determined in this thesis by combining traditional balance techniques with stable isotope tracer techniques. The general protocol of the study is reported in this section. The analytical methods used to differentiate between lipid digestion and absorption were described and evaluated in detail in Chapter 3.

4.1 Study setting

The study was carried out at the Clinical Nutrition & Metabolism Unit, Institute of Human Nutrition at Southampton General Hospital. The facilities at the unit include a bed area for indirect calorimetry, a diet kitchen and laboratory premises for sample handling, storage and analysis. Written consent was obtained from subjects or from parents of the subjects and the study protocol was approved by the Southampton and South West Hampshire Joint Ethics Committee.

4.2 Anthropometry

Weight, height, triceps skinfold thickness, mid-upper-arm circumference and bioelectrical impedance were measured and used to calculate body composition of the subjects and growth parameters of those subjects who were children. Weight was measured using electronic scales (Soehnle Digital S, CMS Weighing Equipment Ltd., London, UK) and height was measured with a self-calibrating electronic stadiometer (Digi-Rod, CMS Weighing Equipment Ltd., London, UK). Holtain skinfold calipers were used for measuring triceps skinfold thickness. Height SD scores and weight SD scores were determined using the British 1990 Growth Reference Database [Child Growth Foundation according to Freeman *et al.* 1995] and proportional weight for height was determined. Body mass index was calculated for adult patients. Mid-upper-arm muscle circumference was calculated using the following formula: Mid-upper-arm muscle circumference = mid-upper-arm circumference (mm) - ($\pi \times$ triceps skinfold thickness (mm)) [Gibson 1990]. Bioeletrical impedance (Bodystat 1500, Bodystat Ltd., Isle of Man, UK) measurements were performed by positioning four electrodes to the skin (hand, wrist, foot and ankle) and passing a small electric current (500 μAmps) between the electrodes. The impedance (resistance) of the

flow to body water was measured, which depends on differences in electrical conductivity of LBM and fat mass [Gibson 1990]. The proportion of fat mass of those subjects who were children was calculated from bioelectrical impedance according to Houtkooper (1992). The LBM was determined as: total body weight (kg) - fat mass (kg).

4.3 Basal metabolic rate

Basal metabolic rate (BMR) was determined from a 30 min indirect calorimetry (Gas Exchange Measurement, PDZ Europa Ltd., Crewe, UK) measurement according to Weir equation [Weir 1949]. The Weir equation uses the rate of O_2 utilisation ($\dot{V}O_2$) and the rate of CO_2 excretion ($\dot{V}CO_2$) on breath for determination of the energy expenditure. Indirect calorimetry measurements were made whilst subjects were in a supine position in a thermoneutral room. The subjects were asked to withhold from food for 12 hours prior to the measurement. Estimated BMR was calculated using World Health Organization (1985) equations, which take into account age, gender and body weight of the subject.

4.4 Bowel habit diary

All subjects completed a daily bowel habit diary (Appendix 3) with parent's help where appropriate to determine the prevalence of gastrointestinal symptoms and bowel habit over the study period. The symptoms recorded by the subjects were summed for the five days and expressed as total amount of symptoms and relative proportion of patients recording symptoms. For example if all the 12 cystic fibrosis patients reported mild abdominal pain every day for the five day recording period, the total n would be $12 \times 5 = 60$ and proportion of patients having mild symptoms over the study period would be 100%. The missing recordings were excluded from the analysis.

4.5 Dietary intake

A 5 day weighed food intake was recorded by all subjects who were provided with electronic scales (Soehnle, CMS Weighing Equipment Ltd., London, UK) and preprinted forms. The subjects were asked to maintain their habitual diet during the study. The amount of supplementary gastrostomy feed taken overnight was recorded by the cystic fibrosis

patients or the parents of the cystic fibrosis patients. The second day of recording when the ^{13}C-labelled meal was administered (See 4.6) does not represent the habitual dietary intake and was therefore excluded from the dietary analysis. A computer program (COMP-EAT, Nutrition Systems, London, UK), based on the food composition tables [Holland *et al.* 1989], was used to analyse the energy and macronutrient (lipid, carbohydrate, protein) content of the diets. The manufacturer's data sheets were used for analysing the energy and macronutrients provided by the overnight feed of cystic fibrosis patients.

The food diaries of cystic fibrosis patients and control subjects for energy and macronutrient content were analysed by Joan Gavin, Paediatric Dietitian at Southampton General Hospital.

The total daily energy requirement of the cystic fibrosis patients was calculated according to the following formula [Ramsey *et al.* 1992]: Total daily energy requirement = [BMR kJ/d + disease coefficient (FEV_1 ≥80%, 1.5 + 0; FEV_1 40%-79%, 1.5 + 0.2 and FEV_1 <40%, 1.5 + 0.3)] × coefficient of lipid absorption (%). For comparative purposes the total daily energy requirement of the control subjects was calculated as BMR kJ/d × 1.5 [Department of Health 1991]. The energy intake from the diet was compared to the estimated total daily energy requirement.

4.6 Administration of [1,1,1-^{13}C] tripalmitin

After an overnight fast 10 mg/kg body weight of [1,1,1-^{13}C] tripalmitin (99 atom % excess; Masstrace, Woburn, USA) was administered as a glucose, sucrose, casein emulsion drink together with a breakfast containing 45 g white bread, 10 g butter and 100 ml sugar free squash. Cystic fibrosis patients omitted pancreatic enzyme preparations with the labelled meal to enable investigation of the residual capacity of the gastrointestinal tract to digest and absorb [1,1,1-^{13}C] tripalmitin.

Crystalline [1,1,1-^{13}C] tripalmitin, purchased from the manufacturer, was emulsified [Emken *et al.* 1993] in order to change the physicochemical properties of the tripalmitin to form, which would enable its handling within the gastrointestinal tract [Murphy *et al.* 1997]. Labelled emulsion drink was prepared by warming double cream, olive oil, sunflower oil and ^{13}C-tripalmitin in water bath at 85°C and mixing this with sucrose, glucose and casein dissolved in warm water (Appendix 4). The emulsion was flavoured with chocolate or strawberry drink powder. The administered dose of [1,1,1-^{13}C] tripalmitin was corrected for

the amount of the label that had remained in the administration vessel after consumption of the drink. The ^{13}C enrichment of the labelled drink that had remained in the administration vessel was analysed by CF-IRMS. It was found that only a small amount, 0.5 to 3.0 mg, of [1,1,1-^{13}C] tripalmitin (0.1% to 0.9% of the administered dose) was left to the administration vessel.

The test meal contained 1960 kJ energy, 29 g lipid and 44 g carbohydrate and was of low natural ^{13}C abundance. Habitual diet was modified for two days prior to administration of the labelled meal and during stool collection period. Subjects were asked to avoid foods that are naturally of high ^{13}C abundance. These are mainly corn and maize products, cane sugar, pineapple and other tropical fruits. The labelled meal was administered following an overnight fast, measurement of BMR (See 4.3) and a baseline breath sample (See 4.8). The labelled drink was either consumed by mouth or administered via the gastrostomy in cystic fibrosis patients and only water was allowed to be consumed until lunch was provided 6 hours later. Lunch included a hamburger or chicken nuggets, french fries and a diet drink. The habitual pancreatic enzyme preparation dose was taken with the lunch. Subjects went home after the 6 hour breath sample and lunch and were asked not to consume any food or drink, except water, until after the 10 hour breath sample.

4.7 Stool collection and analysis

All stools were collected for a three day period labelled by carmine markers. Two carmine capsules (2 × 400 mg) were taken with the labelled meal and another two capsules on the third morning after the labelled meal. Stool collection started at the administration of the labelled meal and finished when the second red carmine marker was passed in stools. One stool sample was collected prior to the administration of the labelled meal for determination of the baseline ^{13}C abundance of stool. Subjects placed stools in polyethene bags immediately into cool boxes containing dry ice. Portable toilet, polyethene bags, marker pen and cool box with dry ice were provided for subjects. Experiments showed that stools were frozen within two hours and lasted frozen up to 120 hours in the provided cool boxes filled with dry ice. Cool boxes were transported to the laboratory twice a week and stools were placed into a -80°C freezer.

Frozen stools were weighed, homogenised with distilled water (1:1.5; stools:distilled water) using a laboratory blender and a weighed aliquot (20 g) was freeze-dried (Genevac,

Ipswich, UK). Stool dry weight and proportion of the stool dry weight from wet weight were determined. Dry stool samples were ground using a mortar and were placed into a -20°C freezer in sealed containers with silica gel to prevent rehydrating the samples.

The laboratory methods for stool sample analysis were described in Chapter 3. All analyses were completed in duplicate. The ^{13}C-enrichments of dried stool samples above the baseline abundance were measured for individual days by CF-IRMS (See 3.2.3.1) and the proportion of the label excreted in stools over the study period was calculated. Total lipids were extracted from the baseline dry stool sample with Protocol 5 using chloroform:methanol (2:1, v:v) and acetic acid (See 3.3.2.3). For cystic fibrosis samples the total lipids were also extracted from the most enriched stool samples as determined by CF-IRMS. The total amount of lipid excreted per day was determined from the proportion of lipid dry stool weight for both groups. Coefficient of lipid absorption was calculated according to Ramsey *et al.* (1992) using the following formula: [(Lipid intake g/d - Stool lipid g/d)/Lipid intake g/d × 100]. Lipid fractions, TAG and FA, were separated by TLC (See 3.2.2; Protocol 2) from the baseline and the most enriched stool sample of cystic fibrosis patients. The weight of TAG and FA in stools was determined gravimetrically and the ^{13}C enrichments of TAG and FA fractions were measured by CF-IRMS. The ^{13}C enrichments were calculated as described in Chapter 3 (See 3.2.3.1). An aliquot of the total lipid, TAG and FA from the baseline sample and the most enriched sample was hydrolysed and methylated (as in Protocol 4, see 3.2.3.2) for determination of the proportional FA composition by gas chromatography with flame detection (GC-FID). The proportional FA composition of total lipid was determined from the baseline stool samples of the control subjects.

Bacteria mass of stools of the cystic fibrosis patients was also measured as part of another project [Gavin, submitted) and was compared to total lipid losses in stools measured in this thesis.

4.8 Breath sample collection and analysis

Breath samples were collected to determine the excretion of ^{13}C-label on breath as $^{13}CO_2$ in order to describe the metabolic disposal of dietary lipid. A single end-tidal breath sample was collected into a breath collection bag (Quintron, Milwaukee, USA) before consumption of the labelled meal (baseline) and at hourly intervals for 6 hours and at 8 and 10 hours after

the meal. Three samples (10 ml) were transferred to evacuated gas sample containers (Exetainers, Isochem, Finchampstead, UK) for subsequent analysis of $^{13}CO_2$ by CF-IRMS (See 3.2.3.1) in duplicate. The analysis of breath sample enrichment is in principal the same as for the stool sample analysis, except that the breath samples were injected into CF-IRMS after the combustion tube (See Figure 3.3). Indirect calorimetry (PDZ Europa Ltd, Crewe, UK) was used to measure CO_2 excretion on breath for 10 minutes at hourly intervals for 6 hours. The ^{13}C excretion on breath as a proportion of the administered dose was calculated using the following equations:

Excretion of ^{13}C (above baseline abundance) on breath as a proportion of the administered dose at time t =

$$\frac{^{13}C \text{ in breath above baseline abundance at time t (mmol)} \times 100}{^{13}C \text{ administered (mmol)}}$$

Where:

1) ^{13}C in breath above baseline abundance at time t (mmol) =

$$\frac{(\text{atom\%}^1 \text{ at time t} - \text{atom\%}^1 \text{ at time } t_0) \times \text{carbon in breath at time t (mmol/h)}^2}{100}$$

[1] From CF-IRMS measurement.

[2] From indirect calorimetry measurement (mmol carbon/h = $\dot{V}CO_2$ (ml/min) × 60 min ÷ 22.4 (l/mol), where 1 mol of gas has a volume of 22.4 liters (Avogadro's principle).

2) 1 mol of [1,1,1-^{13}C] tripalmitin contains 3 mol of ^{13}C, therefore mmol ^{13}C administered =

$$\left(\frac{\text{mg [1,1,1-}^{13}\text{C] tripalmitin administered}}{\text{mol wt of [1,1,1-}^{13}\text{C] tripalmitin}^4}\right) \times 3 \times \left(\frac{\text{isotopic purity of [1,1,1-}^{13}\text{C] tripalmitin}^3}{100}\right)$$

[3] 99%

[4] 810.7 g/mol

The cumulative ^{13}C excretion on breath was calculated as:

$$\left(\frac{(\%\ ^{13}\text{C/h at time t} + \%\ ^{13}\text{C/h at time t-1}) \times \text{change in time (h)}}{2}\right) + \text{cumulative \%}\ ^{13}\text{C at time t-1}$$

4.9 Substrate oxidation

Net substrate oxidation rates were calculated according to Frayn (1983) using the following formulae:

$$\text{Carbohydrate oxidation (g/min)} = (4.55 \times \dot{V}CO_2) - (3.21 \times \dot{V}O_2) - (2.87 \times n)$$

$$\text{Lipid oxidation (g/min)} = (1.67 \times \dot{V}O_2) - (1.67 \times \dot{V}CO_2) - (1.92 \times n)$$

Where $\dot{V}CO_2$ is CO_2 excretion on breath (l/min), $\dot{V}O_2$ is O_2 consumption (l/min) and n is urinary nitrogen excretion (g/min). Indirect calorimetry was used to measure O_2 consumption rate and CO_2 excretion rate for a 30 minute period before administration of the labelled meal and thereafter for 10 minute periods at hourly intervals for 6 hours. Urinary nitrogen excretion was estimated to be 0.01 g/min. The formulae use a typical TAG of stearic acid, palmitic acid and oleic acid to calculate the lipid oxidation rate, glucose to calculate carbohydrate oxidation rate and nitrogen to calculate protein oxidation rate.

4.10 Statistics

Statistical analysis was carried out using the computer program SPSS for Windows v. 9.0 (SPSS Inc., Chicago, USA). Non-parametric tests were used due to the small sample size and skewed distribution of some of the variables. Differences between groups were tested by the Mann-Whitney U test and differences between dependent samples were analysed by the Wilcoxon signed rank test. Correlations were calculated as the Spearman correlation coefficient (ρ). Differences were considered significant at $P < 0.05$. Results of group data are expressed as median, minimum (Min) and maximum (Max) and figures are illustrated as median and minimum and maximum or median and intermediate quartiles. The power of the non-significant differences was calculated to evaluate the probability of rejecting the null hypothesis when a difference between the groups exists (preventing type II error) [Kirkwood 1996].

Chapter 5: Study groups

The enrolment of study groups and clinical characteristics of subjects including anthropometry, BMR, gastrointestinal symptoms and dietary intake will be described in this Chapter.

5.1 Enrolment

All cystic fibrosis patients in the Wessex Region who had gastrostomies for overnight supplementary feeding were asked to participate in the study. From the 24 patients with gastrostomies, 10 patients declined to participate in the study due to ill health or social reasons. From the 14 patients who agreed to take part in the study, 12 patients successfully completed the study and are reported in this thesis. Stool collection was incomplete in one patient and breath data was not collected from one patient who had cerebral palsy in addition to cystic fibrosis. The 12 patients (9 females, 3 males) included 10 children and 2 adults (Median age 14.1 years, Range 7.0 to 30.9 years).

Eight healthy children (5 females and 3 males) were also studied and are referred to as control subjects in this thesis. Control subjects were recruited from friends and family members of patients and staff at Southampton General Hospital. Control subjects did not have gastrointestinal, endocrine or other diseases. The median age was 12.4 years (Range 7.8 to 16.5 years) for control subjects and was not different from the cystic fibrosis patients ($P = 0.488$).

The sample size to be studied was determined by a power analysis [Kirkwood 1996] using previously observed variability (SD) in the total ^{13}C excretion in stools and on breath in cystic fibrosis patients and control subjects following an oral administration of [1,1,1-^{13}C] tripalmitin [Murphy *et al.* 1998]. With the previously observed SD, at 80% power, 12 subjects would be required to detect about 15% difference in the ^{13}C-label excretion in stools and on breath of the administered dose between two groups. To detect a larger difference, about 20%, 10 subjects would be required to detect a difference in the label excretion in stools and 4 subjects would be required to detect a difference in the label excretion on breath between two groups.

5.2 Clinical characteristics of cystic fibrosis patients

The clinical characteristics of the cystic fibrosis patients are shown in Table 5.1. The following tables and figures for individual cystic fibrosis patients will follow the same numbering of the patients. The diagnosis of cystic fibrosis had been confirmed by sweat sodium concentrations greater than 70 mmol/l. Five of the patients had been diagnosed at birth, 6 under the age of 18 months and 1 at the age of 36 months. Most cystic fibrosis patients (10/12) had ΔF508 homozygous genotype and one patient had ΔF508 and S489X genotype. The genotype of 1 patient had not been determined. In addition to cystic fibrosis, one patient had coeliac disease (number 8), two patients had cystic fibrosis related diabetes mellitus (numbers 3 and 11) and one patient had had complete pancreatectomy (number 7, confirmed by personal communication with Consultant Paediatrician Dr. Connett). Pulmonary function (Forced expiratory volume in 1 second = FEV_1) was normal in 4 patients and moderately impaired in 8 patients. A means of assessing pulmonary disease by chest x-rays, the Chrispin-Norman score [Chrispin & Norman 1974], had been determined in 8 patients. Abnormalities in chest x-rays reflect the extent of pulmonary disease such as changes in lung volume, accumulation of mucus in lungs and lobular lesions. Scores ranged from 4 to 22 indicating a wide range of severity of pulmonary complications. All patients routinely received PERT. Daily lipase intake ranged from 4820 IU to 24040 IU (Median 10410 IU) per kg body weight. The duration of gastrostomy use ranged from 5 to 71 months (Median 29 months). At the time of the study cystic fibrosis patients were clinically well and not receiving antibiotic treatment other than those given by nebuliser for chronic *Pseudomonas aeruginosa* infection.

Table 5.1. Clinical characteristics of the cystic fibrosis patients.

Patient	Sex[1]	Age (yr)	SD score		Genotype[2]	FEV_1[3]	C-N score[4]	Bacterial colonisation[5]
			Height	Weight				
1	F	7.0	-0.5	-0.8	ΔF508 H	72	19	PA, SA
2	F	10.0	-1.9	-1.8	ΔF508 H	71	4	PA
3	F	10.3	-0.8	-0.1	ΔF508 H	90	4	PA
4	F	11.5	-3.2	-1.1	ΔF508 H	90	3	PA
5	F	14.1	-0.8	-0.4	ΔF508 H	90	-	PA
6	F	14.1	-1.3	-1.9	-	49	18	PA, SH, PC
7	M	14.2	-2.5	-1.2	ΔF508, S489X	56	16	PA
8	M	14.3	-3.0	-2.3	ΔF508 H	99	-	PA
9	F	15.1	-1.6	-0.8	ΔF508 H	46	18	PA
10	M	16.4	-2.8	-2.5	ΔF508 H	76	22	PA, SA
11	F	26.2			ΔF508 H	66	-	PA
12	F	30.9			ΔF508 H	55	-	PA

[1] F = female, M = male. [2] H = homozygous
[3] % of predicted. Normal lung function ≥80% of predicted, moderate lung disease 40-79% of predicted and severe lung disease <40% of predicted FEV_1.
[4] Chrispin-Norman score. Reference range: 0 to 36, where 0 = normal lung function [Chrispin & Norman 1974].
[5] PA = *Pseudomonas aeruginosa*, SA = *Staphylococcus aureus*, SH = *Staphylococcus haemophilis*, PC = *Pseudomonas cepacia.*
- Not known.

5.3 Anthropometry

Table 5.2 presents the anthropometric measurements of those cystic fibrosis patients who were children (n = 10) compared to control subjects. Cystic fibrosis patients and control subjects were of similar height (cm) and weight (kg). Cystic fibrosis patients were shorter and lighter compared to children of the same age and gender in the population and also compared to the control subjects as evaluated by height and weight SD scores. Height SD scores of 4 patients and weight SD scores of 2 patients were lower than -2 SD (Figure 5.1, Table 5.1). The height and weight of all control subjects was above -1 SD. There was no

difference in the proportional weight for height between the groups. The weight for height of 2 cystic fibrosis patients and 3 control subjects was less than 100%. The body mass indices of the 2 adult cystic fibrosis patients were 19.2 and 20.3 (kg/m^2), which are in the low normal range of healthy individuals (Normal range 20-25 kg/m^2; Gibson 1990).

Cystic fibrosis patients (n = 10) had lower LBM (kg) but similar fat mass (kg) and similar proportional fat mass (%) and proportional LBM (%) compared to control subjects (n = 8). When the comparison between the two groups was carried out including the two adult cystic fibrosis patients (n = 12) there was only a trend for LBM to be lower in cystic fibrosis patients compared to the control subjects (P = 0.064). There was a tendency for mid-upper-arm muscle circumference to be lower in cystic fibrosis patients (n = 10) compared to control subjects, although this did not reach statistical significance (P = 0.062). Similar difference between the groups was obtained when the two adult cystic fibrosis patients (n = 12) were included in the analysis (P = 0.054). However, mid-upper-arm muscle circumference was below the 25th centile for age and gender in the majority of the cystic fibrosis patients (9 children and 2 adults), whereas all control subjects were above the 25th centile.

Table 5.2. Anthropometric measurements in cystic fibrosis patients (n = 10) and control subjects (n = 8).

	Cystic fibrosis patients			Control subjects			
	Median	Min	Max	Median	Min	Max	P value[1]
Height (cm)	141.5	119.2	154.4	151.4	133.4	173.4	0.155
Height (SD score)	-1.8	-3.1	-0.5	0.3	-0.8	1.7	0.001
Weight (kg)	35.9	20.5	48.0	47.2	29.8	70.0	0.131
Weight (SD score)	-1.1	-2.5	-0.1	0.7	-0.1	1.4	0.001
Weight for height (%)	107.5	91	149	100	93	122	0.592
LBM (kg)	28.3	15.6	36.0	35.1	23.6	57.3	0.033
LBM (%)	75.2	68.8	82.3	79.4	66.4	85.6	0.214
Fat mass (kg)	9.5	4.6	15.0	8.6	5.4	20.9	0.824
Fat mass (%)	24.8	17.7	31.3	20.6	14.4	33.6	0.214
MUAMC (mm)[2]	176	135	184	205	159	259	0.062

[1] Difference between the groups, Mann-Whitney U test.
[2] Mid-upper-arm muscle circumference.

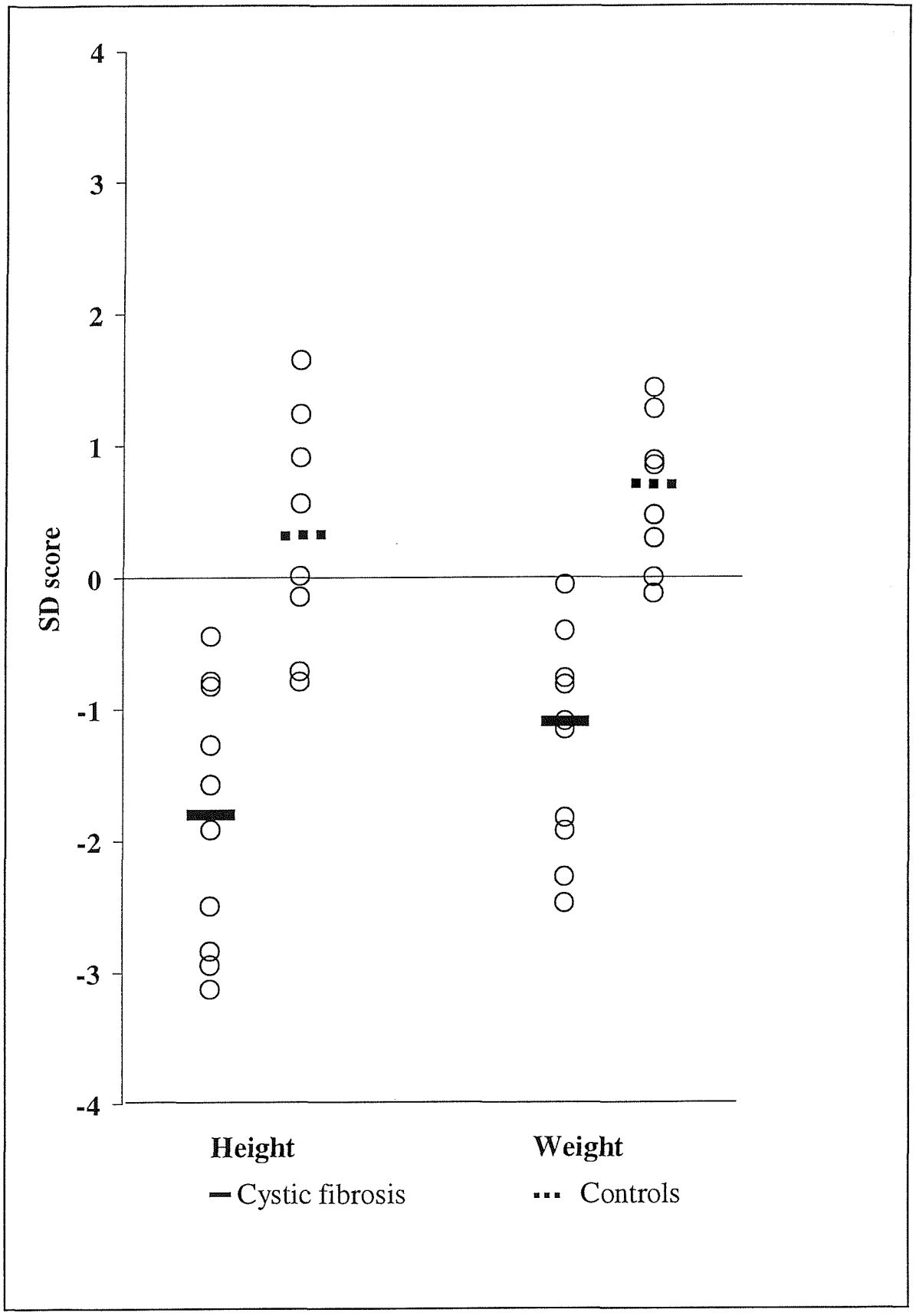


Figure. 5.1 Height and weight SD scores in cystic fibrosis patients (n = 10) and control subjects (n = 8). Median and individual values.

5.4 Basal metabolic rate

The BMR in cystic fibrosis patients and control subjects is shown in Table 5.3. There were no significant differences between cystic fibrosis patients and control subjects in BMR whether expressed kJ/d, kJ/kg body weight/d or kJ/kg LBM/d. Similar difference between the groups was obtained when the two adult cystic fibrosis patients were excluded from the analysis (kJ/d, $P = 0.328$; kJ/kg body weight/d, $P = 0.076$; kJ/kg LBM/d, $P = 0.076$).

The median estimated BMR was 5142 kJ/d (Range 4014 kJ/d to 5903 kJ/d) for cystic fibrosis patients and 5525 kJ/d (Range 4889 kJ/d to 7725 kJ/d) for control subjects ($P = 0.123$, cystic fibrosis patients vs. control subjects). The measured BMR as a proportion of the estimated BMR was similar in both groups (Table 5.3).

Weight ($\rho = 0.757$, $P = 0.030$) and LBM ($\rho = 0.905$, $P = 0.002$) were associated with the BMR in the control subjects but no association was found in the cystic fibrosis patients (Weight $\rho = 0.385$, $P = 0.217$; LBM $\rho = 0.308$, $P = 0.330$). Similar associations were found in the cystic fibrosis patients when the two adult patients were excluded from the analysis ($n = 10$: Weight $\rho = 0.309$, $P = 0.385$; LBM $\rho = 0.347$, $P = 0.327$). However, mid-upper-arm muscle circumference was associated with BMR in both groups (Cystic fibrosis patients $\rho = 0.755$, $P = 0.005$; Control subjects $\rho = 0.738$, $P = 0.037$)

Table 5.3. Basal metabolic rate in cystic fibrosis patients ($n = 12$) and control subjects ($n = 8$).

BMR	Cystic fibrosis patients			Control subjects			
	Median	Min	Max	Median	Min	Max	*P* value[1]
kJ/d	4960	3268	6586	5692	4577	7556	0.316
kJ/kg body weight/d	149	82	173	128	82	158	0.190
kJ/kg LBM/d	202	118	233	163	117	205	0.143
% estimated BMR	99	71	123	97	86	111	1.000

[1] Difference between the groups, Mann-Whitney U test.

5.5 Gastrointestinal symptoms

All subjects completed a daily bowel habit diary with parent's help where appropriate, to determine the prevalence of gastrointestinal symptoms and bowel habit over the study period. Table 5.4 presents the total and proportional prevalence of the gastrointestinal symptoms over the five day recording period. The maximum prevalence for the cystic fibrosis patients was 60 (12 subjects over 5 days) and for control subjects 40 (8 subjects over 5 days). For example the maximum prevalence (100%) for a particular symptoms would have occurred if all 12 cystic fibrosis patients had reported symptoms every day for the five day recording period.

The control subjects reported mainly having no gastrointestinal symptoms. A small proportion of the control subjects reported having abdominal pain, flatulence and nausea during the study period (6%, 16% and 5% respectively). The stool frequency of the control subjects varied from 1 stool per day to 3-5 times per week, which is considered to be a normal stool frequency. Cystic fibrosis patients reported more frequent gastrointestinal symptoms over the study period than control subjects, but the prevalence of severe symptoms was rare. Frequency of passing stools varied from normal (once/day or 3-5 times/wk) to several times per day (4 of the 12 patients). Also stool consistency and colour varied, 38% of patients reporting loose stool and 22% reporting pale or black stool.

Table 5.4. Prevalence of gastrointestinal symptoms and bowel function in cystic fibrosis patients and control subjects. Number and proportion of subjects reporting symptoms over five days.

	Cystic fibrosis (Total $n = 60$)[1]		Controls (Total $n = 40$)[1]	
	n	%	*n*	%
Abdominal pain				
None	37	65	37	95
Mild	13	23	1	3
Moderate	7	12	1	3
Flatulence				
None	37	67	33	85
Mild	15	27	5	13
Moderate	2	4	1	3
Severe	1	2	0	0
Nausea				
None	50	93	36	95
Mild	3	6	0	0
Moderate	1	2	2	5
Heartburn				
None	54	95	38	100
Mild	0	0	0	0
Moderate	1	2	0	0
Severe	1	2	0	0
Stool frequency				
3-5 times/wk	2	17	2	25
Once/d	6	50	6	75
2-5 times/d	4	33	0	0
Stool consistency				
Normal	24	48	26	84
Hard	7	14	3	10
Loose	19	38	2	6
Stool colour				
Brown	33	66	28	100
Pale	9	18	0	0
Black	2	4	0	0
Pain when passing stool				
None	50	88	8	100
Mild	6	11	0	0
Moderate	1	2	0	0

[1] The maximum prevalence refers to 12 patients and 8 controls reporting symptoms over five days. For example the maximum prevalence (60 = 100%) would have occurred if all 12 cystic fibrosis patients had reported mild abdominal pain every day for the five day recording period ($12 \times 5/60 \times 100$).

5.6 Dietary intake

Intake of energy and macronutrients from diet in control subjects and from the diet and supplementary feed in cystic fibrosis patients are shown in Tables 5.5 and 5.6. Cystic fibrosis patients had greater intakes of energy, lipid, carbohydrate and protein both as absolute amounts and when corrected for body weight, except the intake of saturated FA was similar in both groups. When the dietary intake excluding the supplementary feed in cystic fibrosis patients was compared to that of control subjects, there were no differences in the intakes of energy or macronutrients between the groups. The intake of saturated FA as a percentage of energy was lower and the intake of polyunsaturated FA as a percentage of energy was higher in cystic fibrosis patients compared to control subjects. The median intakes of macronutrients as a proportion of the energy were in concordance with the dietary reference values in the both groups (Lipid 35% of energy, carbohydrates 50% of energy and protein 15% of energy; Department of Health 1991). Median energy intake as a proportion of the estimated energy requirements was 116% for cystic fibrosis patients (Range 61% to 185%) and 92% (Range 73% to 112%, $P = 0.031$) for control subjects.

Table 5.5. Intake of energy and macronutrients (/d) from diet in control subjects (n = 8) and from diet and supplementary feed in cystic fibrosis patients (n = 12). Median, minimum and maximum.

	Cystic fibrosis patients						Control subjects			P value[1]	
	Diet			Diet and supplementary feed			Diet			Diet	Diet and feed
Nutrient	Median	Min	Max	Median	Min	Max	Median	Min	Max		
Energy											
kJ	7239	4326	9559	10448	7413	15859	7703	6073	10922	0.247	0.005
kJ/kg body weight	191	110	266	300	214	518	162	114	258	0.758	0.001
Lipid											
g	53	36	109	106	69	158	68	54	110	0.123	0.014
g/kg body weight	1.7	0.8	2.9	2.7	1.5	5.7	1.6	0.9	2.3	0.758	0.002
SAFA[2]											
g	22.3	13.8	47.6	24.8	15.8	37.5	30.1	18.0	42.2	0.123	0.671
g/kg body weight	0.6	0.3	1.4	0.6	0.3	1.6	0.6	0.4	1.0	0.877	0.589
MUFA[3]											
g	17.2	11.3	34.5	30.5	22.1	62.2	20.7	14.8	34.6	0.190	0.004
g/kg body weight	0.6	0.2	1.0	0.9	0.5	1.6	0.5	0.3	0.7	0.537	0.002
PUFA[4]											
g	6.7	2.7	18.9	19.7	10.6	36.9	10.5	5.4	15.2	0.232	0.001
g/kg body weight	0.2	0.1	0.6	0.5	0.3	1.1	0.2	0.1	0.3	0.939	<0.001
Carbohydrate											
g	221	96	321	390	245	495	255	201	345	0.316	0.005
g/kg body weight	6.0	3.2	9.7	10.1	6.2	13.7	4.9	3.9	9.0	0.537	0.001
Protein											
g	48	27	93	100	51	153	63	51	92	0.064	0.014
g/kg body weight	1.4	0.8	18	2.4	1.4	4.5	1.5	1.0	1.9	0.440	0.001

[1] Difference between cystic fibrosis and control groups, Mann-Whitney U test.
[2] Saturated lipid.
[3] Monounsaturated lipid.
[4] Polyunsaturated lipid.

Table 5.6. Intake of macronutrients (% of energy) from diet in control subjects (n = 8) and from diet and supplementary feed in cystic fibrosis patients (n = 12). Median, minimum and maximum.

	Cystic fibrosis patients						Control subjects			P value[1]	
	Diet			Diet and supplementary feed			Diet			Diet	Diet and feed
Nutrient	Median	Min	Max	Median	Min	Max	Median	Min	Max		
Lipid											
% energy	35	24	50	35	30	42	34	28	38	0.643	0.669
SAFA[2]											
% energy	14	7	25	8	0.04	12	15	10	18	0.846	0.001
MUFA[3]											
% energy	11	7	14	11	8	17	11	7	12	0.223	0.146
PUFA[4]											
% energy	5	2	8	8	5	9	5	3	8	0.638	0.008
Carbohydrate											
% energy	54	36	63	52	43	56	51	45	56	0.908	0.614
Protein											
% energy	14	10	18	15	13	17	15	13	17	0.558	0.968

[1] Difference between cystic fibrosis and control groups, Mann-Whitney U test.
[2] Saturated lipid.
[3] Monounsaturated lipid.
[4] Polyunsaturated lipid.

5.7 Discussion

Before presenting the findings concerning stool lipid losses and metabolic disposal of lipid, the clinical characteristics of cystic fibrosis patients were described and anthropometric measurements, gastrointestinal symptoms, BMR and dietary intake of patients were compared to those of controls.

Anthropometry

Cystic fibrosis patients who were receiving supplementary overnight feeding via a gastrostomy were studied. At Wessex Region, patients who are not growing at the expected rate or have difficulties in achieving or maintaining adult weight and have a poor appetite are selected to receive supplementary feeding via a gastrostomy in order to improve their growth and nutritional status. Therefore all the patients studied have previously had an inadequate energy availability of a variable degree compared to energy requirements, which had caused disturbed growth and weight loss and consequently patients received supplementary feeding via a gastrostomy. This study was not designed to examine the effects of supplementary feeding but it has previously been shown that supplementary feeding in cystic fibrosis patients improves growth [Levy *et al.* 1985; Rosenfeld *et al.* 1999]. However, despite supplementary feeding of variable duration (5 to 71 months) prior to the study, the height and weight SD scores of the patients were lower compared to control subjects and were below 2 SD in four patients for height and two patients for weight. Although the weight for height of most patients was more than 100% and similar to control subjects suggesting that supplementary feeding had increased weight gain but had not necessarily increased stature to the same extent. The absolute weights and heights of the two patient groups were comparable which may be of importance when comparing the metabolic disposal of lipids (Chapter 7), since body weight may be associated with energy expenditure [Cunningham 1980]. Those cystic fibrosis patients who were children (n = 10) had a lower LBM compared to the control subjects. When the adults were included in the analysis there was a trend for the LBM and also mid-upper-arm muscle circumference to be lower in the cystic fibrosis patients compared to the control subjects. The trend towards a lower LBM in the cystic fibrosis patients may have resulted from disproportional gain of fat and LBM due to supplementary feeding. However the effects of supplementary feeding on

body composition in cystic fibrosis are poorly understood and studies specifically designed to address this issue are required to optimise weight gain during supplementary feeding. The previous malnourishment and subsequent supplementary feeding may influence the current capacity of these cystic fibrosis patients to metabolise dietary lipid and will be discussed in Chapter 8.

Gastrointestinal symptoms

The prevalence of gastrointestinal symptoms and bowel habit was determined using a bowel habit diary. Compared to control subjects, cystic fibrosis patients reported more frequent gastrointestinal symptoms and bowel habit. However, most cystic fibrosis patients reported having none or having mild gastrointestinal symptoms. Only a small proportion of the patients (2% to 12%) reported moderate or severe gastrointestinal symptoms during the study period. However, 52% of the patients reported abnormal stool consistency (hard or loose stools) and 33% of the patients reported passing stools more than once a day suggesting that they may have some degree of gastrointestinal dysfunction causing excess stool losses or stool lipid losses. It seems that according to their own assessment the prevalence of moderate or severe gastrointestinal symptoms was low in the cystic fibrosis patients studied. The prevalence of mainly mild symptoms or absence of symptoms may have also been due to a selected group of patients that was studied. Only cystic fibrosis patients who had a gastrostomy were asked to participate in the study and those patients who did not feel well enough to participate, declined from the study. Therefore, if it would have been possible to include all the cystic fibrosis patients with gastrostomies to the study, the prevalence of moderate and severe symptoms might have been higher. The extent to which the reported gastrointestinal symptoms and bowel habit by the patients are related to the measured stool and stool lipid losses and the possible applications of using bowel habit diary or bowel habit questionnaire in the clinical management of the patients will be considered in Chapter 8.

Basal metabolic rate

Basal metabolic rate was measured by indirect calorimetry to determine whether cystic fibrosis patients have increased BMR compared to control subjects and to estimate the

energy requirements. Several studies have previously shown higher BMR in cystic fibrosis patients compared to control subjects or predicted values [Vaisman *et al.* 1987; Buchdahl *et al.* 1988; Spicher *et al.* 1991; Grunow *et al.* 1993]. However, others have also reported similar BMR between the two groups [Ward *et al.* 1999]. In the present study no difference in the BMR was observed between cystic fibrosis patients and control subjects. The predicted BMR of 5 cystic fibrosis patients exceeded the upper limit of the 95% confidence interval for controls (104%), which may suggest that the BMR of these patients was increased. The difference in the BMR between the groups should be interpreted with caution, because the power calculations indicated that with the observed SD and observed difference between the groups (720 kJ), 30 patients would have been required to detect a statistically significant difference ($P < 0.05$ at 80% power) between two groups. To detect a statistically significant difference between the two groups with the 12 patients, a difference of 1100 kJ in the BMR would have been required. Therefore it may be that the sample size studied was too small to detect a significant difference between the groups.

According to Cunningham (1980) LBM is the major determinant of the BMR. A trend towards lower LBM in the cystic fibrosis patients in the present study could therefore result in a lower energy expenditure. However no differences in the BMR was found between the groups when expressed as kJ/kg LBM. In the control subjects 82% (ρ^2) and in the cystic fibrosis patients only 9% (ρ^2) of the variability in the BMR could be explained by LBM. Therefore, in cystic fibrosis patients other factors than LBM contribute proportionally more to the BMR than in the control subjects. It has been suggested that an impaired lung function [Vaisman *et al.* 1987; Fried *et al.* 1991] may contribute to the increased BMR in cystic fibrosis. The cystic fibrosis patients participating in this study had varying degrees of severity of pulmonary dysfunction as assessed by FEV_1 and Chrispin-Norman score. However, at the time of the study patients were clinically well and not receiving antibiotics for pulmonary infections, which could explain the similar BMR between the two groups. Furthermore, no association was found between BMR and FEV_1 ($\rho = -0.007$, $P = 0.983$; $n = 12$) or Chrispin-Norman score ($\rho = -0.241$, $P = 0.565$; $n = 8$) in cystic fibrosis patients. However, considering the sample size studied and widely differing ages and body sizes of the subjects it is difficult to interpret whether the BMR measurements truly differ between the groups and what factors influence the BMR in the cystic fibrosis patients.

The present study did not attempt to measure total energy expenditure, but it is recognised that the overall energy requirements are determined by the total energy

expenditure and growth in children. The total energy expenditure in the cystic fibrosis patients may be increased due to a higher BMR, increased work of breathing muscles or infection or could be lowered due to a reduced physical activity as a result of illness. Previous studies have found either similar [Spicher *et al.*1991] or increased [Shepherd *et al.*1988] total energy expenditures in cystic fibrosis patients compared to control subjects. The overall increased energy expenditure in the cystic fibrosis patients could contribute to the development of energy deficiency and therefore growth impairment.

It is debatable whether BMR and total energy expenditure are increased in cystic fibrosis. In the present study there was no evidence for the increased BMR in cystic fibrosis patients compared to control subjects, however the difference between the groups should be interpreted with caution due to the small sample size studied. Increased BMR in the cystic fibrosis patients could result in increased total energy expenditure and increased energy requirements. However, cystic fibrosis patients are likely to be less active especially during disease exacerbation (infections), which would reduce the total daily energy expenditure. In the patients studied, the BMR does not seem to be increased and therefore would not increase the energy requirements of the patients compared to healthy individuals. The estimated total energy requirements are compared to the energy intake from the diet below.

Dietary intake

An inadequate supply of energy and nutrients with respect to demands may contribute to disturbed growth in cystic fibrosis patients. Accordingly an energy intake of 120% to 150% in excess to the recommended for age is commonly advocated [Littlewood & MacDonald 1987], however the evidence-base to support this recommendation is not conclusive. Another approach that may be used to estimate energy requirements and may be more appropriate for cystic fibrosis patients is to determine BMR (measured or estimated for gender and body weight) and to take into account patient's activity, pulmonary status (FEV_1) and degree of lipid losses in stools (coefficient of lipid absorption) [Ramsey *et al.* 1992]. In the present study the energy intake of 9 of the 12 cystic fibrosis patients exceeded the estimated energy requirements [Ramsey *et al.* 1992] when both the diet and the supplementary gastrostomy feed were taken into account. If only diet was taken into account the energy intake of 10 of the 12 patients was below the estimated energy requirement. Previous studies have also reported that the energy intake of cystic fibrosis

patients is similar to or higher compared to the energy intake of control subjects or the recommended energy intake for healthy individuals [Buchdahl *et al.* 1989; Ellis *et al.* 1992].

Cystic fibrosis patients are advised to increase their energy intake by consuming foods with relatively high lipid content (full fat products). In the present study the dietary intake of lipid, carbohydrate and protein as a proportion of the energy intake was similar in the cystic fibrosis patients and the control subjects and was in accordance with the dietary reference values for food energy and nutrients for the UK [Department of Health 1991]. When the total macronutrient intake from the diet and supplementary feed were taken into account the intake of saturated FA was lower and the intake of polyunsaturated FA was higher in cystic fibrosis patients compared to control subjects. The supplementary feed provided on average 42 g (Range 17 g to 73 g) additional lipid to the diet and increased the intake of unsaturated lipids. However, considering that cystic fibrosis patients (See Chapter 6) may have markedly increased lipid losses in stools, it may be inappropriate to compare the dietary intake of the cystic fibrosis patients to the dietary reference values for healthy individuals. Excessive lipid losses in stools would result in different proportions of the macronutrients absorbed compared to the healthy subjects. However, a higher intake of polyunsaturated fatty acids may be beneficial for cystic fibrosis patients, because unsaturated FA have been shown in healthy individuals to be better absorbed than saturated fatty acids [Jones *et al.* 1985b; Jones *et al.* 1999]. However, this has not been confirmed in cystic fibrosis patients.

The accurate estimation of energy requirements is difficult in cystic fibrosis patients due to various contributing factors such as possibly increased BMR, pulmonary infections and increased stool energy losses, which may lead to increased total energy requirements. Using equations for the estimated energy requirements that take into account these factors [Ramsey *et al.* 1992], the total energy intake (diet and supplementary feed) of the cystic fibrosis patients in this study was adequate or in surplus compared to the estimated energy requirements. Therefore, it seems that the availability of energy from the diet does not seem to be the major constraint that could affect growth and nutritional status in these patients. At the time of the study, most patients were to some extent overfed compared to their requirements. However, the energy intake of the cystic fibrosis patients from the diet is likely to vary considerably at different times and is likely to reduce when the patients have exacerbation of the disease such as infections. In these situations the supplementary feed will become an important source of energy for the patients. However, the extent to which the ingested nutrients are digested and absorbed in the gastrointestinal tract may be a more

significant factor affecting the energy availability in cystic fibrosis patients. The compensation of energy losses in stools may be difficult by increasing the energy intake from the diet and even supplementary feeding may not be sufficient to compensate stool losses if they are markedly increased. The extent of lipid losses in stools in these patients will be discussed in Chapter 6.

5.8 Summary

The clinical characteristics of cystic fibrosis patients were described and anthropometric measurements, gastrointestinal symptoms, BMR and dietary intake of patients were compared to those of the control subjects and are summarised as:

1) Cystic fibrosis patients received supplementary overnight feeding via a gastrostomy and PERT as part of their clinical management. Patients had a wide range of severity of pulmonary complications and gastrointestinal symptoms but were clinically relatively well and not receiving antibiotics at the time of the study.
2) The height and weight SD scores of those cystic fibrosis patients who were children were lower compared to control subjects, but weight for height (%) and absolute weight (kg) and height (cm) were similar in both groups. The LBM was lower in those cystic fibrosis patients who were children compared to the controls. When the two adult patients were included in the analysis there was only a trend for the LBM to be lower in cystic fibrosis patients.
3) The measured BMR and measured BMR as a proportion of the estimated BMR were similar in cystic fibrosis patients compared to control subjects.
4) The energy and macronutrient intake from diet only was similar in cystic fibrosis patients compared to control subjects. Taking into account the supplementary feed the energy and macronutrient intake of patients exceeded that of controls. The total energy intake (diet + supplementary feed) of 9 of the 12 patients exceeded the estimated energy requirements, but energy intake from the diet alone was insufficient to meet the requirements.

Chapter 6: Do cystic fibrosis patients have increased stool lipid losses and is it due to maldigestion or malabsorption?

6.1 Introduction

The extent of stool lipid losses in the cystic fibrosis patients compared to the control subjects was determined by the traditional technique (total lipid losses in stools) and by the novel stable isotope technique, where the ^{13}C-label excretion in stools was measured. The methods developed and evaluated in Chapter 3 to differentiate between lipid maldigestion and malabsorption by measuring the ^{13}C-label excretion in stool TAG and FA fractions following administration of ^{13}C-labelled TAG was used. The general methods were described in Chapter 4 and the study groups in Chapter 5. The main outcome variables investigated were:

1) Excretion of total lipid in stools, which was used to assess whether cystic fibrosis patients have increased stool lipid compared to control subjects and to evaluate whether stool lipid is associated with the amount of stools passed and with the intake of lipid from diet.
2) Excretion of TAG and FA in stools, which was used to assess the extent to which stool total TAG and total FA may be used to differentiate between maldigestion and malabsorption of lipids in cystic fibrosis patients.
3) Relative fatty acid composition of stool lipid, which was compared between the groups to determine whether stool fatty acid composition of the cystic fibrosis patients differs from that of the control subjects.
4) The excretion of ^{13}C in total stools, which was used to determine the residual capacity (without pancreatic enzyme preparations) of the gastrointestinal tract to digest and absorb [1,1,1-^{13}C] tripalmitin in cystic fibrosis patients compared to control subjects. The ^{13}C-label excretion in stools was compared to total lipid excretion in stools and to coefficient of lipid absorption.
5) Excretion of ^{13}C in stool TAG and FA fractions, which was used to determine the extent to which the administered [1,1,1-^{13}C] tripalmitin and therefore dietary lipid is maldigested (TAG) and malabsorbed (FA) in cystic fibrosis patients.

In addition to presenting the results, the factors that could affect the interpretation of the amount and type of stool lipid losses will also be discussed and the results will be compared to the previous studies. The possible explanations for the findings and implications of the findings for the clinical management of the patients will be discussed in Chapter 8.

6.2 Results

6.2.1 Excretion of total lipid in stools

Table 6.1 shows stool weight and lipid excretion in stools in cystic fibrosis patients and control subjects. Cystic fibrosis patients passed more stools per day compared to control subjects. Lipid excretion in stools of the control subjects was within the normal range (0 to 7 g/d) [Wollaeger *et al.* 1947; Murphy *et al.* 1991]. In contrast cystic fibrosis patients had elevated stool lipid losses. The stool lipid excretion of one cystic fibrosis patient (5.7 g/d) was similar to that of the control subjects. The proportion of stool lipid of wet stool weight was also higher in cystic fibrosis patients compared to control subjects. During the stool collection period cystic fibrosis patients omitted pancreatic enzyme preparations with the labelled meal, which may have caused an increase in lipid excretion in stools. The median lipid excretion at baseline was 16.8 g/d (Range: 4.3 to 36.5 g/d), which was not different to the lipid excreted in stools following the administration of the labelled meal (Median 22.0 g/d, range: 5.7 to 54.8 g/d, $P = 0.084$). Although, there was a tendency for the lipid excretion in stools to be greater when the enzyme preparations were omitted with the labelled meal. It is likely that the total lipid excretion in stools reflects the lipid losses with enzyme preparations rather than the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid.

The total stool lipid weight increased with stool weight in cystic fibrosis patients ($\rho = 0.860$, $P < 0.001$), but not in control subjects ($\rho = 0.611$, $P = 0.108$, Figure 6.1). The stool lipid excretion was not related to the intake of lipid from diet in control subjects ($\rho = 0.143$, $P = 0.736$) and from diet and enteral feed in cystic fibrosis patients ($\rho = 0.504$, $P = 0.094$, Figure 6.2). The coefficient of lipid absorption ranged from 91% to 98% in control subjects and from 47% to 92% in cystic fibrosis patients (Table 6.1). All patients had excessive lipid losses in stools according to definition by Ramsey *et al.* (1992) that coefficient of lipid absorption less than 93% indicates excessive lipid losses in stools.

Table 6.1. Stool weight (g/d), stool lipid (g/d), proportion of lipid of stool weight and coefficient of lipid absorption in cystic fibrosis patients (n = 12) and control subjects (n = 8).

	Cystic fibrosis patients			Control subjects			
	Median	Min	Max	Median	Min	Max	P value[1]
Stool (g/d)	132.0	55.8	332.5	66.6	41.0	152.9	0.037
Stool lipid (g/d)	22.0	5.7	54.8	2.6	1.9	5.4	<0.001
Stool lipid % of wet stool weight	16.2	10.2	23.5	3.5	3.0	6.6	<0.001
Coefficient of lipid absorption (%)[2]	80	47	92	96	91	98	<0.001

[1] Difference between cystic fibrosis and control group, Mann-Whitney U test.
[2] [(Lipid intake g/d - Stool lipid g/d)/Lipid intake g/d × 100].

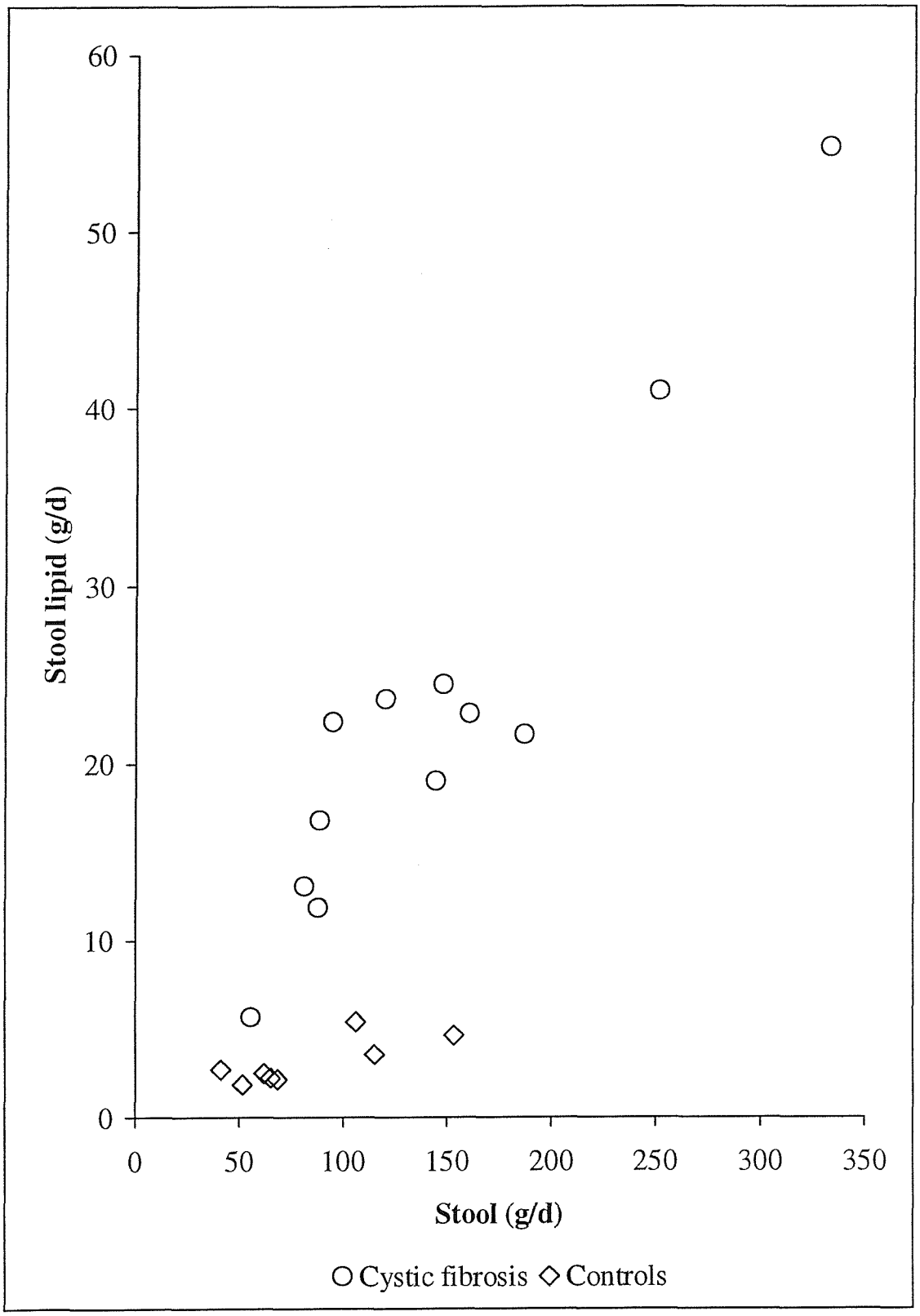


Figure 6.1. Association between stool total lipid excretion (g/d) and stool excretion (g/d) in cystic fibrosis patients (n = 12) and control subjects (n = 8).

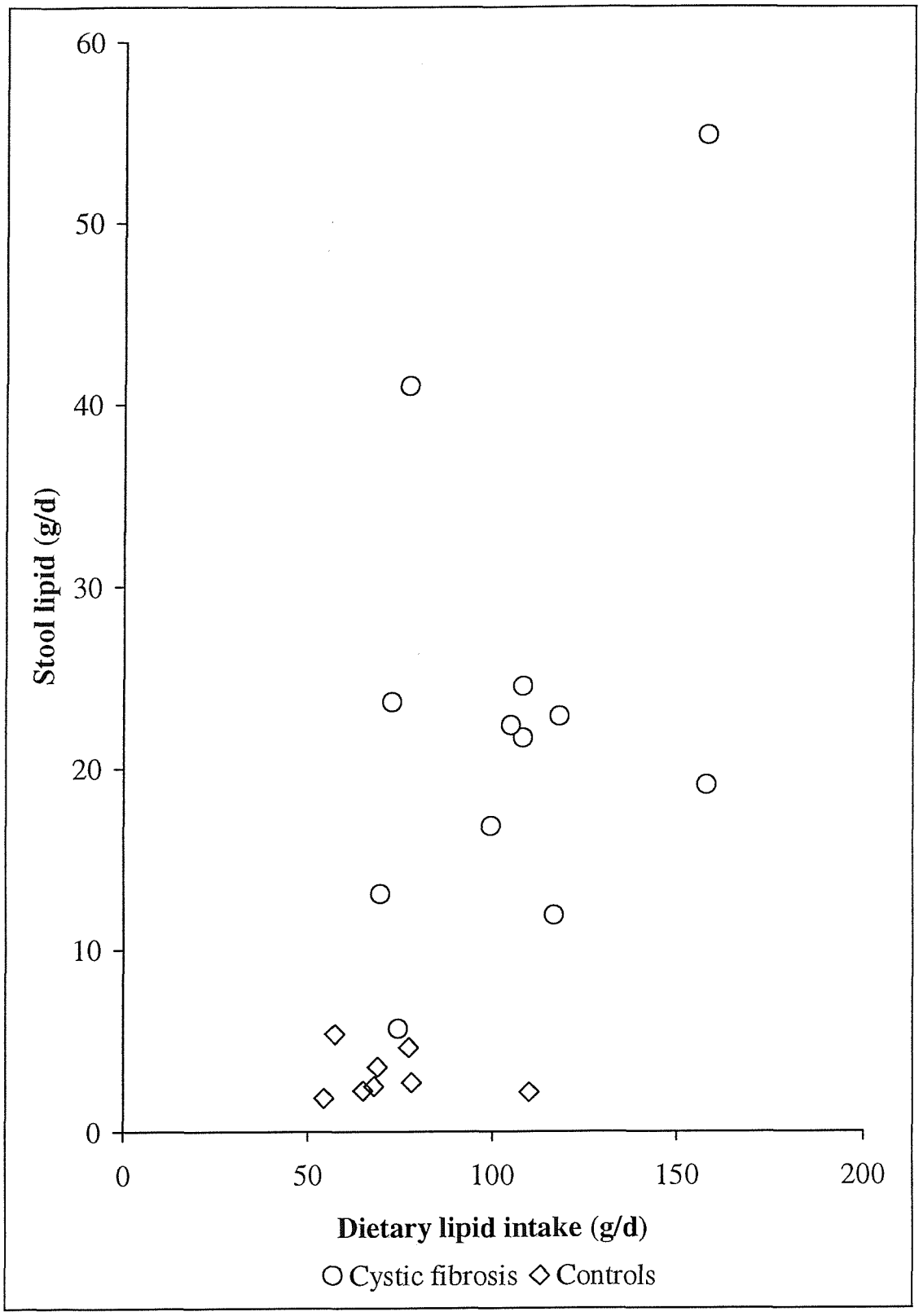


Figure 6.2. Association between stool total lipid excretion (g/d) and total (diet and supplementary feed) dietary lipid intake (g/d) in cystic fibrosis patients (n = 12) and control subjects (n = 8).

6.2.2 Excretion of TAG and FA in stools

The TAG and FA fractions were only measured in the stool samples of cystic fibrosis patients. The control subjects had markedly lower lipid losses in stools than cystic fibrosis patients and also similar stool lipid losses compared to previous studies. Therefore, control subjects were considered not to have lipid maldigestion or malabsorption that would be of clinical relevance. In the absence of the control data for stool TAG and FA content, the upper limit of the 95% confidence interval for total lipid losses in stools in the control subjects (4.2g/d) was used as a criteria to determine the excessive lipid losses in stools in the cystic fibrosis patients. The lipid losses in stools in the control subjects may be derived either from maldigested lipid or from malabsorbed lipid and therefore the TAG or FA losses in stools in excess of 4.2 g/d were considered as maldigestion and malabsorption of lipid, respectively. The stool TAG and FA contents over the study period were estimated by extrapolating the TAG and FA content of the most enriched stool sample to the total lipid losses and were expressed as g/day. The approach of using stool TAG and FA fractions to define maldigestion and malabsorption of lipids will be discussed in 6.3.

The results for excretion of total lipid, TAG and FA in the most enriched stool sample (g/sample) and the concentration of lipid, TAG and FA in stools (mg/g stool) are presented in Table 6.2. Triacylglycerols and FA fractions were detected in the stool samples of all cystic fibrosis patients. The TAG content ranged from 2.7 g to 12.6 g in the sample and from 14.6 mg to 125 mg/g stool and FA content ranged from 3.7 g to 26.3 g in the sample and from 57.7 mg to 157.4 mg/g stool. The sum of TAG and FA accounted for 52% to 99% of the stool total lipid in the sample. Figure 6.3 shows daily total lipid, TAG and FA losses in stools, extrapolated for total stool losses and expressed as g/d in individual cystic fibrosis patients in ascending order for the total lipid. The dotted line represents the upper limit of the 95% confidence interval for total lipid excretion in stools observed in the control subjects (4.2 g/d). The total TAG excretion in stools exceeded 4.2 g/d in 8 of the 12 patients indicating maldigestion of lipids and the total FA excretion in stools exceeded 4.2 g/d in 11 patients indicating malabsorption of lipids.

Table 6.2. Stool lipid, TAG and FA content (g/stool sample and mg/g stool) and the sum of TAG and FA as a proportion of total lipid in the most enriched stool sample in individual cystic fibrosis patients (n = 12).

Patient	Stool	Lipid		TAG		FA		Sum of TAG and FA % of lipid
	g / sample	g / sample	mg / g stool	g / sample	mg / g stool	g / sample	mg / g stool	
1	186	24.5	132.2	10.8	58.2	11.8	63.6	92
2	73	11.9	163.4	6.3	86.7	5.5	75.0	99
3	65	7.2	111.2	3.0	46.7	3.7	57.7	94
4	97	15.3	157.9	4.6	47.5	8.1	83.5	83
5	183	24.1	131.3	5.2	28.5	11.3	61.5	69
6	134	21.6	161.2	12.6	93.6	8.0	59.6	95
7	48	10.1	211.8	5.9	125.0	3.8	80.4	97
8	207	36.0	174.0	8.3	40.1	26.3	127	96
9	185	33.3	180.0	2.7	14.6	14.7	79.2	52
10	124	23.1	185.8	4.9	39.5	9.0	72.8	60
11	51	19.8	218.5	4.0	44.6	14.2	157.4	92
12	151	22.2	147.0	6.6	43.6	14.9	98.7	97
Median	129	21.9	162.3	5.6	45.7	10.2	77.1	93

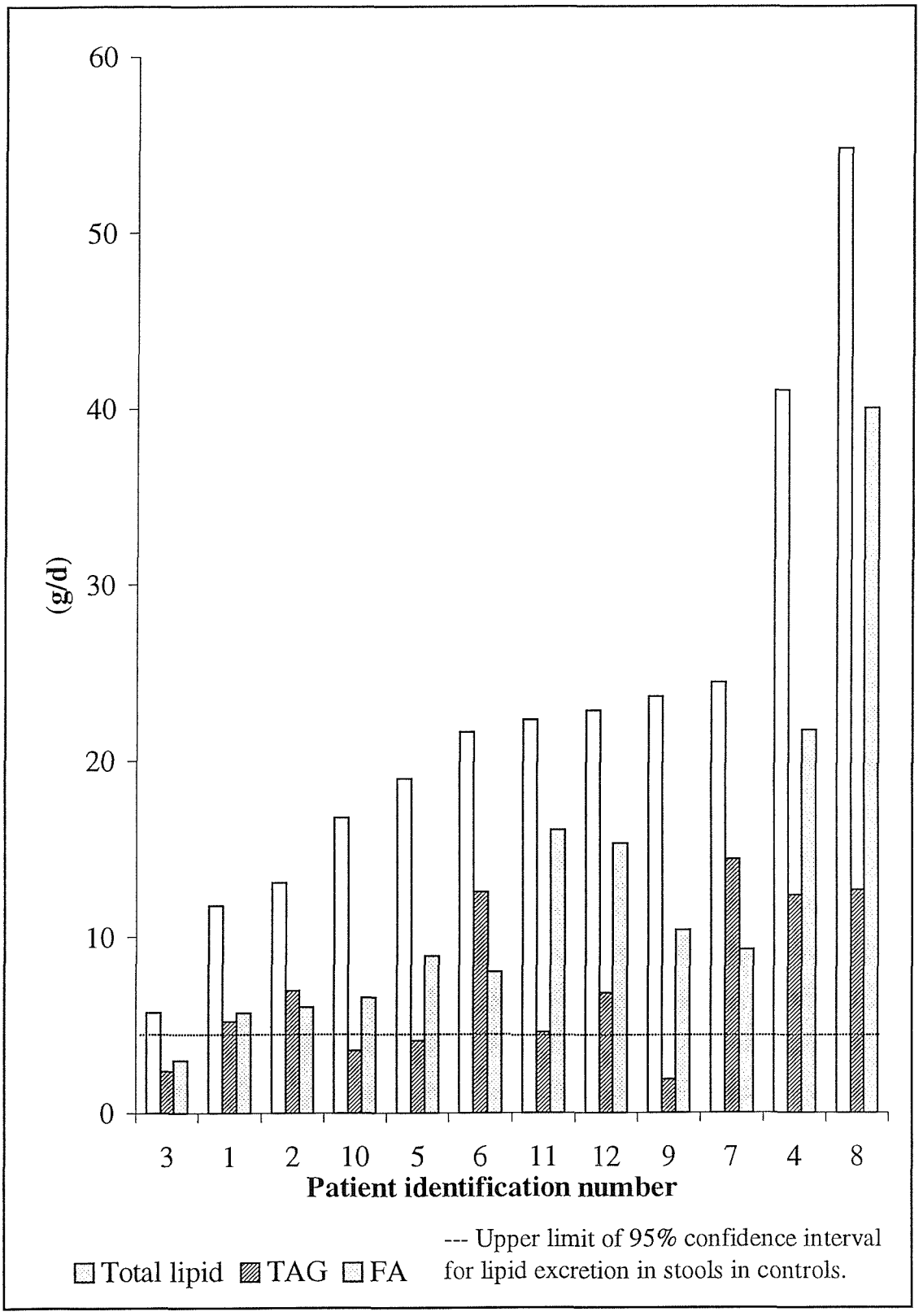


Figure 6.3. Excretion of total lipid, TAG and FA in stools (extrapolated to total stool lipid) expressed as g/d in individual cystic fibrosis patients shown in ascending order for the total lipid in stools. The dotted line represents the upper limit of 95% confidence interval for lipid excretion in stools (g/d) observed in control subjects.

6.2.3 Fatty acid composition of stool lipid

Table 6.3 shows the relative proportions of C16:0, C18:0, C18:1 and C18:2 in stool total lipid and TAG and FA fractions as a proportion of total fatty acids (Sum of C16:0, C18:0, C18:1 and C18:2) in each fraction. The proportion of C16:0 and C18:2 in stool total lipid was similar in cystic fibrosis patients and control subjects, but cystic fibrosis patients had proportionally less C18:0 and more C18:1 in stool total lipid compared to control subjects. In cystic fibrosis patients there was more C16:0 in baseline FA fraction compared to baseline TAG fraction, but less C18:1 and C18:2. The same differences remained when enriched samples were compared. In addition there was less C18:0 in the enriched TAG compared to enriched FA samples. In the comparison of FA compositions between the baseline samples and the enriched samples in cystic fibrosis patients only small differences were observed but there was proportionately more C18:2 in the baseline TAG samples compared to enriched TAG samples.

Table 6.3. Fatty acid composition (% total FA[1]) of stool lipid, TAG and FA fractions in cystic fibrosis patients (n = 12) and control subjects (n = 8).

	Control subjects			Cystic fibrosis patients					
	Baseline sample			Baseline sample			The most enriched sample		
	Median	Min	Max	Median	Min	Max	Median	Min	Max
Total lipid									
C16:0 (%)	33.4	26.4	48.4	36.8	14.9	42.5	30.2	19.5	34.4
C18:0 (%)	40.6	30.4	56.9	19.2[2]	7.9	53.6	19.2[2]	11.31	23.9
C18:1 (%)	16.0	10.7	27,2	32.3[2]	9.2	67.0	40.7[2]	32.0	56.7
C18:2 (%)	7.8	1.9	16.1	8.6	0	21.5	10.2	5.3	14.7
TAG									
C16:0 (%)	-			19.0[4]	13.7	29.9	21.3[5]	11.4	28.8
C18:0 (%)	-			13.8	5.4	38.8	9.2[5]	5.5	15.0
C18:1 (%)	-			43.3[4]	14.8	69.5	50.9[5]	44.0	63.4
C18:2 (%)	-			19.7[4]	10.4	32.1	16.1[3,5]	9.4	25.1
FA									
C16:0 (%)	-			37.6	18.9	44.5	34.3	26.3	38.3
C18:0 (%)	-			18.7	8.9	52.0	22.2	16.0	35.2
C18:1 (%)	-			30.6	11.0	62.9	34.3	25.7	47.8
C18:2 (%)	-			9.2	1.9	16.4	8.7	6.5	12.2

[1] Sum of C16:0, C18:0, C18:1 and C18:2.
[2] Different from controls, P<0.05, Mann-Whitney U test.
[3] Different from the baseline TAG, P<0.05, Wilcoxon Signed Rank Test.
[4] Different from the baseline FA, P<0.05, Wilcoxon Signed Rank Test.
[5] Different from the enriched FA, P<0.05, Wilcoxon Signed Rank Test.
- Not analysed.

6.2.4 Excretion of ^{13}C in total stools

The administered [1,1,1-^{13}C] tripalmitin was well digested and absorbed in control subjects. Only 0% to 10.9% of the administered ^{13}C-label (Median 1.9%) was excreted in stools indicating a bioavailability of the [1,1,1-^{13}C] tripalmitin in the range from 89.1% to 100%. In cystic fibrosis patients 6.8% to 77.9% of the label (Median 51.2%, $P < 0.001$) was excreted in stools indicating a bioavailability of the [1,1,1-^{13}C] tripalmitin in the range from 22.1% to 93.2%. Figure 6.4 illustrates the label excretion in stools of individual cystic fibrosis patients and control subjects. Two cystic fibrosis patients had a label excretion in stools within the range of control subjects (6.8% and 9.7% of the administered dose), despite not receiving pancreatic enzyme preparations with the labelled meal. The stool collection period varied from 4 to 5 days by which time the stool ^{13}C enrichment had returned to the baseline level. Most of the label was excreted in stools during the first three days of the stool collection in both groups.

There was an association between total ^{13}C-label excretion in stools over the study period and amount of lipid excreted in stools per day in control subjects ($\rho = 0.719$, $P = 0.045$), but no association was found in cystic fibrosis patients ($\rho = 0.336$, $P = 0.286$, Figure 6.5). However, in cystic fibrosis patients the amount of lipid excreted in stools (g/d) correlated positively with the amount of bacteria in stools (g/d; $\rho = 0.840$, $P = 0.02$, $n = 10$). It is likely that bacterial lipid contributes to the stool lipid in cystic fibrosis samples and therefore ^{13}C excretion in stools is a better indicator of dietary lipid losses than total stool lipid. Stool ^{13}C excretion was not related to the coefficient of lipid absorption in either groups (Cystic fibrosis patients: $\rho = -0.420$, $P = 0.175$; control subjects $\rho = -0.683$, $P = 0.062$, Figure 6.6).

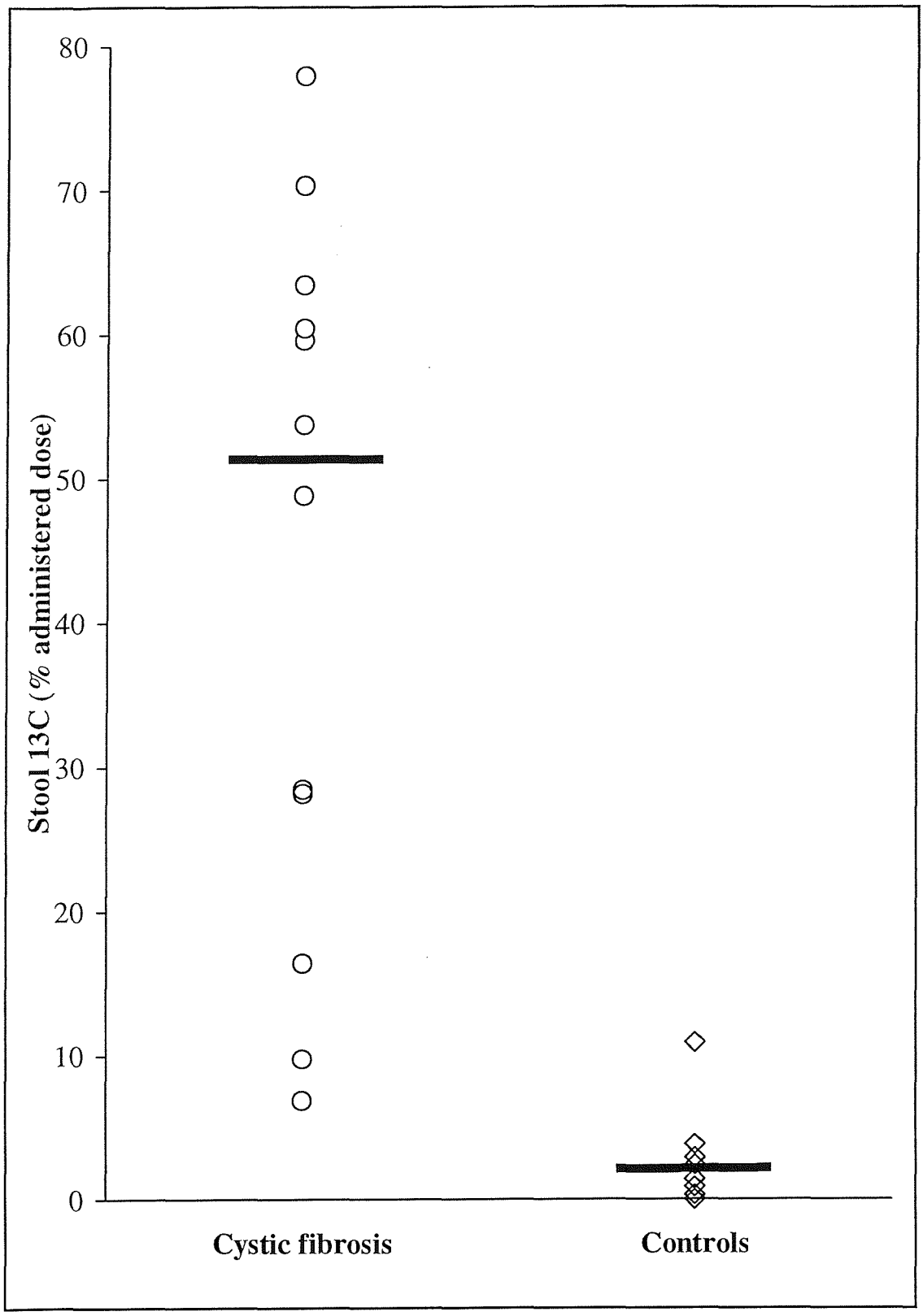


Figure 6.4. Excretion of ^{13}C in stools (% administered dose) over the study period following administration of ^{13}C-labelled meal in cystic fibrosis patients and control subjects. Median and individual values.

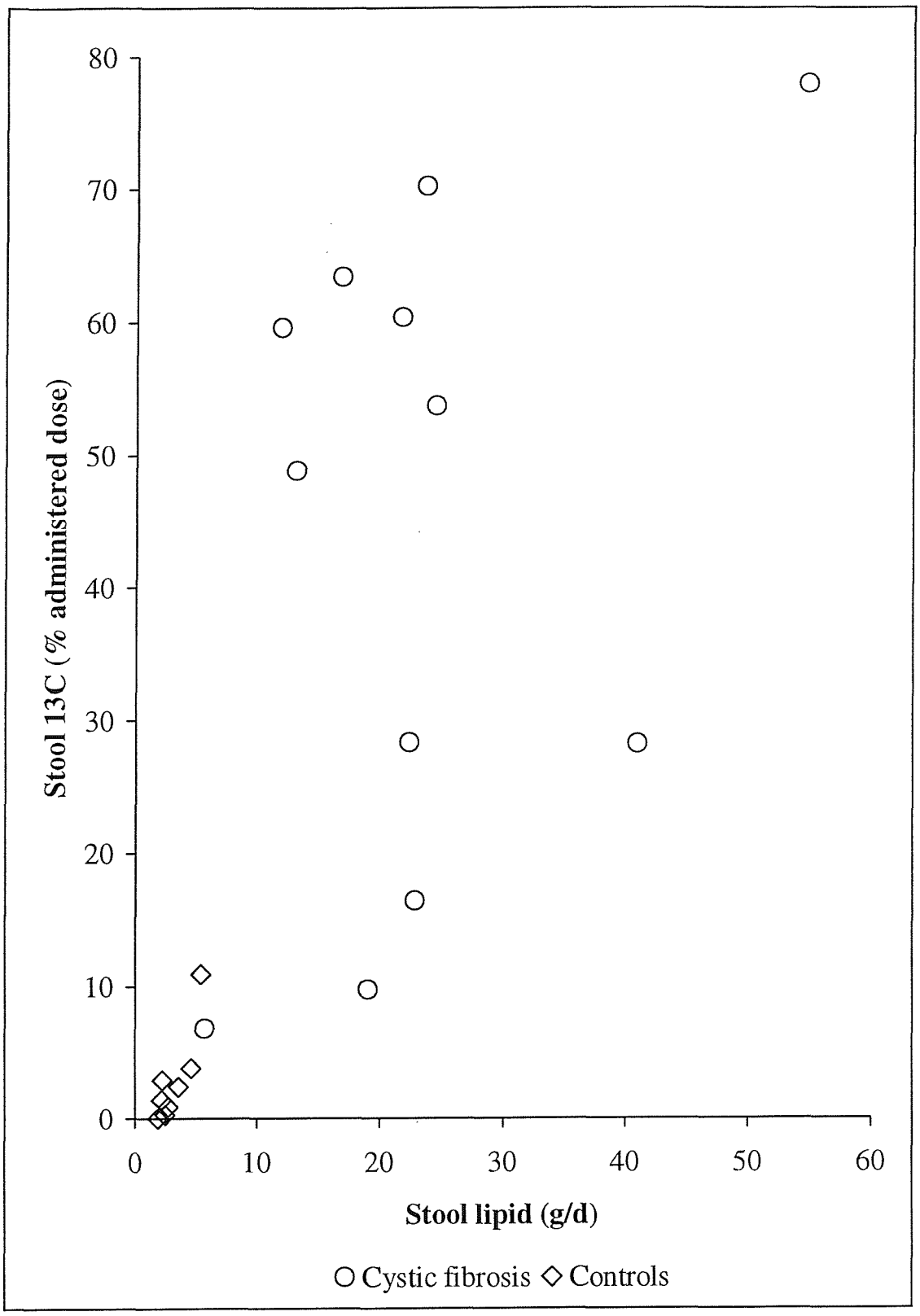


Figure 6.5. Association between excretion of ^{13}C in stools (% administered dose) over the study period and excretion of total lipid in stools per day (g) in cystic fibrosis patients (n = 12) and control subjects (n = 8).

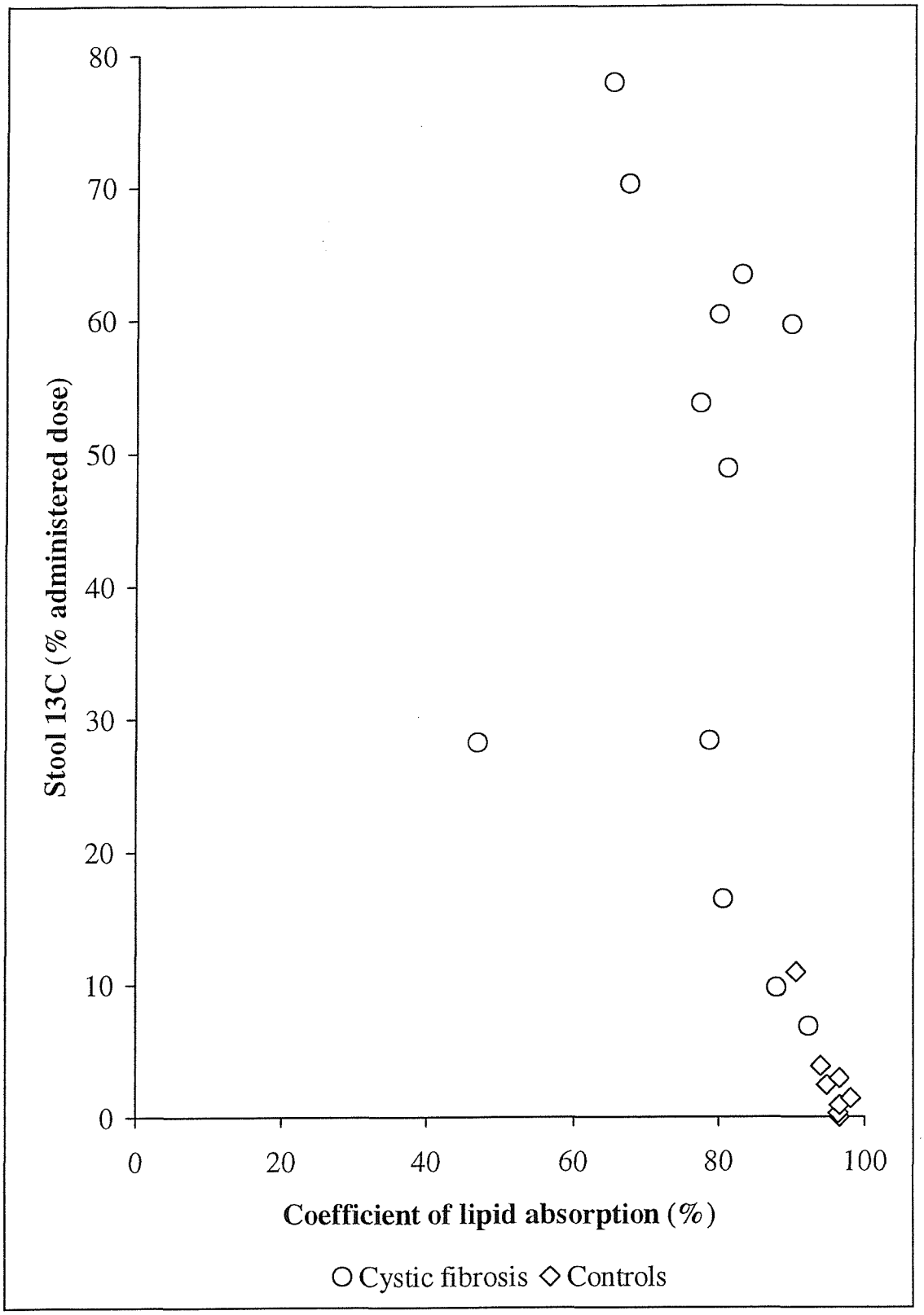


Figure 6.6. Association between excretion of ^{13}C in stools (% administered dose) over the study period and coefficient of lipid absorption (%) in cystic fibrosis patients (n = 12) and control subjects (n = 8).

6.2.5 Excretion of ^{13}C in TAG and FA fractions

Similar criteria as for total TAG and FA losses in stools described in 6.2.2, were used to determine whether cystic fibrosis patients had increased excretion of ^{13}C-label in total stools and TAG and FA fractions. The upper limit of the 95% confidence interval for total ^{13}C-label losses in stools in the control subjects (5.8% of the administered dose) was used to determine the excessive ^{13}C-label losses in the cystic fibrosis patients. Therefore, excretion of ^{13}C-label in the TAG or FA fractions in excess of 5.8% of the administered dose was considered as maldigestion and malabsorption of the administered [1,1,1-^{13}C] tripalmitin, respectively. The label excretion in the TAG and FA fractions was measured from the most enriched stool samples, because they were assumed to best describe the residual (without enzyme preparations) capacity of the gastrointestinal tract to digest and absorb lipids (See 6.3). The ^{13}C-label excretion in TAG and FA fractions over the study period were estimated by extrapolating the ^{13}C excretion in TAG and FA fractions of the most enriched stool sample to the total label losses in stools over the study period. The excretion of the label in TAG and FA fractions was expressed as % of the administered dose. The approach of using ^{13}C-label excretion in TAG and FA fractions to define maldigestion and malabsorption of lipids will be discussed in 6.3.

Table 6.4 shows the excretion of ^{13}C (% administered dose) in total stools, the most enriched stool sample and TAG and FA fractions of the most enriched stool sample in cystic fibrosis patients. The ^{13}C enrichment was detected in FA fraction in all cystic fibrosis patients and in TAG fraction in 11 patients. The most enriched sample contained 35% to 97% of the total ^{13}C enrichment in stools over the study period. The proportion of ^{13}C-label within stool as TAG ranged from 0% to 58% (Median 18%) and within FA fraction from 11% to 123% (Median 60%) of the stool enrichment. This is equivalent to 0% to 23.3% (Median 1.9%) of the administered dose in TAG and 2.6% to 28.4% (Median 10.2%) of administered dose in FA. Sum of the ^{13}C enrichment in TAG and FA accounted for 56% to 123% (Median 77%) of the label in stool. Figure 6.7 shows excretion of ^{13}C-label in total stools and TAG and FA fractions, extrapolated for total stool losses and expressed as % of the administered dose in individual cystic fibrosis patients in ascending order for the label excretion in total stools. The dotted line represents the upper limit of the 95% confidence interval for the excretion of total ^{13}C in stools observed in control subjects (5.8% of the administered dose). All cystic fibrosis patients had greater ^{13}C-label losses in stools than

5.8% of the administered dose. The ^{13}C-label excretion in TAG fraction exceeded 5.8% in 5 of the 12 patients indicating maldigestion of lipids and the ^{13}C-label excretion in FA fraction in stools exceeded 5.8% in 11 of the patients indicating malabsorption of lipids.

Table 6.4. Excretion of ^{13}C (% administered dose) in total stools, the most enriched stool sample and TAG and FA fractions of the most enriched stool sample in individual cystic fibrosis patients.

Patient	Total stools (%)	The most enriched stool sample		
		Stool (%)	TAG (%)	FA (%)
1	59.6	57.9	17.3	28.0
2	48.8	27.0	11.9	8.5
3	6.8	5.2	0.9	2.6
4	28.2	15.8	1.0	8.7
5	9.7	7.9	0	9.7
6	60.4	51.9	23.3	5.6
7	53.7	32.8	19.0	10.8
8	77.9	38.6	0.3	24.9
9	70.3	40.5	0.4	28.4
10	63.4	22.1	4.0	16.3
11	28.3	22.2	0.2	21.7
12	16.4	9.3	2.8	7.9
Median	51.2	24.6	1.9	10.2

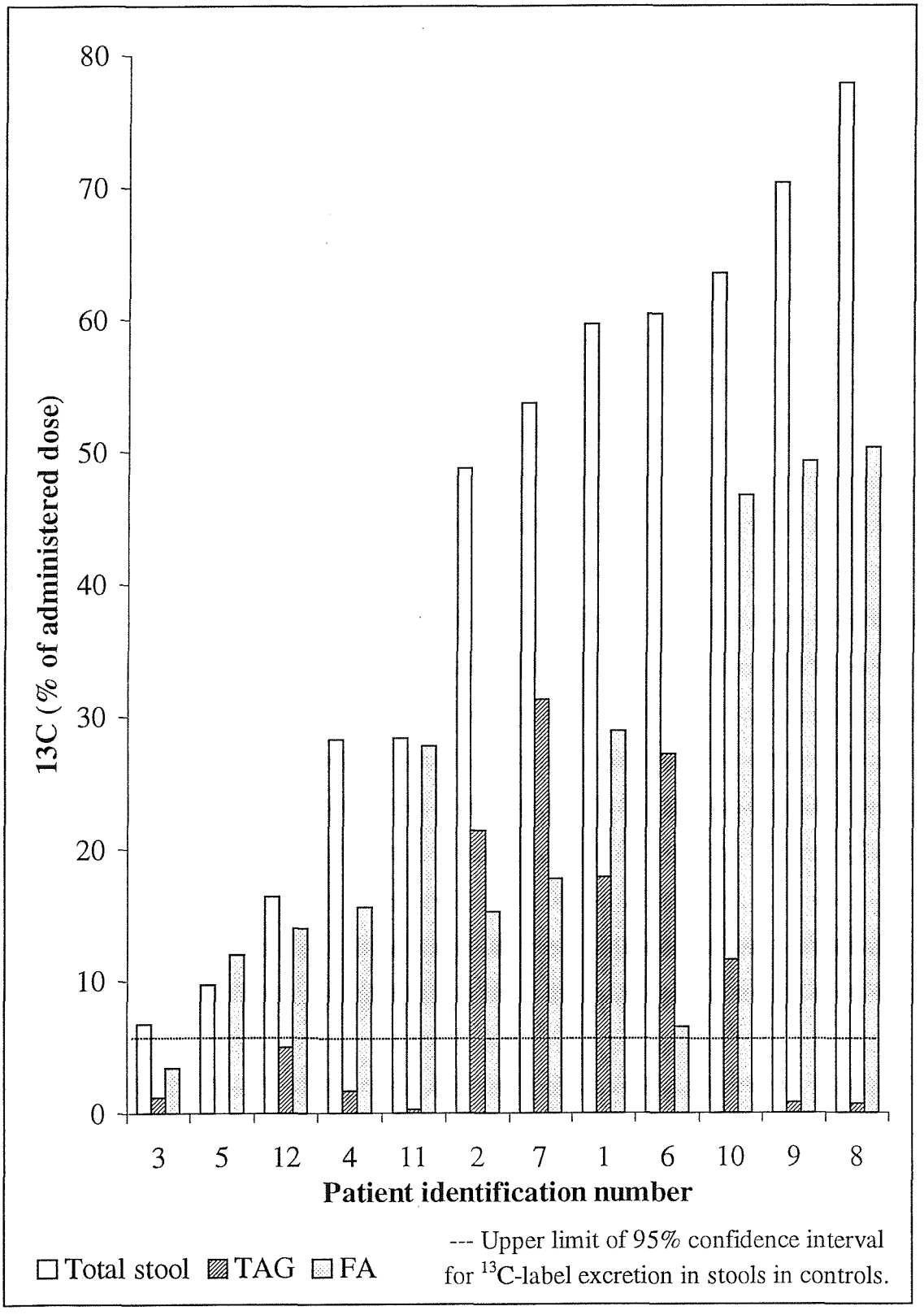


Figure 6.7. Excretion of ^{13}C (% administered dose) in total stools and TAG and FA fractions (extrapolated to the total stool ^{13}C losses) in individual cystic fibrosis patients shown in ascending order for total stools. The dotted line represents the upper limit of 95% confidence interval for total ^{13}C-label excretion in stools observed in the control subjects.

6.3 Discussion

The amount and type of lipid losses in stools in cystic fibrosis patients compared to control subjects were investigated. The traditional method of analysing total stool lipid was combined with stable isotope techniques where the stool losses of the ^{13}C-label were measured after administration of [1,1,1-^{13}C] tripalmitin. The excretion of ^{13}C-label in stool TAG and FA fractions was used to differentiate between maldigestion and malabsorption of dietary lipid. Label excreted in stool TAG indicates maldigestion of dietary lipid and label excretion in stool FA indicates that lipid has been digested but not absorbed. For comparative purposes, the total TAG and FA excretion in stools were also analysed. The primary aim was to investigate the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid and therefore cystic fibrosis patients did not take pancreatic enzyme preparations with the labelled meal.

In this section the use of stool TAG and FA fractions (both unlabelled and labelled) to differentiate between digestion and absorption of dietary lipid and the factors that that could affect the interpretation of the results are discussed and the findings are compared with other published studies. The possible explanations for maldigestion and malabsorption of dietary lipid and their implications for the clinical management of the patients will be discussed in Chapter 8.

6.3.1 Do stool TAG and FA fractions differentiate between maldigestion and malabsorption of lipids and what factors could affect the interpretation of results?

Does [1,1,1-^{13}C] tripalmitin reflect digestion and absorption of all dietary lipid?

A TAG, tripalmitin, where the carboxyl carbon of each FA was labelled with ^{13}C, was chosen for the present study to trace the digestion and absorption of dietary lipid. Is this substrate suitable for tracing the digestion and absorption of all dietary lipid? It was firstly important to determine whether tripalmitin is digested and absorbed in the gastrointestinal tract by the healthy individuals. The low total label excretion in stools in the control subjects (0 to 10.9% of the administered dose) demonstrated that the administered ^{13}C-labelled tripalmitin was well digested and absorbed in healthy individuals. The labelled tripalmitin was administered as an emulsion drink, which is the physicochemical form of dietary lipid

[Emken *et al.* 1993]. Emulsification of the administered lipid was necessary, since lipids administered in crystalline form may be poorly digested and absorbed even in healthy individuals [Murphy *et al.* 1995].

Palmitic acid is the predominant dietary saturated FA and is therefore suitable for tracing the metabolism of a large proportion of dietary FA. Other FA such as oleic acid, which is the predominant dietary unsaturated FA could have been chosen, but it was considered important to use tripalmitin, which would enable comparisons with previous studies carried out with cystic fibrosis patients [Murphy *et al.* 1997; Murphy *et al.* 1998]. The most physiological approach to trace the digestion and absorption of dietary lipid would be to use TAG consisting of a range of FA. Natural dietary TAG is typically composed of combination of palmitic acid, stearic acid and oleic acid and also α-linoleic acid in vegetable oil sources [Gurr & Harwood 1991]. However, such ^{13}C-labelled mixed TAG was unnecessarily costly for the purposes of this study in comparison to tripalmitin. In cystic fibrosis patients there are no previous studies comparing the digestion and absorption of different fatty acids and the metabolism of long-chain FA especially that of saturated FA is poorly understood.

The digestion and absorption of tripalmitin does not represent digestion and absorption of all dietary lipid, because different lipases (phospholipase A_2 and cholesterol ester hydrolase) are required for digestion of dietary phospholipids and cholesterol esters. Tripalmitin does not represent medium chain TAG and FA either, because in contrast to long-chain FA, medium chain FA do not require micelle formation for absorption and are absorbed directly to portal blood compared to long-chain FA, which are transported via lymphatic system. However, TAG constitute the major form of dietary lipid (on average 100g) and an average diet contains only 2 to 3 g phospholipids, less than 1 g cholesterol and cholesterol esters and minute amounts of medium chain TAG [Gregory *et al.* 1990]. Therefore the [1,1,1-^{13}C] tripalmitin, which was used to trace the gastrointestinal handling of dietary lipid adequately represents the bulk of the dietary lipid and enabled comparison to previous studies where the metabolism of palmitic acid and tripalmitin in cystic fibrosis patients has been studied.

Is it necessary to use labelled lipids to assess stool lipld losses and to differentiate between lipid maldigestion and malabsorption?

The main aim of this thesis was to differentiate between digestion and absorption of dietary lipid by measuring the excretion of ^{13}C-label in stool TAG and FA fractions following administration of [1,1,1-^{13}C] tripalmitin. Is it necessary to use ^{13}C-labelled lipid, which increases the cost of the analysis, to determine the stool lipid losses and to differentiate between digestion and absorption of lipids, or would it be adequate to analyse the total TAG and FA excretion in stools? Stable isotope technique makes it possible to trace the gastrointestinal handling of dietary lipid and to differentiate between lipid losses in stools derived from maldigested and malabsorbed dietary lipid from that derived from endogenous secretions and bacterial lipid. This is especially important when cystic fibrosis patients are studied, since it has been shown previously that cystic fibrosis patients have increased stool bacteria mass compared to control subjects [Murphy *et al.* 1991]. The patients in the present study also had increased stool bacteria mass compared to values previously reported for healthy subjects [Gavin, submitted]. Bacterial lipid is included in the total stool lipid, which therefore may overestimate the dietary lipid losses.

Before discussing what possible advantages the use of ^{13}C-labelled lipid might bring to the analysis of the amount and type of stool lipid losses, the criteria for defining increased total lipid losses in stools and to differentiate between maldigestion and malabsorption is discussed. It was found that cystic fibrosis patients had both higher total lipid losses in stools (g/d) and ^{13}C-label losses in stools (% administered dose) compared to the control subjects. The upper limit of the 95% confidence interval for the control subjects was used to define whether the individual cystic fibrosis patients had increased stool total lipid and stool TAG and FA excretion and increased ^{13}C-label losses in total stools and TAG and FA fractions. Using these criteria all the cystic fibrosis patients studied had increased stool total lipid and stool label losses. Analysing more control subjects would increase the confidence of using the upper limit of the 95% confidence intervals as criteria for defining excess stool total lipid (4.2 g/d) and total ^{13}C-label losses (5.8% of the administered dose). However, it has been shown previously on several occasions, that stool lipid excretion in the control subjects is low and does not normally exceed 7 g/day [Wollaeger *et al.* 1947; Murphy *et al.* 1991; Murphy *et al.* 1998]. It has also been shown in a similar study design as in the present study that [1,1,1-^{13}C] tripalmitin is well digested and absorbed in healthy subjects (stool

label losses 1% to 12.7% of the administered dose) [Murphy *et al.* 1998]. However, it seems that stool total lipid excretion of one patient (5.7 g/d) and stool total ^{13}C-label excretion of two patients (6.8% and 9.7% of the administered dose) were within the range of control subjects despite exceeding the upper limit of the 95% confidence interval for control subjects. Therefore these patients could be considered to have similar lipid and label losses in stools compared to healthy individuals.

The control subjects had markedly lower total lipid losses and total label losses in stools compared to cystic fibrosis patients and also compared to criteria that is used to diagnose clinically significant stool total lipid losses. Therefore, control subjects were considered not to have lipid maldigestion or malabsorption that would be of clinical relevance and therefore the TAG and FA excretion in stools or the ^{13}C-label excretion in TAG and FA fractions was not analysed from the stool samples of the control subjects. The upper limit of the 95% confidence interval for total lipid and total label losses in stools in the control subjects was used to define the excessive TAG and FA excretion in stools and excessive ^{13}C-label excretion in stools in the cystic fibrosis patients. The approach chosen to define whether cystic fibrosis patients have lipid maldigestion or malabsorption seems reasonable, because similar stool lipid losses in the cystic fibrosis patients compared to control subjects would not be considered clinically significant. However, having information about the TAG and FA excretion in stools in the control subjects would make the direct comparisons between the groups possible. If the TAG and FA excretion in stools in the control subjects was known it could be possible to evaluate whether TAG to FA ratio may be used to define maldigestion or malabsorption of lipids. However, this approach would overlook a combination of maldigestion and malabsorption of lipids and might not be suitable to evaluate the digestion and absorption of lipids in cystic fibrosis patients who may have both maldigestion and malabsorption as was shown in the present study.

The total ^{13}C enrichment of stools was analysed from individual dry stool samples instead of pooled samples, because both the enrichment (atom %) and carbon content of the sample are required for calculation of the proportion of the administered dose excreted in stools. Pooling samples could decrease the enrichment resulting from a dilution effect by unenriched stool and therefore would underestimate the label excretion in stools. However, TAG and FA excretion in stools was measured only from the most enriched stool sample. The aim of this study was to investigate the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid and therefore pancreatic enzyme preparations were not taken

with the labelled meal. It was not possible to withdraw enzyme preparations from more than one meal due to inevitable increases in the gastrointestinal symptoms. It was therefore assumed that the label excretion in TAG and FA fractions of the most enriched stool sample would best describe the residual (without enzyme preparations) gastrointestinal handling of dietary lipid. The TAG and FA excretions in stools over the study period were estimated by extrapolating the TAG and FA content of the most enriched stool sample to the total daily lipid losses over the study period and were expressed as g/d. Similarly the ^{13}C-label excretion in TAG and FA fractions over the study period were estimated by extrapolating label excretion in TAG and FA fractions of the most enriched stool sample to the total label losses in stools over the study period and were expressed as % of the administered dose. In the present study the most enriched stool sample contained 35% to 97% of the total enrichment in stools and 11 of the 12 samples contained more than 50% of the total stool enrichment. Therefore the label in the most enriched stool sample represented the majority of the label excreted in stools.

Using the upper limit of the 95% confidence interval of the control subjects for total lipid losses and label losses in stools, all 12 cystic fibrosis patients had increased lipid and label losses in stools. However, examining the individual subjects, for example patient number 5 had increased stool total lipid losses, but the label losses were relatively low and similarly patient number 4 had high stool lipid losses, but comparatively low label losses (Figures 6.3 and 6.7). Both of these patients had increased stool bacteria mass (10 g/d and 19g/d, patients 5 and 4 respectively) compared to that previously reported in healthy individuals [Murphy *et al.* 1991], which may have resulted in an overestimation of stool lipid. Lipid losses by the labelled data were generally higher, which reflects the omission of pancreatic enzyme preparations from the labelled meal, whereas unlabelled total lipid excretion in stools describes the lipid losses whilst on the enzyme preparations. Therefore, the labelled (stool total ^{13}C) and unlabelled (stool total lipid) data may not be directly comparable.

Eight of the 12 cystic fibrosis patients had higher TAG excretion in stools than the upper limit of the 95% confidence interval for total lipid losses in stools of the control subjects. Using the labelled data, five patients had higher ^{13}C-label losses in the TAG fraction than the upper limit of the 95% confidence interval for total label losses in stools of the control subjects. Whereas 11 of the 12 patients had higher FA excretion in stools or label excretion in FA fractions than the upper limit of the 95% confidence interval for the

control subjects. These comparisons suggest that use of the total TAG and FA fractions to differentiate between lipid maldigestion and malabsorption leads to different results than using the ^{13}C-label excretion in the TAG and FA fractions and is illustrated in Figures 6.8 and 6.9. Figure 6.8 shows the excretion of ^{13}C-label in TAG and FA fractions as a proportion of the total ^{13}C-label in the most enriched stool sample and Figure 6.9 shows stool total TAG and FA fractions as a proportion of the total lipid in the most enriched stool sample. The fraction other refers to the proportion of the label or lipid in stools that was not confined to either TAG or FA fraction. Comparing the proportional contribution of the TAG and FA fractions in total stools or total stool lipid makes it possible to compare the labelled and unlabelled results, which are independent from the units (% of administered dose and g/d). It can be seen that the unlabelled data generally overestimates the proportion of TAG in stools. Despite TAG was excreted in stools, very little label was measured in the TAG fraction in patients 4, 5, 8, 9 and 11 resulting in an overestimation of lipid maldigestion. Therefore, for quantitative evaluation of the maldigested and malabsorbed lipid it is necessary to use labelled lipids.

Sum of TAG and FA as a proportion of total lipid was on average 86%. Samples of two patients (9 and 10, Figure 6.9) had a relatively low recovery of TAG and FA, the sum being 52% and 60% of the total lipid. The low recovery may have been due to analytical imprecision but more likely explanations are the presence of other lipid fractions than TAG and FA such as MAG, phospholipids and especially bacterial lipid in the total stool lipid. Sum of the ^{13}C-label in TAG and FA fractions exceeded the total label in stools in two patients (5 and 12, Figure 6.8). These samples were of low ^{13}C enrichment and the error is likely to be due to analytical imprecision. These samples can have the same absolute error due to the analytical imprecision as the samples with high enrichment, but when expressed as a proportion, the relative error seems bigger compared to the samples with high enrichment. It is unlikely that the interpretation of the proportion of lipid that is maldigested or malabsorbed by the two patients is affected, because the amount of ^{13}C in the FA fraction compared to the TAG fraction was excessive in both samples. Recovery of the ^{13}C-label in lipid fractions will be discussed in more detail in the next section.

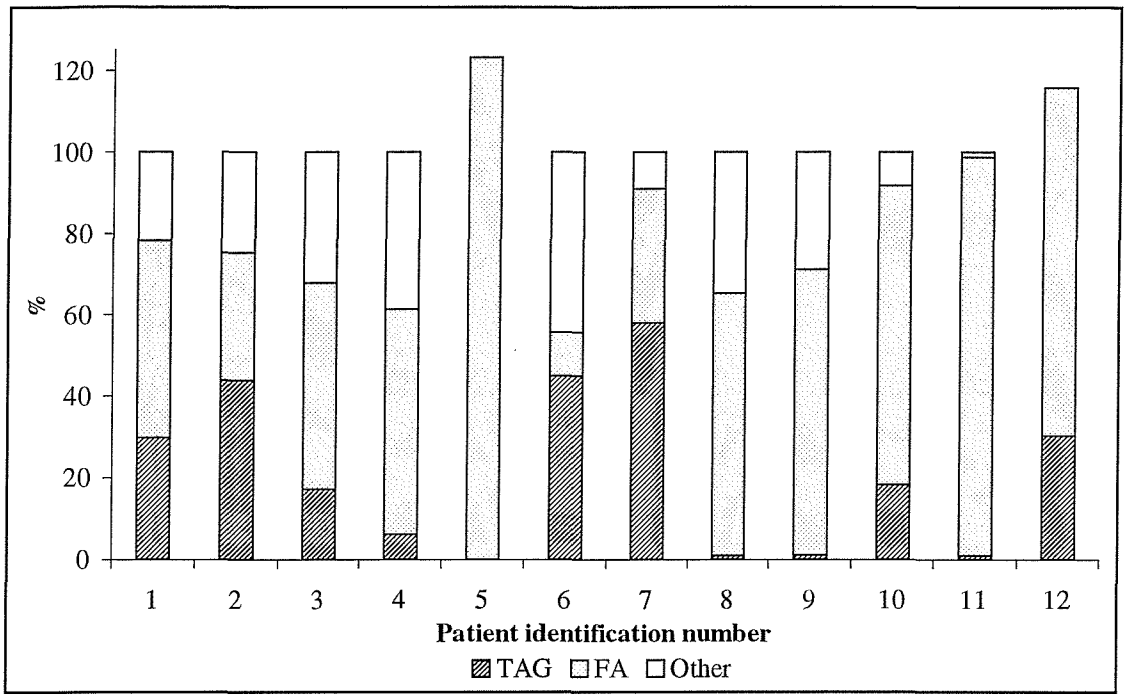


Figure 6.8. Excretion of ^{13}C-label in TAG and FA fractions expressed as a proportion of the total ^{13}C-label excretion in the most enriched stool sample. The fraction other refers to proportion of the label in stools that was not confined to either TAG or FA fraction.

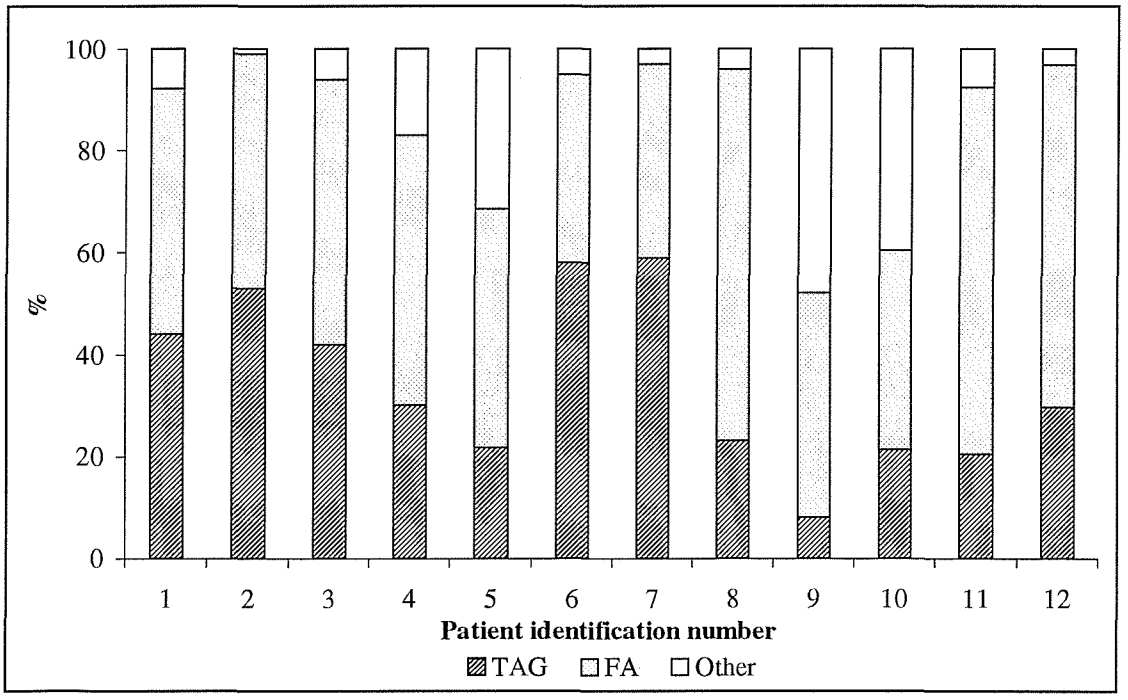


Figure 6.9. Stool TAG and FA expressed as a proportion of the total lipid in the most enriched stool sample. The fraction other refers to proportion of the lipid that was not confined to either TAG or FA fraction.

Is all ^{13}C-label in stools recovered in TAG and FA fractions?

In the present study the ^{13}C-label recovered in the TAG and the FA fractions expressed as a proportion of the total stool enrichment ranged from 56% to 123% (median 77%). Thus up to 44% of the stool label was not recovered in the TAG or the FA fractions. Should other lipid fractions than TAG and FA be measured to differentiate between lipid maldigestion and malabsorption and what is the metabolic fate of the label that is not recovered in TAG and FA fractions? The completeness of the extraction of ^{13}C-labelled lipids from stools was evaluated by measuring the ^{13}C enrichments of non-lipid residues in comparison to non-lipid residues of the unenriched (baseline) samples from the same patient. No significant enrichment was found in the non-lipid residue and therefore all ^{13}C-labelled lipids were extracted from stools. Consequently the label, which was not recovered in TAG and FA fractions should be present in other lipid fractions.

Some of the label may be found in other lipid digestion products than FA fraction namely MAG and DAG fractions. The ^{13}C-label excretion in the MAG fraction was measured previously during the validation of the analytical methods (Chapter 3) and was found to be very low, only 0.2% to 2% of the total ^{13}C-label was found within the MAG fraction in highly ^{13}C enriched stool samples. Therefore, it is unlikely that measuring the label excretion in MAG fraction would be required to differentiate between maldigestion and malabsorption of lipids. Alternatively dietary TAG may be incompletely hydrolysed and the main digestion product would be DAG, which if not directly measured would result in underestimation of lipid maldigestion. In healthy individuals MAG and FA are the major end products of TAG digestion [Borgström 1954], but it is not known whether this process is altered in cystic fibrosis patients.

The ^{13}C-label could also be incorporated in metabolites other than TAG and TAG digestion products within the gastrointestinal tract, such as esterified with cholesterol to form cholesterol esters, which might also be lost in stools. In addition, cholesterol esters are formed in the intestinal mucosa and are subsequently transported to the liver in chylomicrons. In the liver, cholesterol esters are either incorporated in lipoproteins or cholesterol is released, so that the FA can be utilised for oxidation. Labelled FA could re-enter the gastrointestinal tract if the acetyl-CoA, derived from the β-oxidation of FA, is utilised for cholesterol and bile salt production and therefore excreted in the gastrointestinal tract. It is possible that some of the label is incorporated with cholesterol and bile salt

metabolism because the bile salt pool turn over rate is high, 3 to 4 times per day. Another possible explanation for incomplete recovery of the stool label in TAG and FA fractions could be incorporation of the label in the phospholipids, which are formed through phosphorylation of the *sn*-3 glycerol of TAG. However, it is unlikely that any of these other metabolites could significantly contribute to the label loss in stools due to the dilution effect of the label entering body pools, which results in a very small amount of the label that can return to the gastrointestinal tract. Furthermore, enzymes required for re-esterification of the FA to e.g. phospholipids are not present within the gastrointestinal tract. Therefore, the label excretion in any of the other metabolites than TAG and TAG digestion products is likely to be beyond the limit of detection and is not likely to be significant for differentiation between maldigestion and malabsorption of dietary lipid.

Some of the label in the gastrointestinal tract could also be incorporated in bacteria within colon. It would be possible to investigate the capacity of the bacteria to incorporate the ^{13}C-labelled lipids by separating bacteria from stools and by measuring the ^{13}C enrichment of the bacteria. However, the capacity of the bacteria to store lipids is not likely to be large and the types of lipids in the bacterial-wall are mainly glycolipids and phospholipids [Gunstone *et al.* 1994]. The effect of bacteria on stool lipid will be discussed in more detail below.

Therefore, it appears that a combination of factors discussed in this section may contribute to the incomplete recovery of stool label in TAG and FA fractions, which was typically about 30%. However, the majority of the label in stools was found in TAG and FA fractions and therefore it seems that they are the most appropriate fractions to differentiate between lipid maldigestion and malabsorption.

Was administered TAG hydrolysed by pancreatic enzyme preparations?

The cystic fibrosis patients studied normally use PERT. However the enzyme preparations were omitted from the labelled meal to investigate the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid. Could the enzyme preparations ingested before or after the labelled meal was consumed hydrolyse the [1,1,1-^{13}C] tripalmitin, in which case the label excretion in stools would not describe the residual capacity of the gastrointestinal tract? It has been shown that pancreatic enzyme preparations (microspheres) may be emptied from the stomach even more rapidly than food and that in

most patients enzymes taken orally reach the terminal ileum by two hours after the ingestion of the preparations [Taylor *et al.* 1999]. Therefore, it is unlikely that the enzymes taken by the patients the previous evening before administration of the labelled meal could have remained active in the small intestine and hydrolysed lipids in the labelled meal. Nevertheless, decreased secretion of pancreatic bicarbonate in cystic fibrosis patients [Barraclough & Taylor 1996] may result in duodenal hyperacidity, which might delay the release of enzymes from the preparations. In that case the enzymes might not be released from the preparations until in the terminal ileum where the pH of the gastrointestinal content rises. However, it is not likely that the enzyme release would have been delayed by 10 to 12 hours, which was the time difference between the consumption of the last enzyme preparations and the labelled meal.

The period between consumption of the labelled meal and lunch when the enzyme preparations were taken was shorter, 6 hours, compared to the overnight withdrawal from the enzyme preparations. Previous studies have reported similar gastric emptying times in cystic fibrosis patients compared to controls, half-emptying time being in average 14 minutes for liquids (orange squash) [Smith *et al.* 1990], 28 minutes for corn oil-dextrose drink [Roulet *et al.* 1980] and 103 minutes for pancake and baked bean meal. None of these meals was exactly comparable to the meal consumed in the present study (emulsion drink, buttered toast and orange squash), but it can be estimated from the previous studies that the gastric emptying time of a meal may vary from 20 to 200 minutes. It has been shown that small intestinal transit time may be prolonged in cystic fibrosis patients compared to controls ranging from 140 to 390 minutes [Bali *et al.* 1983; Taylor *et al.* 1999]. Hence, it can be estimated that small intestinal transit in cystic fibrosis patients may take up to 6 ½ hours and therefore it is possible that a proportion of the labelled meal was still retained in the small intestine when the lunch with the enzyme preparations was consumed. However, it is more likely that by the time the lunch was consumed, the meal had reached the colon and the enzyme preparations taken with the lunch would not have been available for the hydrolysis of TAG. Furthermore, in healthy individuals only 1% of the lipase activity in the duodenum survives to the terminal ileum [Layer *et al.* 1986] and considering the additional constraints in the cystic fibrosis patients, such as acidity of the gastrointestinal content, it is unlikely that lipase from enzyme preparations or from lingual, gastric or pancreatic sources could have hydrolysed the administered TAG to any significant extent after the meal had reached the colon.

Do colonic bacteria affect stool lipid composition?

Cystic fibrosis patients studied had increased stool bacteria compared to values measured previously in healthy individuals [Gavin, submitted]. In principle, colonic bacteria could affect stool lipid in two ways, firstly bacteria itself contain lipid and secondly bacteria may metabolise the lipid that is present in the colon. In the present study, an association was found between stool total lipid and stool bacteria, but not between stool ^{13}C-label and stool lipid in cystic fibrosis patients. A weak association between stool ^{13}C-label and stool total lipid in cystic fibrosis patients following administration of labelled tripalmitin has also been shown previously by Murphy *et al.* (1998). However, in this study an association was found between the stool ^{13}C-label and stool lipid in the control subjects. These findings suggest that an increased colonic bacteria and therefore bacterial lipid may contribute to the total lipid losses in stools in the cystic fibrosis patients, which would explain the lack of association between label excretion in stools and total stool lipid. This highlights the potential advantages of using stable isotopes to trace the digestion and absorption of dietary lipid.

The possible effects of bacteria on maldigested TAG or malabsorbed FA entering the colon are shown in Figure 6.10. It has been shown in an *in vitro* study that certain bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus, Serratia marcescens and Bacillus subtilis*) can produce bacterial lipase [Kouker & Jaeger 1987]. In healthy individuals the main stool bacteria are bacteroides and bifidobacteria [Drasar *et al.* 1969] but the composition of colonic bacteria in cystic fibrosis is not well known, however *Pseudomonas aeruginosa* has been detected in stools of the cystic fibrosis patients [Döring *et al.* 1989; Taylor *et al.* 1992]. The presence of these bacteria in the colon could therefore hydrolyse maldigested TAG entering the colon. However, the extent to which bacterial lipase could hydrolyse the maldigested lipid in the colon is not known. One study compared the proportions of TAG and FA in the contents of terminal ileum to those in stools in one patient with pancreatic insufficiency [Bliss & Small 1970]. It was found that the proportion of TAG fell from 71% in terminal ileum aspirate to 22% in stools and the proportion of FA increased from 15% in aspirate to 31% in stools. The study was completed only in one patient and was presented as an abstract, which does not allow a careful consideration of the methodology. Therefore the hydrolysing effects of bacteria in the colon remain unclear. In the present study only five patients exhibited pronounced label losses in stool TAG as

assessed by the ^{13}C-label excretion in the TAG fraction despite omitting enzyme preparations with the labelled meal. Therefore the main concern is that if the maldigested TAG was hydrolysed in the colon by bacteria the lipid maldigestion would be underestimated and the lipid malabsorption would be overestimated. Although possible, at the present time there is little evidence in the literature to support the view that bacteria in the colon hydrolyse the maldigested TAG entering the colon. The most direct, although invasive, way to investigate the effects of the colonic function on the maldigested and malabsorbed dietary lipid would be to compare the lipid composition of the intestinal aspirate collected from the terminal ileum to the lipid composition in stools.

In addition to hydrolysing lipid, bacteria could transform the unabsorbed lipid by desaturation, saturation or synthesis of isomers [Webb *et al.* 1963]. Fatty acids, such as hydroxy acids that are not normally present in dietary lipid have been detected in stools of patients with steatorrhoea, which suggests that these lipids have been synthesised in the gastrointestinal tract [James *et al.* 1961; Webb *et al.* 1963]. The influence of the colonic function on the composition of lipid in the gastrointestinal tract has been investigated by comparing FA composition of ileostomy dejecta to the dietary FA composition, which were found to be similar [Wiggins *et al.* 1969], whilst stool FA composition differed from the dietary FA composition [James *et al.* 1961; Webb *et al.* 1963; Wiggins *et al.* 1969]. However, it has been shown that in patients with excessive stool lipid losses, the stool lipid composition resembles more dietary lipid composition than in healthy individuals [Webb *et al.* 1963]. It may be that an increased transit time reduces the opportunity of bacteria to metabolise lipids. It has also been shown that in patients with steatorrhoea, an alteration of dietary lipid composition changes the stool lipid composition [Webb *et al.* 1963] suggesting that stool lipid reflects dietary lipid. The ^{13}C excretion in total FA fraction was measured in the present study and therefore an alteration of the malabsorbed lipid that enters the colon is not of significance, because ^{13}C-label incorporated with these metabolites would be included in the total ^{13}C-label excretion in the FA fraction. It was also shown during the validation of the methods (Chapter 3) that the ^{13}C-label was detected only in palmitic acid (GC-C-IRMS analysis of stool lipid) suggesting that the hydrolysed FA from the administered ^{13}C-labelled tripalmitin was not altered in the colon.

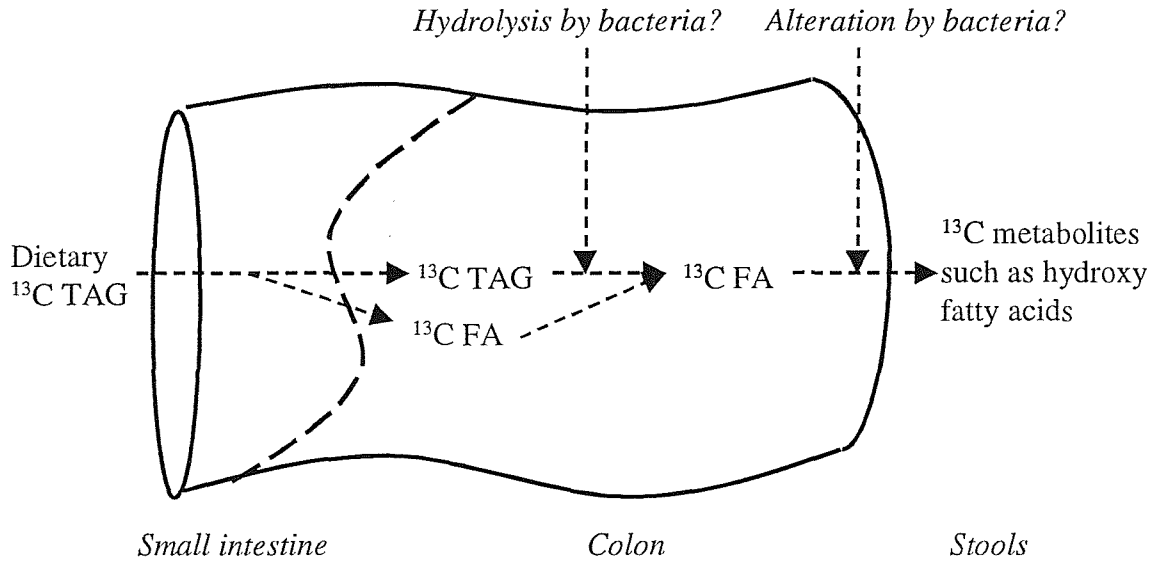


Figure 6.10. Possible effects of bacteria on maldigested TAG or malabsorbed FA entering the colon.

6.3.2 How do stool lipid losses in the present study compare to previous studies?

Total lipid and total ^{13}C-label excretion in stools

In the present study, total lipid excretion in stools ranged from 5.7 to 54.8 g/d in cystic fibrosis patients, which exceeded the total lipid excretion in stools of the control subjects. It has been shown previously that excretion of total lipid in stools in healthy individuals does not normally exceed 7 g/d [Wollaeger *et al.* 1947; Murphy *et al.* 1991] which was also shown in the present study. Although stool lipid losses were higher in cystic fibrosis patients compared to control subjects, one patient had lipid excretion in stools less than 7 g/d, which suggests that this patient did not have increased stool lipid losses. Like the present study, several previous studies have shown marked variability (5 to 59 g/d) in stool lipid losses in cystic fibrosis patients, even when receiving PERT (See Table 2.7). Therefore, the average stool lipid losses per day in the present study were similar to those previously reported in cystic fibrosis patients. The interpretation of stool lipid losses and comparison to other studies is complicated by different ways of expressing the stool lipid losses, namely g/day,

proportion of daily lipid intake [Stool lipid losses / Dietary lipid intake × 100] and coefficient of lipid absorption [(Dietary lipid intake - Stool lipid losses) / Dietary lipid intake × 100]. Expression of the results both as a proportion of daily lipid intake and as a coefficient of lipid absorption requires an accurate analysis of dietary lipid intake in addition to analysis of stool lipid, which introduces an additional error for the estimation of lipid losses in stools. The stool lipid losses expressed as g/d are well reported in healthy individuals and it might be more appropriate to compare stool lipid losses of patients to those of healthy individuals. In the present study the dietary lipid intake was analysed from the weighed food diaries and the coefficient of lipid absorption was calculated in order to compare these findings with those of others. In controls the coefficient of lipid absorption ranged from 91% to 98%, whilst in cystic fibrosis patients it ranged from 47% to 92%. Ramsey *et al.* (1992) defined that a coefficient of lipid absorption less than 93% indicates excessive stool lipid losses (steatorrhoea) and according to this definition all cystic fibrosis patients studied had elevated stool lipid losses. The range of the coefficient of lipid absorption was also similar to those observed in previous studies (See Table 2.7).

The total ^{13}C-label excretion in stools varied from 6.8% to 77.9% of the administered dose in the cystic fibrosis patients studied, which were higher than the label losses in stools of the control subjects. Similar to the findings of present study, the only other study that has investigated label losses in stools in cystic fibrosis patients following an administration of ^{13}C-labelled tripalmitin also found a large range of label losses in stools, 0% to 64% of the administered dose [Murphy *et al.* 1998]. In the study of Murphy and co-workers the usual dose of pancreatic enzyme preparations was taken with the labelled meal, whereas enzyme preparations were omitted in the present study. Omission of the enzyme preparations probably explains the higher median stool label losses in the present study (51% of administered dose) compared to Murphy *et al.* (28% of administered dose). The label losses in stools in control subjects were similar in both studies [1.0% to 12.7% of administered dose and 0% to 10.9% of administered dose Murphy *et al.* (1998) and present study, respectively].

The total lipid losses in stools in the present study were comparable to those observed in previous studies. The lipid losses in stools examined by the total ^{13}C-label losses tended to be higher in the present study compared to previous findings, which is likely to reflect the omission of enzyme preparations from the labelled meal. The total lipid or label losses in stools do not describe the extent to which these patients have maldigestion and/or

malabsorption of lipid and this was addressed by measuring the type of stool lipid losses and is discussed in the next section. The reasons for the increased stool lipid losses and their implications in the clinical management of the cystic fibrosis patients will be discussed in Chapter 8.

Stool TAG and FA losses

Use of stool TAG to differentiate between stool lipid losses that are caused by maldigestion or malabsorption of lipid was first introduced by Thompson *et al.* (1969b) who showed that stool TAG excretion both as absolute amount (g/d) and as concentration (g/100 g stool) differentiated between patients with excessive lipid losses due to pancreatic disease ($n = 14$) and due to intestinal disease ($n = 19$; $P < 0.001$). The patients with pancreatic disease mainly had cystic fibrosis and chronic pancreatitis and the majority of the patients with intestinal disease had untreated coeliac disease. Twenty years later Khouri *et al.* (1989b) investigated the same approach of measuring stool TAG and FA concentrations to detect maldigestion, but did not show any differences in the TAG concentration (mg/g stool) between patients with chronic pancreatitis (alcohol induced chronic pancreatitis, $n = 6$) and controls ($n = 6$). Nevertheless the concentration of FA was five times higher in chronic pancreatitis patients compared to controls. This was interpreted as maldigested TAG being hydrolysed beyond the absorptive site within the gastrointestinal tract. Despite these two studies, no others appear to have used this approach to differentiate between maldigestion and malabsorption. In this thesis the principle of measuring TAG and FA fractions in stools to differentiate between maldigestion and malabsorption was further developed by applying stable isotope techniques.

The excretion of TAG and FA in stools in the present study compared to previous studies is presented in Table 6.5. Although Thompson *et al.* (1969b) and Khouri *et al.* (1989b) measured TAG and FA excretion in stools, the present study was the first to investigate the excretion of TAG and FA in stools in a larger series of cystic fibrosis patients and to measure the ^{13}C-label excretion in the fractions. In the present study the TAG excretion in stools, both in absolute amounts and as concentration, in the cystic fibrosis patients was of similar range to the study of Thompson *et al.* (1969b), whereas Khouri *et al.* (1989b) reported lower stool TAG concentrations. The large range in the study of Thompson *et al.* (0.4 to 40 g TAG in stools/d) may be explained by the inclusion of cystic

fibrosis patients in the pancreatic insufficiency group, since they may have malabsorption in addition to maldigestion. It remains unclear why the TAG concentrations obtained by Khouri *et al.* (1989b) were lower compared to those of Thompson *et al.* (1969b) or present study. Some of the difference may be explained by sample handling and preparation, which could result in hydrolysis of TAG, or by colonic hydrolysis of TAG as speculated by the authors. Khouri *et al.* (1989b) also reported lower total lipid (Sum of TAG and FA) concentration in stools compared to the other two studies (Khouri *et al.* 1989b: 2.2% to 3.4%; Thompson *et al.* 1969b: 2.8% to 10.3%; Present: 10.2% to 23.5% lipid of stool weight), which may explain the lower values also for TAG and FA concentrations.

In the present study the ^{13}C-label excretion in the TAG fraction was higher in four cystic fibrosis patients compared to other patients studied. These patients had higher TAG concentration in stools, ranging from 58 to 125 mg/g stool, compared to the other 8 patients, ranging from 15 to 48 mg/g stool. Figure 6.11 shows an association ($\rho = 0.776$, $P = 0.003$) between stool TAG concentration and ^{13}C-label excretion in stool TAG suggesting that it might be possible to identify lipid maldigestion simply by measuring stool TAG concentration. Although, an association was found between excretion of ^{13}C-label in the TAG fraction and concentration of TAG in stools, the label excretion in the TAG fraction was low (less than 10% of the administered dose) for a variable range of the TAG concentrations (about 15 to 50 mg/g stool). The reasons for the observation remain unknown but it may be that there is a cut-off point above which the TAG concentration distinguishes patients with lipid maldigestion. However, further studies are required in patients with known deficiency of lipolytic activity within the gastrointestinal tract to confirm whether the TAG concentration could be used as a marker for lipid maldigestion. The stool FA concentrations in cystic fibrosis patients in the present study were higher than those reported by Khouri *et al.* (1989b) in both patients with pancreatic insufficiency and control subjects. Similarly to the TAG concentration, the low FA concentration in the study of Khouri *et al.* (1989b) may be explained by the lower total lipid concentration compared to the present study. However, there are no comparative studies of stool FA concentration in patients with defined lipid malabsorption or cystic fibrosis. Only a weak association between stool FA concentration and ^{13}C excretion in stool FA fraction ($\rho = 0.545$, $P = 0.067$) was found (Figure 6.12). Reasons for the absence of association are unknown, but it may be that FA retains more water than TAG as suggested by Bo-Linn and Fordtran (1984), which could cause similar concentrations of FA in stools despite variable losses of

^{13}C-label in the FA fraction. It has been suggested previously that total lipid concentration in stools may be used to differentiate between maldigestion and malabsorption [Bo-Linn & Fordtran 1984; Thorsgaard Pedersen *et al.* 1987]. Cut-off points for maldigestion (>10% lipid in stools, w/w), malabsorption (5.1% to 10%, w/w) and for normal lipid assimilation (<5%, w/w) were defined by comparing stool lipid concentrations amongst patients with pancreatic disease (e.g. chronic pancreatitis), intestinal disease (e.g. Crohn's disease and intestinal resection) and control subjects. However, in patients who may have both maldigestion and malabsorption, stool total lipid concentration alone may not differentiate between maldigestion and malabsorption of lipids. Therefore, TAG or FA concentration may be a more useful tool, but at present time there is not enough evidence to draw definitive conclusions about their usage to differentiate between maldigestion and malabsorption of lipids.

Table 6.5. Excretion of TAG and FA in stools in the present study compared to previous studies.

Study	Present study		Thompson *et al.* (1969b)[1]		Khouri *et al.* (1989b)	
Subject group	**TAG**					
	g/d[2]	(mg/g stool)[2]	g/d[2]	(mg/g stool)[2]	g/d	(mg/g stool)[3]
Cystic fibrosis	1.9 - 14.4	(15 - 125)	-		-	
Pancreatic insufficiency	-		0.4 - 40	(30 - 80)	-	(2.6 ± 1)
Intestinal disease	-		0 - 0.5	(0 - 2)	-	
Controls	-		-	(0.1 - 14)	-	(2.8 ± 0.4)
	FA					
Cystic fibrosis	3 - 40	(58 - 157)	-		-	
Pancreatic insufficiency	-		-		-	(28.3 ± 4.7)
Intestinal disease	-		-		-	
Controls	-		-		-	(5.9 ± 0.9)

[1] Estimated from the figures in Thompson *et al.* (1969b).
[2] Range.
[3] Mean ± SD.
- Not measured.

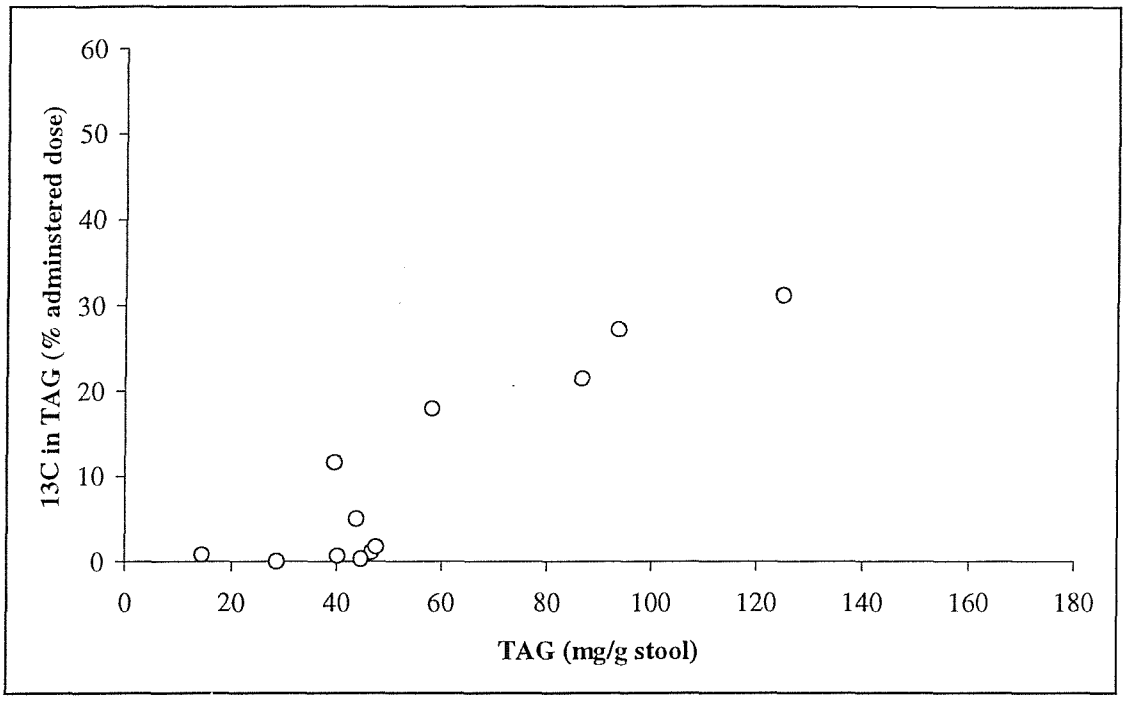


Figure 6.11. Association between ^{13}C-label excretion in stool TAG fraction (% administered dose) and stool total TAG concentration (mg/g stool) in cystic fibrosis patients.

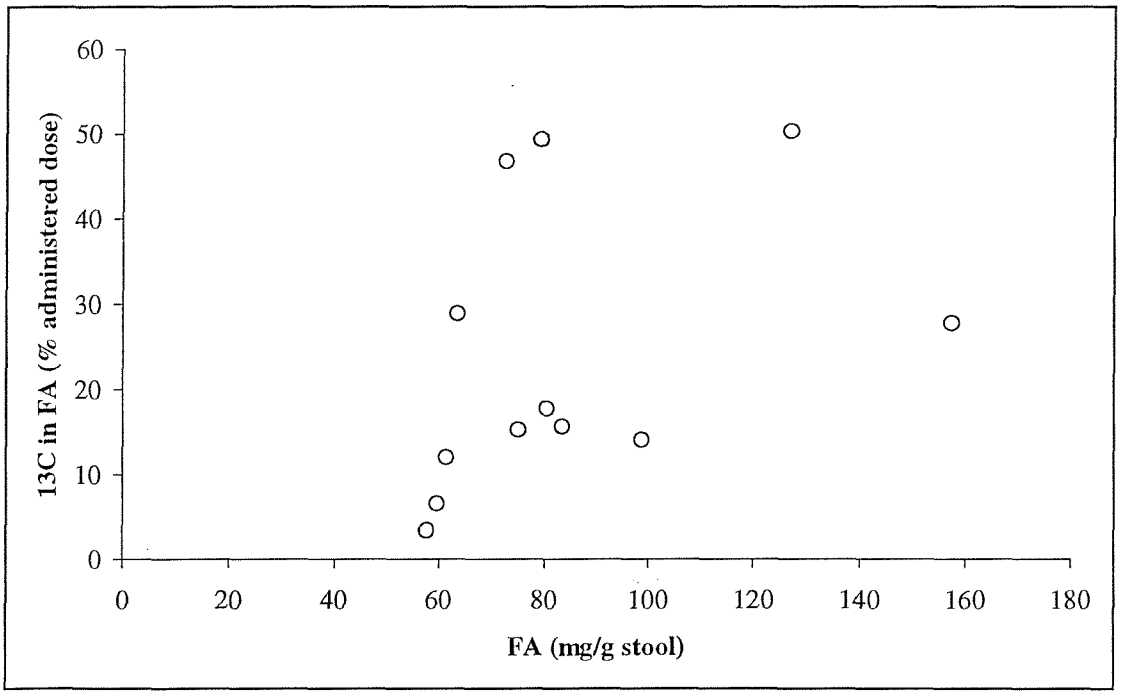


Figure 6.12. Association between ^{13}C-label excretion in stool FA fraction (% administered dose) and stool total FA concentration (mg/g stool) in cystic fibrosis patients.

The extent to which cystic fibrosis patients may have malabsorption of lipids was previously investigated by measuring ^{13}C-label excretion in stools following an administration of ^{13}C-labelled palmitic acid [Murphy *et al.* 1997]. Surprisingly, the proportion of the label excreted in stools of the administered dose was lower in cystic fibrosis patients ranging from 1% to 12% compared to control subjects ranging from 11% to 65%, which would suggest that cystic fibrosis patients did not have lipid malabsorption. However, palmitic acid was administered as a crystalline form, which has different physicochemical properties such as a higher melting point than dietary lipids. It is possible that due to altered gastrointestinal luminal environment the administered crystalline free FA were better absorbed in cystic fibrosis patients compared to control subjects. One possible explanation is that in the control subjects the administered free ^{13}C-labelled FA formed salts in the gastrointestinal tract resulting in higher label losses in stools. Whilst due to higher acidity in the gastrointestinal tract of the cystic fibrosis patients, the administered ^{13}C-labelled FA formed micelles and subsequently the FA were absorbed to a greater extent than in the control subjects. Poor availability of the crystalline free acid in the control subjects demonstrated that the physicochemical properties of the administered lipid were different to the properties of dietary lipids, which are emulsions. Jones *et al.* (1999) showed couple of years later that palmitic acid administered as an emulsion was well absorbed from the gastrointestinal tract in healthy women, stool lipid losses of the label being on average 1.2% of the administered dose (SD 0.9%). In the present study the labelled tripalmitin was administered as an emulsion, which was well digested and absorbed in the control subjects. Furthermore, the FA administered as free acid may be differently handled in the gastrointestinal tract than FA esterified to TAG. The administered lipid (TAG) in this study is a more physiological approach to investigate the digestion and absorption of dietary lipid than administering free FA because the dietary lipids are mainly TAG.

One previous study investigated the metabolism of ^{13}C-labelled linoleic acid in cystic fibrosis patients by measuring the proportion of the label excreted in stools, on breath over 6 hours and in plasma at 8 hour time point [Kalivianakis *et al.* 1999]. The investigators found a negative association between the 8 hour plasma ^{13}C-linoleic acid concentration and excretion of lipid in stools and concluded that a low label recovery in plasma was due to impaired intestinal uptake of long-chain FA. The possibility that maldigestion had occurred in the same patients was excluded following a mixed TAG breath test (1,3 distearoyl, 2[^{13}C]octanoyl glycerol), since no association was found between the label excretion on

breath and excretion of lipid in stools. However, in the conclusions the measured ^{13}C-label excretion in stools following administration of ^{13}C-labelled linoleic acid, 0% to 1.8% of the administered dose, were omitted. In response to a comment on their paper [Murphy *et al.* 1999], the authors questioned the use of ^{13}C-label in stools as a marker for dietary lipid due to a weak association observed between ^{13}C-label excretion in stools and total lipid in stools [Verkade 1999]. However, why this should occur in cystic fibrosis patients (increased stool bacteria) was not considered by the authors. The low label excretion in stools observed by Kalivianakis *et al.* (1999) would suggest that the administered lipid was well absorbed, however the total lipids were extracted from freeze-dried stools by the method of Bligh and Dyer (1959) which uses chloroform and methanol without acidification to extract lipid from stools. It has been shown in this thesis, described in Chapter 3, that stool samples need to be acidified in order to extract FA that are bound as salts in stools. Therefore the extraction of the labelled lipids might have been incomplete in the study of Kalivianakis *et al.* (1999), resulting in the underestimation of the label excretion in stools.

Therefore, the previous studies do not support the findings of the present study that cystic fibrosis patients may have malabsorption of lipids to a considerable extent. However, the methodological issues discussed above make it difficult to interpret the results of the previous studies. In the present study the issues related to the form of administered label (emulsion) and analysis of the extraction of lipids from stools (extraction of the main lipid fractions in stools: TAG, FA and FA salts) were carefully considered and increase the reliability of the findings.

Stool fatty acid composition

In the present study the relative FA composition of stool total lipid was analysed to determine whether the stool FA composition is different in cystic fibrosis patients compared to control subjects. A different stool FA composition in cystic fibrosis patients could either indicate that the overall gastrointestinal function is altered in cystic fibrosis patients compared to controls or it could simply reflect different FA composition of the diet. It was found that the FA composition of stool total lipid differed between the two groups, cystic fibrosis patients having proportionately less C18:0 and more C18:1 in stool total lipid compared to controls. In addition the FA composition of TAG and FA fractions was compared in cystic fibrosis patients and it was found that the TAG fraction contained

proportionately less C16:0 and more C18:1 and C18:2 than the FA fraction. The FA composition in total lipid, TAG and FA of the most enriched sample of the cystic fibrosis patients was measured to explore the affects of omitting pancreatic enzymes on the FA composition. The only difference found was that the most enriched TAG sample had proportionately less C18:2 than the baseline sample.

The FA composition of stool total lipid has not been previously investigated in cystic fibrosis patients, but there is an agreement between the FA composition of stool lipid in the controls to that previously reported, the typical stool FA being been C16:0, C18:0, C18:1 and C18:2 [Gompertz & Sammons 1963; Webb *et al.* 1963]. The stool FA composition of healthy individuals on a habitual mixed diet has previously been compared to that of patients with steatorrhoea and was found not to differ between the two groups [Gompertz & Sammons 1963]. It is possible that dietary intake of lipids explains some of the differences in the stool FA composition between control subjects and cystic fibrosis patients studied. Although, the intake of individual FA from the diet was not investigated, the absolute intake of monounsaturated and polyunsaturated lipids and the proportional intake of polyunsaturated lipids from the diet were higher in cystic fibrosis patients compared to control subjects. This could partially explain why cystic fibrosis patients had higher relative content of C18:1 in stool lipid compared to the control subjects. It has been shown previously that a change in the dietary lipid composition (addition of corn oil) changes stool lipid composition to a larger extent in patients with steatorrhoea (increased C18:2), compared to controls [Webb *et al.* 1963]. The differences in the proportional stool FA composition between cystic fibrosis patients and controls may also be explained by differential absorption of different FA. It has been shown previously using ^{13}C-labelled FA that excretion of C16:0 and C18:1 in stools was lower than that of C18:0 in healthy women [Jones *et al.* 1999]. However, the differences in the absorption of various FA in cystic fibrosis patients have not been studied. The reasons for differences in the relative FA composition of TAG and FA fractions are unclear, but they may be related to the variations in different FA that are esterified to *sn* positions in TAG. It may be that unsaturated FA are preferentially esterified in *sn*-2 position of TAG and therefore would end up to MAG not to FA after digestion within the gastrointestinal tract. This could explain why the TAG fraction contained relatively more C18:1 and C18:2 compared to the FA fraction.

6.4 Summary

The extent of lipid losses in stools and maldigestion and malabsorption of dietary lipid were determined by combining traditional gross balance techniques with novel stable isotope techniques by measuring ^{13}C-label excretion in total stools and in TAG and FA fractions after administration of [1,1,1-^{13}C] tripalmitin. The stable isotope approach enabled tracing the metabolism of dietary lipid compared to stool total lipid, which includes endogenous lipid losses and bacterial lipid. Pancreatic enzyme preparations were omitted with the labelled meal to investigate the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid in cystic fibrosis patients. The results demonstrate that:

1) Total lipid excretion in stools was elevated in cystic fibrosis patients compared to control subjects, however one patient had similar total stool lipid compared to control subjects.
2) The total ^{13}C-label excretion in stools was elevated in cystic fibrosis patients compared to control subjects. The stool ^{13}C-label losses were markedly variable in cystic fibrosis patients ranging from similar losses to control subjects to very high losses (6.8% to 77.9% of the administered dose) despite omitting enzyme preparations with the labelled meal. Although, the total label excretion in stools of all cystic fibrosis patients exceeded the upper limit of the 95% confidence interval for the control subjects, the label losses in stools of two cystic fibrosis patients were within the range of those in control subjects.
3) In order to quantitatively determine the extent of maldigestion and malabsorption it was necessary to trace the metabolism of dietary lipid by ^{13}C-labelled lipids. The ^{13}C-label excretion in TAG fraction of 5 of the12 patients and the ^{13}C-label excretion in FA fraction of 11 of the 12 patients exceeded the upper limit of the 95% confidence interval of the control subjects for the total label excretion in stools. The increased label excretion in the TAG fraction indicated maldigestion of lipid and the increased label excretion in the FA fraction indicated malabsorption of lipid in these cystic fibrosis patients.
4) The FA composition of stool total lipid differed between the two groups with cystic fibrosis patients having proportionately less C18:0 and more C18:1 in stool total lipid compared to control subjects, which may reflect differences in the FA composition of the diet or differential absorption of FA.

5) In cystic fibrosis patients the FA composition of stool TAG and FA differed so that the TAG fraction contained proportionately less C16:0 and more C18:1 and C18:2 than the FA fraction. Only minor changes in the proportional FA composition was found when baseline and enriched total lipid or TAG or FA fractions were compared.

Chapter 7: Are metabolic disposal of dietary lipid and net substrate oxidation rates altered in cystic fibrosis patients?

7.1 Introduction

Metabolic disposal of [1,1,1-^{13}C] tripalmitin and net substrate oxidation rates were investigated in cystic fibrosis patients compared to control subjects by combining stable isotope techniques (excretion of ^{13}C-label on breath) with indirect calorimetry measurements (net lipid and net carbohydrate oxidation). The methods were described in Chapter 4 and study groups in Chapter 5. In this chapter the results for the metabolic disposal of the labelled lipid and net substrate oxidation rates both during postabsorptive and postprandial period (following administration of the labelled meal) are compared between cystic fibrosis patients and control subjects. The following outcome variables were investigated:

1) Cumulative excretion of ^{13}C-label on breath over 10 hours corrected for stool losses of the label (% absorbed dose) following administration of a meal labelled with [1,1,1-^{13}C] tripalmitin, which was used to evaluate the oxidation of lipid that is derived from the diet in cystic fibrosis patients compared to control subjects.
2) Cumulative excretion of ^{13}C-label on breath expressed as a proportion of the administered dose, which was compared to the stool losses of the label to investigate the extent to which ^{13}C-label excretion on breath reflects the gastrointestinal handling of the administered lipid.
3) Postabsorptive and postprandial (over 6 hours) net lipid and net carbohydrate oxidation rates, which were measured to evaluate whether the net substrate utilisation of the cystic fibrosis patients differs from that of the control subjects.

In addition to presenting the results of the metabolic disposal of dietary lipid, the extent to which ^{13}C-label excretion on breath quantitatively reflects the oxidation of dietary lipid and the factors that could affect the net substrate oxidation rate measurements will be discussed and the findings of the present study will be compared to the previous studies. The possible explanations for the findings and implications of the findings for the clinical management of the cystic fibrosis patients will be discussed in Chapter 8.

7.2 Results

7.2.1 Excretion of ^{13}C on breath

Figures 7.1 and 7.2 illustrate the time course of the label excretion on breath over 10 hours following administration of [1,1,1-^{13}C] tripalmitin in cystic fibrosis patients and control subjects. In control subjects label appeared fairly rapidly on breath after administration of the labelled meal peaking at 4 to 6 hours after the label administration. In contrast very little label was excreted on breath in cystic fibrosis patients and the peak time varied from 2 to 8 hours. The maximum label excretion on breath (% of absorbed dose) per hour was lower in cystic fibrosis patients (Median 0.9%, range 0% to 2.6%) compared to control subjects (Median 3.8%, range 3.0% to 7.3%; $P<0.001$). The pattern of the label excretion on breath was similar whether expressed as a proportion of the administered dose (Figure 7.1) or as a proportion of the absorbed dose (Figure 7.2).

Table 7.1 shows the cumulative ^{13}C excretion on breath over 10 hours following administration of [1,1,1-^{13}C] tripalmitin in cystic fibrosis patients and control subjects. The cumulative label excretion on breath as a proportion of the administered dose was higher in control subjects (Median 22.3%) compared to cystic fibrosis patients (Median 2.5%, $P < 0.001$). The difference between the groups persisted even when the label excretion on breath was corrected for stool losses of the label. Whilst control subjects excreted 22.6% (Median) of the absorbed dose on breath, cystic fibrosis patients excreted only 5.6% (Median, $P < 0.001$). Correcting the label excretion on breath for stool losses of the label did not normalise the label excretion on breath in cystic fibrosis patients. The label excretion on breath remained lower in cystic fibrosis patients. If the difference in the label excretion on breath could be completely explained by the impaired gastrointestinal handling of the lipids, no difference would be expected between the groups when the label excretion on breath is expressed as a proportion of the absorbed dose. The expression of the data as a proportion of the absorbed dose still showed a lower label excretion on breath in patients suggesting that the oxidation of dietary lipid was lower in the patients compared to control subjects. The label excretion on breath of one cystic fibrosis patient was of similar magnitude to control subjects.

There was no association between label excretion in stools and on breath (% administered dose) in either group (Cystic fibrosis patients: $\rho = -0.26$, $P = 0.416$ and

Control subjects $\rho = 0.38$, $P = 0.352$, Figure 7.3). In other words the variable stool losses of the label were not reflected in the label excretion on breath. This observation may have important implications for the use of ^{13}C-label excretion on breath to characterise the gastrointestinal handling of lipids and will be discussed in detail in Chapter 8.

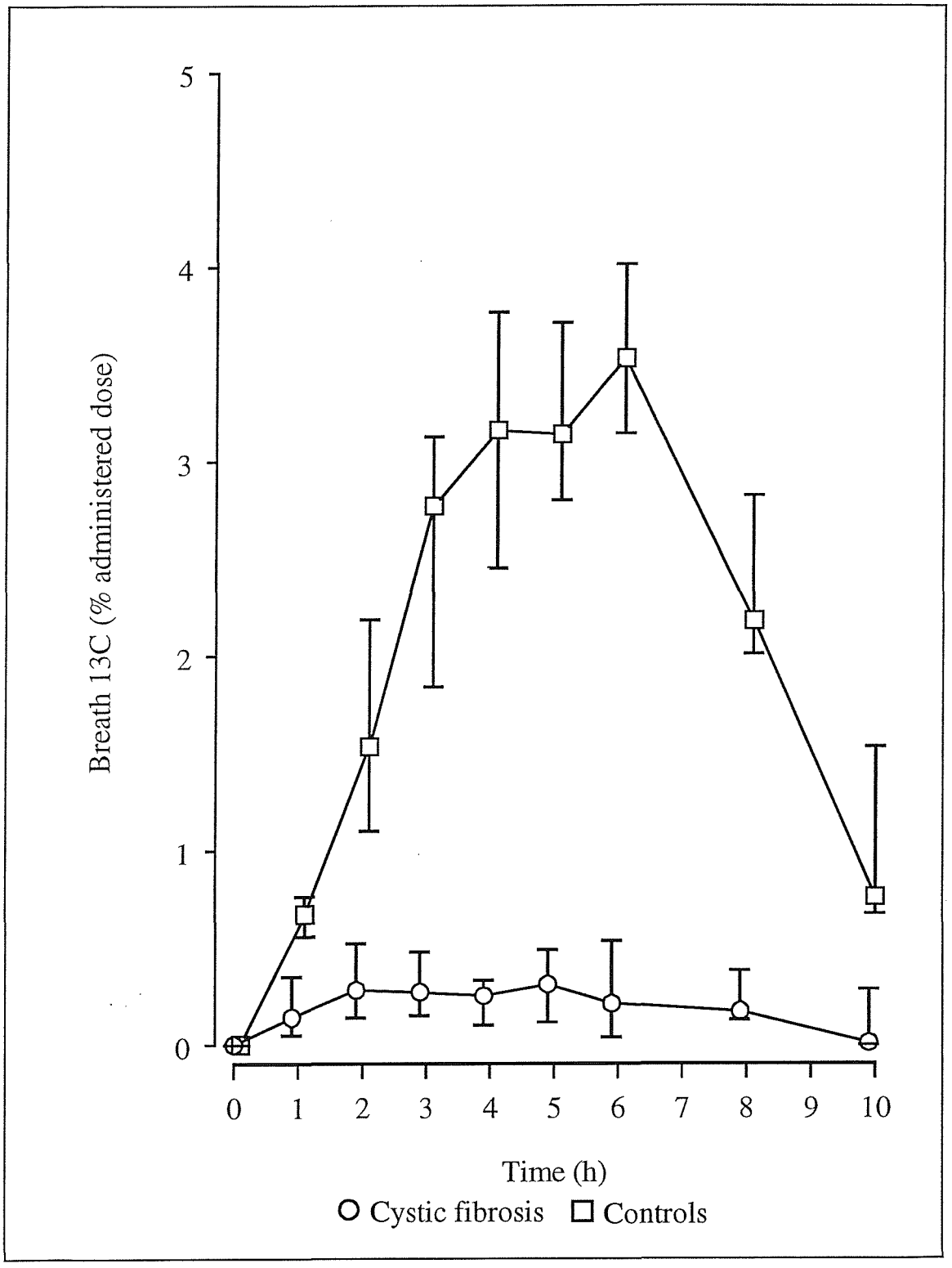


Figure 7.1. Excretion of ^{13}C (% administered dose) on breath over 10 hours following administration of ^{13}C-labelled meal in cystic fibrosis patients (n = 12) and control subjects (n = 8). Median and interquartile range.

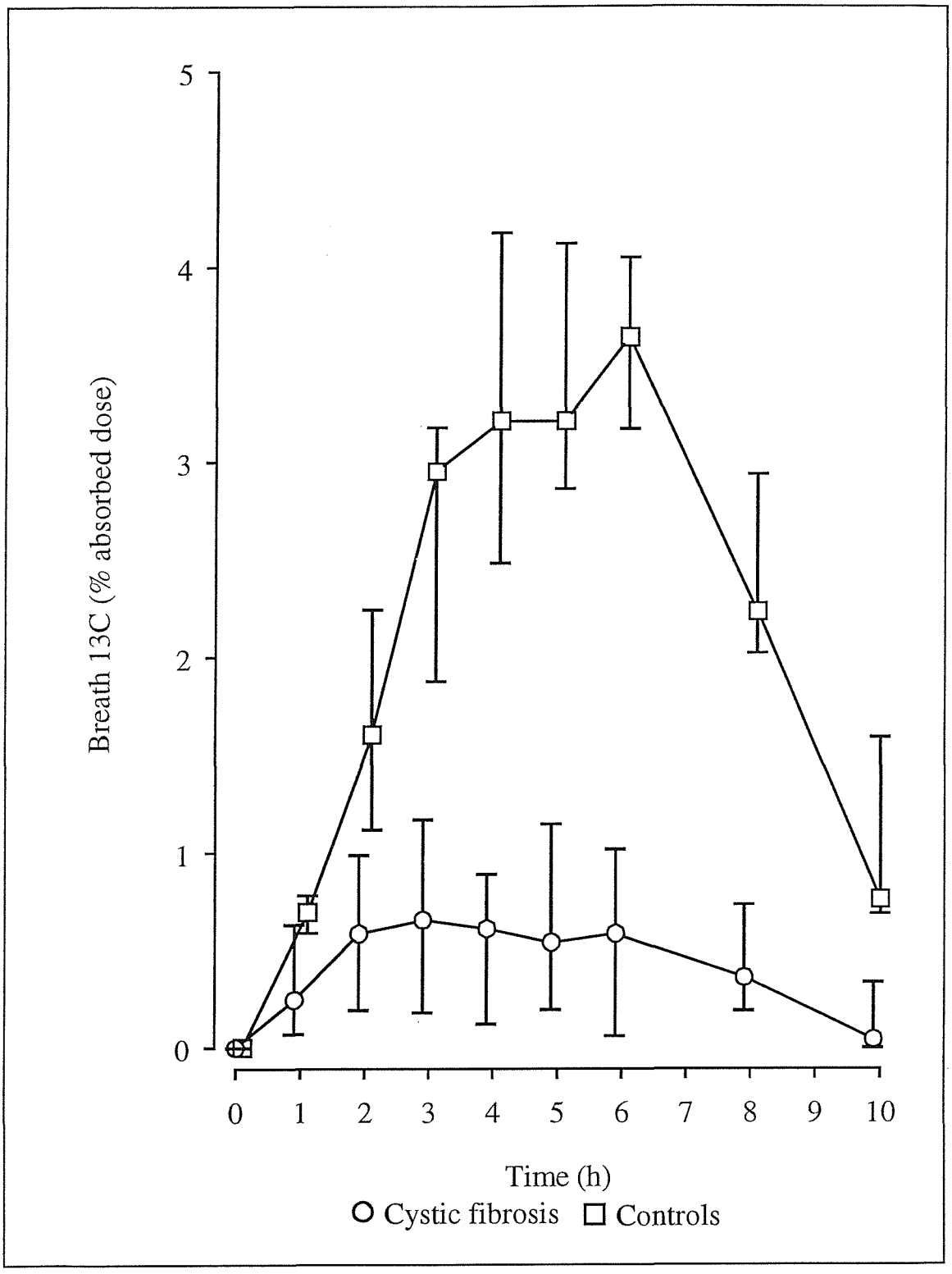


Figure 7.2. Excretion of ^{13}C (% absorbed dose) on breath over 10 hours following administration of ^{13}C-labelled meal in cystic fibrosis patients (n = 12) and control subjects (n = 8). Median and interquartile range.

Table 7.1. Cumulative excretion of ^{13}C on breath over 10 hours following administration of [1,1,1-^{13}C] tripalmitin in cystic fibrosis patients and control subjects.

	^{13}C in breath (% administered dose)	^{13}C in breath (% absorbed dose)
Cystic fibrosis patients		
1	2.4	6.2
2	2.6	5.1
3	3.9	4.2
4	0	0
5	14.3	15.9
6	3.3	8.6
7	3.9	8.8
8	0	0
9	1.7	6.0
10	3.3	9.3
11	1.6	2.3
12	1.2	1.5
Median	2.5	5.6
Control subjects		
1	16.9	17.5
2	18.2	18.3
3	19.7	19.9
4	21.9	22.6
5	22.7	22.7
6	25.4	26.5
7	29.7	30.2
8	31.0	33.7
Median	22.3 [1]	22.6 [1]

[1] Different from cystic fibrosis patients, $P < 0.001$, Mann-Whitney U test.

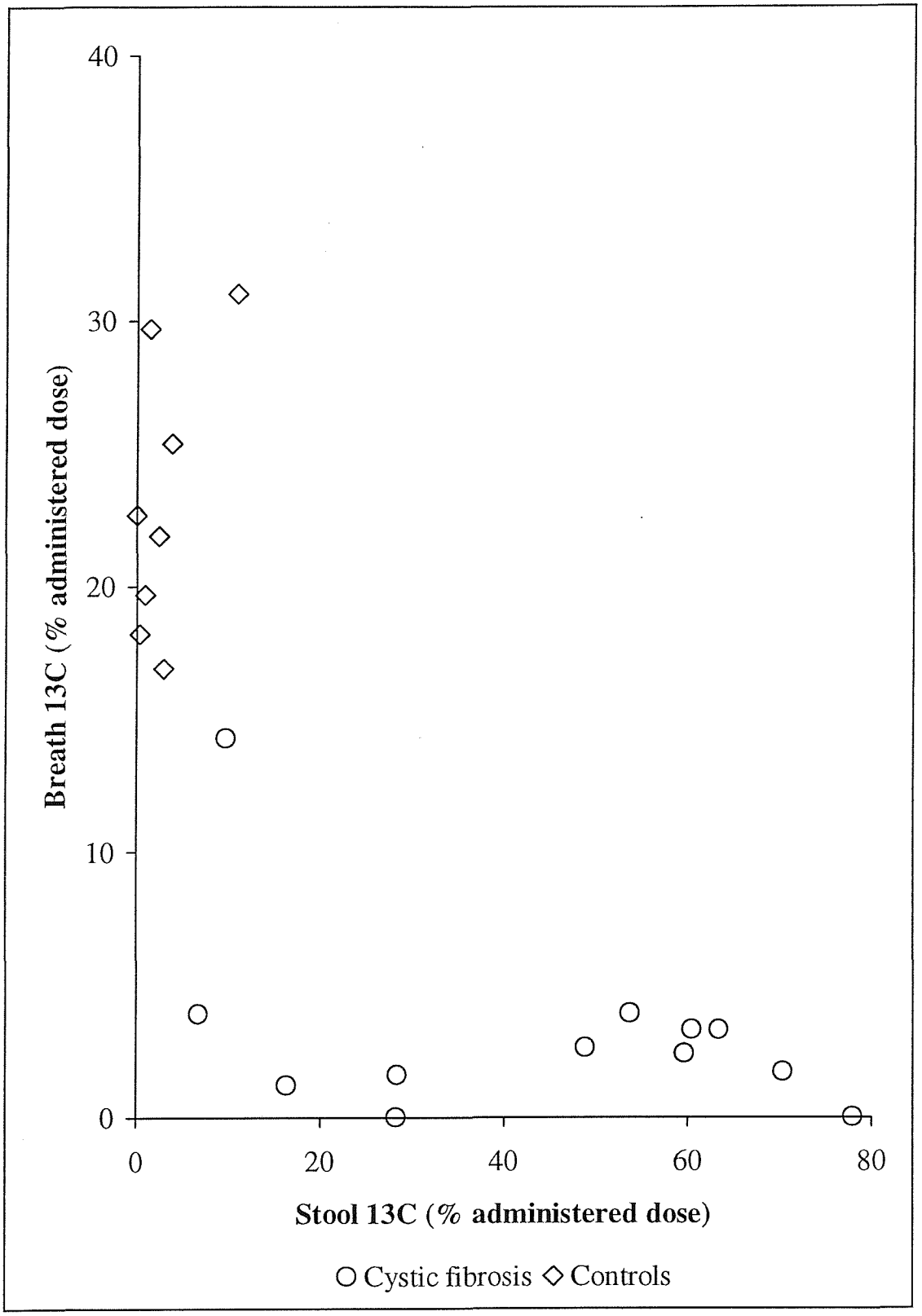


Figure 7.3. Association between excretion of ^{13}C on breath (% administered dose) and excretion of ^{13}C in stool (% administered dose) following administration of ^{13}C-labelled meal in cystic fibrosis patients (n = 12) and control subjects (n = 8).

7.2.2 Substrate oxidation

Figures 7.4 and 7.5 illustrate the pattern of net lipid and net carbohydrate oxidation rates from the postabsorptive state over the 6 hour posptrandial period in cystic fibrosis patients and control subjects. The net lipid oxidation decreased slightly in both groups following the meal and started to increase again at 3 to 4 hours postprandially. The net carbohydrate oxidation increased in both groups following the meal administration and declined after 2 to 3 hours following the meal.

Table 7.2 shows postabsorptive respiratory exchange ratio (RER) and net lipid and net carbohydrate oxidation rates in cystic fibrosis patients and control subjects. There was no difference in the postabsorptive RER or postabsorptive net lipid and net carbohydrate oxidation rates between the groups. The postprandial net lipid and net carbohydrate oxidation rates were also similar in both groups whether expressed as g/kg body weight or as g/kg LBM.

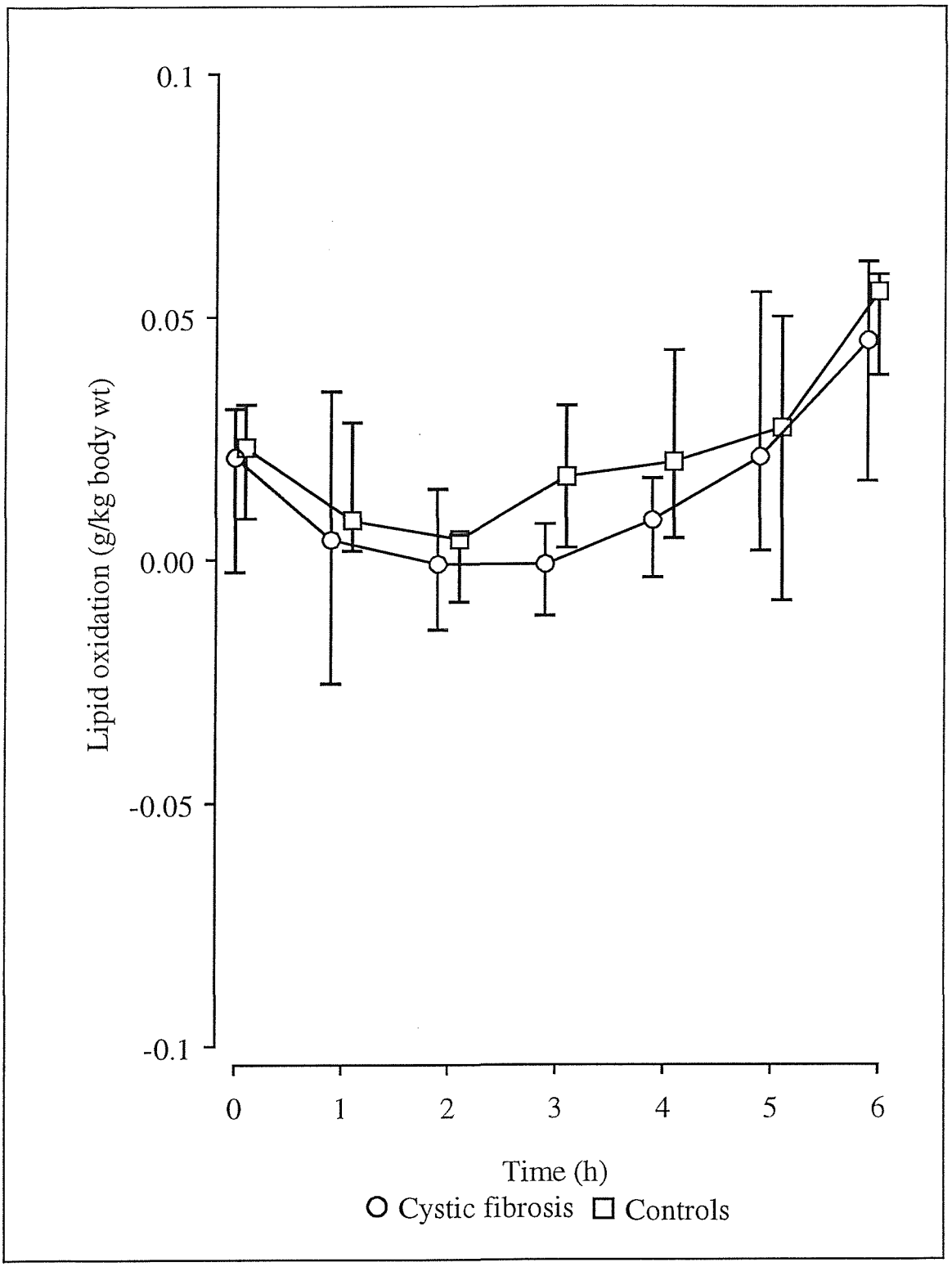


Figure 7.4. Net lipid oxidation (g/kg body wt) over 6 hours following administration of ^{13}C-labelled meal in cystic fibrosis patients (n = 12) and control subjects (n = 8). Median and interquartile range.

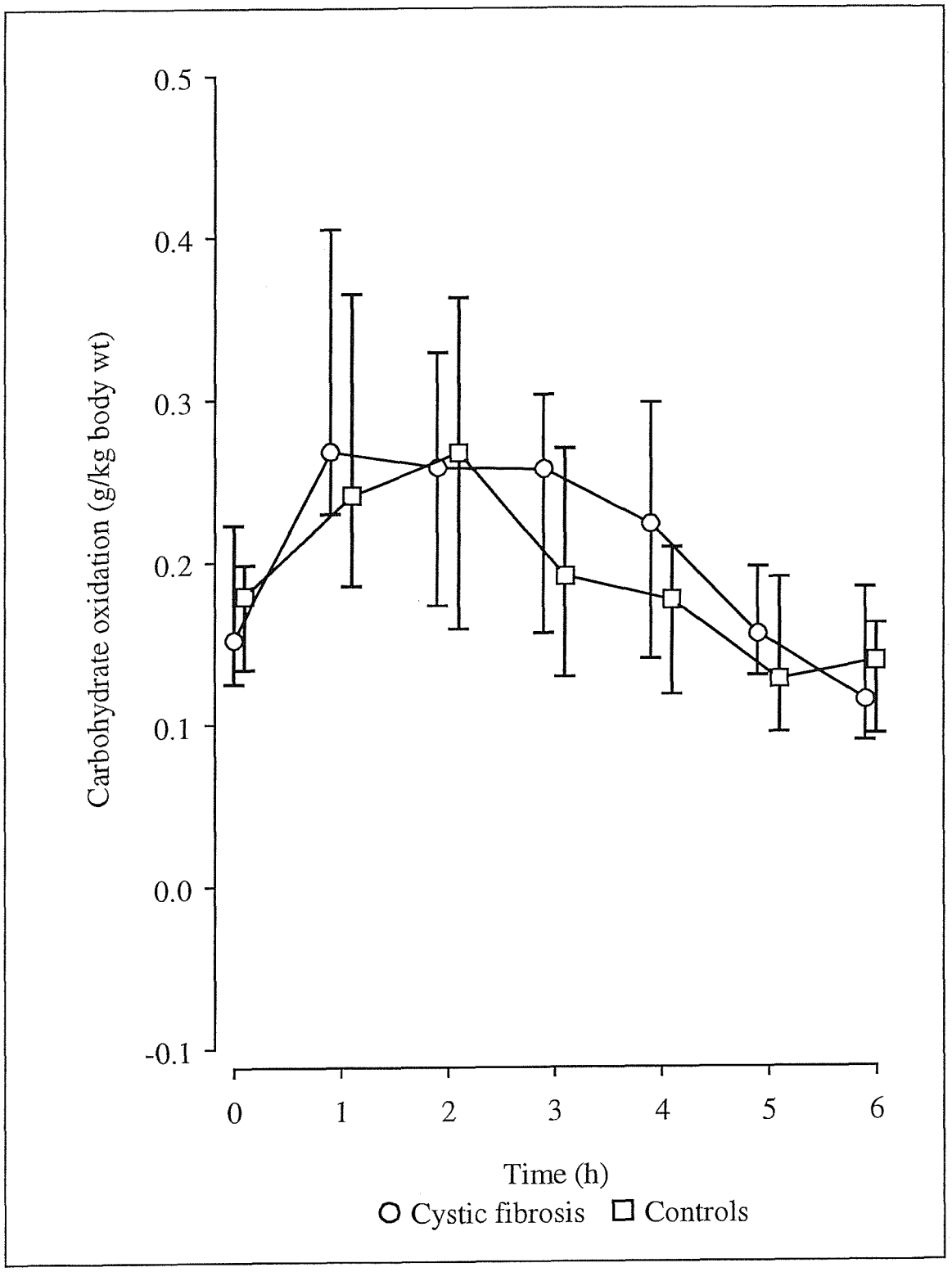


Figure 7.5. Net carbohydrate oxidation (g/kg body wt) over 6 hours following administration of the ^{13}C-labelled meal in cystic fibrosis patients (n = 12) and control subjects (n = 8). Median and interquartile range.

Table 7.2. Postabsorptive and postprandial net substrate oxidation rates in cystic fibrosis patients (n = 12) and control subjects (n = 8).

	Cystic fibrosis patients			Control subjects			
	Median	Min	Max	Median	Min	Max	P value[1]
Postabsorptive RER[2]	0.88	0.79	1.05	0.88	0.86	0.93	0.817
Postabsorptive net lipid oxidation (g/kg body weight/h)	0.02	-0.06	0.09	0.02	0.004	0.04	0.488
Postabsorptive net lipid oxidation (g/kg LBM/h)	0.03	-0.08	0.13	0.03	0.01	0.05	0.589
Postabsorptive net carbohydrate oxidation (g/kg body weight/h)	0.15	0.09	0.49	0.18	0.12	0.25	0.877
Postabsorptive net carbohydrate oxidation (g/kg LBM/h)	0.22	0.13	0.59	0.22	0.17	0.29	0.877
Postprandial net lipid oxidation (g/kg body weight/6h[3])	0.06	-0.40	0.20	0.10	-0.24	0.28	0.076
Postprandial net lipid oxidation (g/kg LBM/6h[3])	0.08	-0.49	0.27	0.14	-0.28	0.33	0.143
Postprandial net carbohydrate oxidation (g/kg body weight/6h[3])	1.38	0.78	3.10	1.12	0.72	2.48	0.384
Postprandial net carbohydrate oxidation (g/kg LBM/6h[3])	1.85	1.02	3.77	1.35	0.86	2.90	0.427

[1] Difference between cystic fibrosis and control groups, Mann-Whitney U test.
[2] Respiratory Exchange Ratio.
[3] Area under the curve over the 6 hour postprandial period.

7.3 Discussion

At all times a mixture of substrates, lipids, carbohydrates and protein, is oxidised to provide energy for the metabolic requirements of the body. At the postabsorptive state the substrates for oxidation are mainly derived from the endogenous sources of the body (adipose tissue and glycogen stores) and at posprandial state the substrates are primarily derived from the diet. Although it has been suggested that cystic fibrosis patients may preferentially use carbohydrates for energy production during postabsorptive state [Bowler *et al.* 1993], it is generally assumed that the metabolic disposal of lipids in cystic fibrosis patients is similar to that of control subjects. In the present study, no differences were found in either postabsorptive or postprandial (over 6 hours) net lipid or net carbohydrate oxidation between the groups. These findings suggest that neither the postabsorptive nor the postprandial lipid oxidation was altered in the cystic fibrosis patients. However, by combining stable isotope tracer techniques with the traditional substrate oxidation measurements by indirect calorimetry, it was shown that the cumulative ^{13}C-label excretion on breath over 10 hours following administration of a meal labelled with [1,1,1-^{13}C] tripalmitin was lower in the cystic fibrosis patients compared to the control subjects. The difference between the groups persisted even when the label excretion on breath was corrected for stool losses of the label indicating that the oxidation of the consumed ^{13}C-labelled lipid and therefore oxidation of dietary lipid was reduced in cystic fibrosis patients compared to the control subjects. These findings suggested that following consumption of a mixed meal, the control subjects preferentially oxidised the lipid derived from the diet, whilst the cystic fibrosis patients preferentially oxidised endogenous lipid.

The factors that could affect the measurements of substrate oxidation and the extent to which ^{13}C-label excretion on breath quantitatively reflects the oxidation of dietary lipid will be discussed in this section. The findings of the present study will also be compared to the previous studies. The possible explanations for the lower excretion of ^{13}C-label on breath in cystic fibrosis patients following administration of ^{13}C-labelled meal and implications of the findings will be discussed in Chapter 8.

7.3.1 Does breath CO_2 quantitatively reflect oxidation of dietary lipid and what factors could influence substrate oxidation?

Does ^{13}C-label excretion on breath quantitatively reflect oxidation of ingested lipid?

In the present study the excretion of ^{13}C-label on breath was measured at hourly intervals for 6 hours and at 8 and 10 hours after consumption of the ^{13}C-labelled meal. The principle of the methodological approach was that the label excretion on breath reflects the proportion of the dietary lipid oxidised over the time period. For the label to appear on the breath the administered [1,1,1-^{13}C] tripalmitin has to be digested, absorbed in the gastrointestinal tract and transported to muscle or liver, where the labelled palmitic acid can enter mitochondria for oxidation. It was assumed that the labelled palmitic acid originating from the administered meal was assimilated to the body pools to the same extent as the unlabelled FA from the meal. The differences in the digestion and absorption of the labelled lipids between the groups were corrected by expressing the label excretion on breath as a proportion of the absorbed dose.

How well does the ^{13}C-label excretion on breath following an administration of the ^{13}C-labelled tripalmitin reflect the oxidation of all dietary FA? It has previously been shown in healthy individuals that unsaturated FA oleic acid and linoleic acid were oxidised to a greater extent than saturated FA stearic acid [Jones *et al.* 1985c], although in another study only small differences were found between the oxidation of palmitic acid, stearic acid and oleic acid suggesting that FA chain length and degree of unsaturation have only modest effects on lipid oxidation [Jones *et al.* 1999]. Palmitic acid is a long-chain saturated FA and therefore best describes the oxidation of dietary saturated FA, but if only modest differences in the oxidation exist between various FA, the palmitic acid may describe the overall oxidation of dietary FA in healthy individuals. However, the oxidation of different FA corrected for variability in the digestion and absorption of the lipid has not been investigated in cystic fibrosis patients.

It has previously been shown that the excretion of ^{13}C-label on breath usually returns to baseline abundance by 24 hours after administration of labelled tripalmitin [Murphy *et al.* 1998] indicating that the oxidation of the administered lipid has been completed and any remaining labelled lipid has been incorporated within body pools. In the present study the breath samples were collected only for 10 hours because it would have been unreasonable

to require the cystic fibrosis patients to withdraw from the supplementary gastrostomy feeding for another night and consumption of the feed could have artificially increased the ^{13}C excretion on breath. Consequently, the breath ^{13}C enrichment did not return to the baseline by the end of the breath sample collection period and the cumulative label excretion on breath over the 10 hours was an underestimation of the total dietary lipid oxidation. However, the maximum label excretion on breath occurred during the 10 hour period and the ^{13}C enrichments of the breath samples were close to the baseline abundance by 10 hours. It is unlikely that the label excretion on breath would have started to rise again after the collection of breath samples was stopped at 10 hours after the meal consumption causing the observed difference in the label excretion on breath between cystic fibrosis patients and control subjects.

Calculation of the ^{13}C-label excretion on breath requires information of the carbon excretion on breath and of the ^{13}C enrichment of breath carbon. The ^{13}C-label excretion on breath was measured by CF-IRMS in duplicate from breath samples collected at hourly intervals for 6 hours and then at 8 and 10 hours. The enrichment values were interpolated to derive the cumulative label excretion on breath over the 10 hour period. For comparative reasons the intervals for breath sample collection were chosen to be the same as in the previous studies. The intermittent collection of the breath samples could result in either overestimation or underestimation of the label excretion on breath, but it was previously shown in an experiment with one healthy subject that the cumulative label excretion on breath over the 10 hour period was not altered whether the breath samples were collected at hourly or at 15 minute intervals [Bennoson 2000].

The CO_2 excretion on breath can either be predicted based on body size or can be measured by indirect calorimetry. In the present study the CO_2 excretion on breath was measured before administration of the labelled meal and at hourly intervals for 6 hours after the meal by indirect calorimetry. It has been shown previously that the predicted CO_2 excretion rate on breath may result in either an underestimation or an overestimation of the CO_2 excretion rate compared to the measured values [Jones 1996]. In cystic fibrosis patients, an increased rate of CO_2 excretion on breath has been shown compared to the predicted values or compared to the measured values of healthy individuals [Amarri *et al.* 1998]. In the present study, the difference between measured and predicted (calculated from Shreeve *et al.* 1976 using body surface area by Haycock *et al.* 1978) postabsorptive CO_2 excretion on breath expressed as a percentage of measured CO_2 excretion in cystic fibrosis

patients ranged from -30% to 37% and in control subjects ranged from -18% to 22%. A lower predicted CO_2 excretion on breath would result in an underestimation of the ^{13}C-label excretion on breath and a higher predicted CO_2 excretion on breath would result in an overestimation of the ^{13}C-label excretion on breath. The use of predicted values for CO_2 excretion on breath instead of measured values would have lead to errors in determining the excretion of ^{13}C on breath up to 10%. The continuous measurement of the CO_2 excretion on breath is not feasible in studies with children who easily become uncomfortable during measurements. The approach used in the present study for measuring CO_2 excretion on breath at hourly intervals reduced the errors that are associated with the estimated or less frequent measurements of the CO_2 excretion on breath.

Both the substrate oxidation measurements and tracer studies presume that the rate at which CO_2 is excreted on breath directly reflects the rate of whole body CO_2 production. However, an additional error in the analysis of substrate oxidation rates and ^{13}C-label excretion on breath could arise if the generated CO_2 was retained within body pools instead of exhaled on breath. The extent to which breath CO_2 excretion reflects the whole body CO_2 production can be measured by administering subjects labelled bicarbonate (sodium bicarbonate) and measuring the subsequent label excretion on breath. Bicarbonate combines with hydrogen in the gastrointestinal tract and forms carbonic acid, which dissociates to water and CO_2. The formed CO_2 is readily absorbed into blood and is subsequently expired on breath [Guyton & Hall 1996]. The average recovery of the labelled CO_2 following an oral or intravenous administration of bicarbonate ranges from 66% to 87% as reviewed by Leijssen and Elia (1996). Generated CO_2 can also be excreted via non-respiratory routes such as stools, urine or sweat, although these contribute only 1% to 2% of the administered dose in healthy individuals [Elia *et al.* 1992; Jones 1996]. The CO_2 may also be incorporated into metabolic products or retained within body CO_2 pools [Irwing *et al.* 1983; Elia *et al.* 1992]. As shown by the bicarbonate studies, excretion of CO_2 on breath is incomplete and therefore results in an underestimation of the substrate oxidation rates. Within a similar study design as in the present study, Jones (1996) showed that the substrate oxidation rates were underestimated by a third if not corrected for the CO_2 retention. A correction factor to adjust for retained CO_2 could be used but was not applied in the present study and consequently the substrate oxidation rates remain underestimates. The interest in the present study however, was to compare the substrate oxidation rates between cystic fibrosis patients and control subjects and the key question is whether CO_2 retention is similar

in the both groups.

Cystic fibrosis patients typically have decreased pulmonary capacity and frequent respiratory infections, which could influence the CO_2 retention. The cystic fibrosis patients studied had a wide range of severity of pulmonary complications assessed both by FEV_1 and Chrispin-Norman score. At the time of study all patients were clinically well and not receiving antibiotics and therefore did not have acute respiratory infections, which might have affected the CO_2 retention. It has been reported previously that cystic fibrosis patients may have an increased O_2 consumption and minute ventilation compared to control subjects [Hirsch *et al.* 1989], which could result in increased body CO_2 pools and therefore increased CO_2 retention. However, it is not likely that the observed four fold difference in the ^{13}C-label excretion on breath between the two groups in the present study could be explained by higher retention of the ^{13}C within CO_2 pools in the cystic fibrosis patients compared to the control subjects. The cystic fibrosis group studied contained both adults (n = 2) and children (n = 10), whereas all the control subjects were children. Previous studies have not found differences in the CO_2 retention within body pools between children and adults [Armon *et al.* 1990; Jones1996]. Although the sample size in the present study is not sufficient to draw conclusions between children and adults, the substrate oxidation rates of the two adults did not seem to differ from those of the children suggesting similar retention of CO_2 within body pools in both children and adults. However, no bicarbonate metabolism studies have been done in cystic fibrosis patients and such studies are required to clarify whether excessive CO_2 retention occurs in cystic fibrosis.

It has been suggested that an acetate recovery factor should be used for correction of lipid oxidation rates [Sidossis *et al.* 1995; Schrauwen *et al.* 1998]. Acetate, like FA, is converted to acetyl-CoA, which enters the citric acid cycle. Part of the administered label can therefore accumulate in the products of the citric acid cycle: glucose, glutamate and glutamine. The ^{13}C recovery on breath from the infused labelled acetate vary from 50% to 80% of the administered dose [Wolfe & Jahoor 1990; Sidossis *et al.* 1995]. Sidossis and co-workers (1995) suggested that the acetate correction factor would be advantageous over the bicarbonate correction factor, because it accounts for the label fixation that might occur at any step between the entrance of the labelled acetyl-CoA into the citric acid cycle until the recovery of the label on breath CO_2. The recovery of the label on breath after administration of ^{13}C-labelled acetate has not been studied in cystic fibrosis patients.

Both retention of the label in body bicarbonate pools and in the products of the citric

acid cycle would result in an incomplete recovery of the ^{13}C-label on breath. At present time, it is unknown whether cystic fibrosis patients have a higher CO_2 retention in body pools compared to the healthy subjects. Although some of the difference in the ^{13}C-label excretion on breath between the groups may be explained by the higher retention of the ^{13}C-label in body pools by the cystic fibrosis patients, it is unlikely to explain the four fold difference between the groups in the present study.

What metabolic factors could influence substrate oxidation rates?

In the present study the net substrate oxidation rates (lipid and carbohydrate) were calculated from breath gaseous exchange measurements based on the theoretical principles of the relation of oxygen consumption and CO_2 production as a substrate is being oxidised [Frayn 1983]. No differences were found between the cystic fibrosis patients and control subjects in either postabsorptive or postprandial net lipid and net carbohydrate oxidation rates. The postprandial net substrate oxidation rate is the sum of dietary (exogenous) substrate oxidation and endogenous substrate oxidation. Once the dietary substrate has been incorporated within body pools such as adipose tissue, it may be considered as an endogenous substrate. The calculations of the lipid and carbohydrate oxidation rates are based on stoichiometry of the oxidation of TAG (palmitoyl-stearoyl-oleoyl-glycerol based on a typical FA composition of adipose tissue) and on the oxidation of glucose [Frayn 1983]. The choice of the substrate for the calculations could differ from truly oxidised substrate, but the calculations are generally applicable to situations where other intervening metabolic processes are not present.

In addition to gaseous exchange measurements the rate of urinary nitrogen excretion, which is used for estimation of protein oxidation, is required for calculation of the substrate oxidation rates. In the present study the urinary nitrogen excretion was estimated, which could potentially result in an error in the substrate oxidation rates. An average value of 0.01 g/min for the urinary nitrogen excretion was used assuming that 1 g nitrogen arises from the oxidation of 6.25 g protein. The dietary intake of protein was variable in both cystic fibrosis (Range 51 to 153 g/d) and control (Range 51 to 92 g/d) groups, which could result in large differences in urinary nitrogen excretion. However, relatively large errors (10%) in the estimate of the urinary nitrogen excretion would result only in a modest error (2%) in the substrate oxidation rates. The protocol of the present study was demanding for the subjects

even without 24 hour urinary collections, therefore an error that would arise from the estimation of the urinary nitrogen excretion on the substrate oxidation rate was regarded as acceptable. The differences in both lipid and carbohydrate oxidation rates between the groups were negligible and it is unlikely that an error associated with the estimation of the urinary nitrogen excretion would have altered the observed difference between the groups.

A mixture of substrates, rather than only lipids or only carbohydrates, is metabolised at all times and the net substrate oxidation measurement is the sum of all the oxidative processes occurring within the body. The stoichiometry of the calculations is well defined for the oxidation of substrates in the isolated conditions but intervening metabolic processes, mainly lipogenesis and gluconeogenesis, could influence the substrate oxidation rates. In extreme conditions such as in chronic malnourishment, individuals may have gluconeogenesis and ketogenesis and during high-rate glucose infusion in surgical patients may have lipogenesis [Frayn 1983]. All subjects were measured under similar resting conditions and following an overnight fast. No differences were found between the two groups in the net lipid or net substrate oxidation rates in the present study indicating that if such processes were occurring they were likely to be similar in both groups.

The difference between cystic fibrosis patients and control subjects in the net substrate oxidation rates should be interpreted with caution, because the power analysis showed that with the observed SD, 280 patients for the postabsorptive lipid oxidation, 260 patients for the postabsorptive carbohydrate oxidation, 130 patients for the postprandial lipid oxidation (area under the curve over 6 hours) and 3200 patients for the postprandial carbohydrate oxidation would have been required to detect a statistically significant difference ($P < 0.05$ at 80% power) between two groups. However, only 17 patients for the postabsorptive lipid oxidation, 17 patients for the postabsorptive carbohydrate oxidation, 4 patients for the postprandial lipid oxidation (over 6 hours) and 5 patients for the postprandial carbohydrate oxidation would have been required to observe a significant difference of 2 SD from the control group (mean of the control subjects ± 2SD). The magnitude of the differences between cystic fibrosis patients and control subjects for the substrate oxidation rates that would be of clinical relevance is not known and the possibility that a difference in the oxidative capacity, especially during the postabsorptive period, might not have been detected due to a small sample size cannot be excluded. However, the observed differences in the net substrate oxidation between the groups were marginal compared to the differences in the ^{13}C-label excretion on breath suggesting that net substrate oxidation rates

were similar in both groups, but the excretion of ^{13}C-label on breath was lower in cystic fibrosis patients.

7.3.2 Comparison to previous studies

Excretion of ^{13}C on breath

Only one study has previously investigated ^{13}C-label excretion on breath following an oral administration of [1,1,1-^{13}C] tripalmitin in children with cystic fibrosis and has corrected the label excretion on breath for stool losses of the label [Murphy *et al.* 1998]. The ^{13}C-label excretion on breath over 24 hours was lower in the cystic fibrosis patients compared to the control subjects, although when corrected for stool losses of the label, there was no difference in the label excretion on breath between the two groups. The cystic fibrosis patients studied by Murphy *et al.* (1998) were conventionally managed cystic fibrosis patients, whereas the patients in the present study were patients receiving supplementary feeding via a gastrostomy. The clinical characteristics of the cystic fibrosis patients in the present study, such as adaptive responses to previous malnourishment, body composition and energy intake in excess to the requirements from the diet and supplementary feed, could explain part of the difference in the label excretion on breath (Chapter 8).

Two other studies have investigated ^{13}C-label excretion on breath over a 6 hour period after administration of ^{13}C-labelled palmitic acid in healthy children [Watkins *et al.* 1982; Jones *et al.* 1998]. For comparison to these two studies the data from the present study and from Murphy *et al.* (1998) were also expressed for the 6 hour period and are presented in Table 7.3. Comparing the four studies, the mean label excretion on breath was similar in the control subjects, except Watkins *et al.* (1982) reported lower label excretion on breath in the control children compared with the other three studies. The control children in the study of Watkins *et al.* (1982) were younger (2.5 to 8 years) which might contribute to the lower label excretion on breath. However, the label excretion on breath of the control children studied by Watkins *et al.* (1982) was similar to the label excretion on breath of the cystic fibrosis patients studied by Murphy *et al.* (1998). Whereas the label excretion on breath of the cystic fibrosis patients in the present study was lower compared to both cystic fibrosis patients [Murphy *et al.* 1988] and control subjects [Watkins *et al.* 1982; Jones *et al.* 1998; Murphy *et al.* 1988] studied previously. The reasons for the differences between the

studies are unclear. Part of the difference in the label excretion on breath may have been due to the clinical characteristics of the patients in the present study, but also age, body composition and physical activity of the subjects could have influenced the label recovery on breath. Although, the present study and also the other three studies were carried out under resting conditions and after an overnight fast, which reduce the variability in the label excretion on breath caused by factors other than the consumption of the ^{13}C-labelled meal.

Table 7.3. Cumulative excretion of ^{13}C-label on breath over 6 hours following administration of ^{13}C-labelled lipid in the present study compared to previous studies.

Study	Subjects	*n*	Age, yr (Range)	Substrate	Excretion of ^{13}C on breath[1]	
					% administered dose	% absorbed dose
Present Study	Cystic fibrosis	12	7 - 31	TP[2]	1.9	3.6
	Controls	8	8 - 17		13.6	14.1
Murphy *Et al.* (1998)	Cystic fibrosis	7	4 - 11	TP[2]	7.1	9.2
	Controls	9	5 - 8		14.4	15.3
Watkins *Et al.* (1982)	Controls	10	2.5 - 8	PA[3]	6.6	
Jones *Et al.* (1998)	Controls	12	5 - 10	PA[3]	13	

[1] Mean
[2] [1,1,1-^{13}C] tripalmitin
[3] [1-^{13}C] palmitic acid

Net substrate oxidation

No difference was found in either postabsorptive or postprandial net lipid or net carbohydrate oxidation between cystic fibrosis patients and control subjects in the present study. Also the patterns of both lipid and carbohydrate oxidation after the meal consumption were similar in the both groups. A slight suppression of the net lipid oxidation was observed after the meal consumption returning back to the baseline oxidation levels by

6 hours after the meal. The net carbohydrate oxidation increased rapidly following the meal consumption and decreased at or below the postabsorptive values by 5 to 6 hours. Jones *et al.* (1998) found a similar pattern of the net lipid and net carbohydrate oxidation in healthy children and adults following consumption of a mixed meal. The net lipid oxidation decreased following a meal with the lowest rates occurring at 3 hours postprandially and by 6 hours the net lipid oxidation increased and exceeded the postabsorptive net lipid oxidation. Net carbohydrate oxidation increased following the meal peaking at 1 hour and returned to postabsorptive values by 6 hours [Jones *et al.* 1998]. The patterns of both the postprandial net lipid and net carbohydrate oxidation rates after consumption of a mixed meal in the present study were comparable to the previous findings in healthy subjects. Following a mixed meal, containing lipid, carbohydrate and protein, a mixture of substrates is being oxidised, but immediately after the meal carbohydrates are preferentially oxidised and net lipid oxidation is suppressed.

Postprandial substrate oxidation rates have not previously been studied in cystic fibrosis patients, but three studies have compared the postabsorptive substrate oxidation rates between cystic fibrosis patients and control subjects. Similarly to the findings of the present study, Hardin *et al.* (1999) and Ward *et al.* (1999) found no differences in either postabsorptive net lipid or net carbohydrate oxidation rates between cystic fibrosis patients and control subjects. One study reported that a greater proportion of the BMR was derived from the net carbohydrate oxidation in cystic fibrosis patients compared to control subjects [Bowler *et al.* 1993]. Bowler *et al.* (1993) suggested that the greater contribution of carbohydrate oxidation to the BMR was due to a higher intake of carbohydrates from the diet (% energy) by the cystic fibrosis patients. In the present study there was no difference in the postabsorptive net carbohydrate oxidation between cystic fibrosis patients and control subjects even when expressed as a proportion of the BMR (Cystic fibrosis patients, Median 48%; Control subjects, Median 52%, $P = 0.678$). The absolute intake of lipid and carbohydrate from the diet and supplementary feed of the cystic fibrosis patients was higher compared to the control subjects, but the intake of lipid and carbohydrate as a proportion of the energy were similar between the groups in this study. The extent to which the absolute or proportional (% of energy) intake of macronutrients determine the net lipid and carbohydrate oxidation is unclear. However, the difference between the groups in the net substrate oxidation rates in the present study should be interpreted with caution, because a larger sample size would have been required to detect a statistically significant difference

between the groups. The sample size of Ward *et al.* (1999) was also relatively small (10 cystic fibrosis patients and 10 control subjects). Hardin *et al.* (1999) studied 29 cystic fibrosis patients and 14 control subjects but did not still find a difference in the postabsorptive substrate oxidation between cystic fibrosis patients and control subjects.

7.4 Summary

The metabolic disposal of [1,1,1-^{13}C] tripalmitin and net lipid and net carbohydrate oxidation were investigated in cystic fibrosis patients compared to control subjects. The results demonstrate that:

1) Excretion of ^{13}C on breath as a proportion of the administered dose over 10 hour study period was markedly lower in cystic fibrosis patients compared to control subjects.
2) Excretion of ^{13}C on breath remained markedly lower in cystic fibrosis patients even when the label excretion on breath was corrected for differences in stool losses of the label (% absorbed dose).
3) No association was found between excretion of ^{13}C on breath (% administered dose) and excretion of ^{13}C in stools (% administered dose).
4) Postabsorptive and postprandial net lipid oxidation and net carbohydrate oxidation were similar in both groups.
5) The lower label excretion on breath and similar net lipid oxidation suggests that the oxidation of the dietary lipid was lower in cystic fibrosis patients compared to control subjects.

Chapter 8: General discussion

8.1 Introduction

Despite the current clinical management of cystic fibrosis patients including PERT and nutritional advice, some patients continue to have excessive lipid losses in stools and disturbed growth or difficulties in maintaining weight. These observations led to the hypothesis of this thesis that poor nutritional status or disturbed growth in cystic fibrosis patients is associated with two metabolic disturbances: 1) increased lipid losses in stools due to malabsorption of dietary lipid in addition to maldigestion, 2) altered metabolism of absorbed lipid.

In order to investigate the hypothesis, it was necessary to develop and evaluate a method which could be used to differentiate between maldigestion and malabsorption of dietary lipid (Chapter 3). This involved development of a method to extract total lipids from stools and evaluation of methods to separate TAG and FA fractions from total lipid and analysis of ^{13}C enrichment in the fractions. Following administration of [1,1,1-^{13}C] tripalmitin, the excretion of the label in TAG fraction represented lipid that has not been digested, and excretion of the label in FA fraction represented lipid that has been digested but has failed absorption. The methodology was then applied to investigate digestion and absorption of [1,1,1-^{13}C] tripalmitin in cystic fibrosis patients habitually receiving supplementary feeding via a gastrostomy compared to healthy control subjects. In addition, the stool lipid losses were evaluated by traditional (unlabelled) techniques. The metabolic disposal of dietary lipid was determined by measuring the excretion of ^{13}C-label on breath following administration of [1,1,1-^{13}C] tripalmitin and net lipid and net carbohydrate oxidation rates were estimated from indirect calorimetry measurements.

In this chapter the underlying causes of the maldigestion and malabsorption of dietary lipid and of the reduced oxidation of dietary lipid during postprandial period and their implications for growth and clinical management of the patients will be discussed. The ultimate aim is to suggest a scheme for nutritional assessment and management of cystic fibrosis patients with weight loss or disturbed growth.

8.2 Why is growth disturbed in some cystic fibrosis patients?

It is now widely accepted that poor growth and weight management in cystic fibrosis patients are not necessarily due to inherent features of the disease, but result from an energy deficit. Figure 8.1 summarises factors that may contribute to an energy deficit and therefore disturb growth and cause weight loss in cystic fibrosis patients. Reduced energy availability originates from increased stool losses, mainly lipid losses caused by maldigestion and malabsorption and reduced dietary intake due to gastrointestinal symptoms and poor appetite. The occurrence of pulmonary infections is frequent in cystic fibrosis and the medication used to treat the infections may further increase lipid malabsorption by increasing gastrointestinal transit. Medication may also reduce appetite resulting in a reduced dietary intake, although some medication (corticosteroids) may also increase appetite. Energy requirements may be increased due to an increased BMR inherent to disease or due to increased work of breathing muscles resulting from reduced pulmonary function. The resulting energy deficit may cause an overall deterioration of nutritional status, which in turn may be related to respiratory and immune function and although causal relations have not been proven, possibly to the overall survival of the patients. Therefore improvement or maintenance of a good nutritional status in the cystic fibrosis patients may advance growth, sustain respiratory function and may improve the quality of life and the overall prognosis of the disease.

In the present study the energy and macronutrient intake from diet and supplementary feed in cystic fibrosis patients exceeded the intake of control subjects and also the estimated energy requirements in most patients (Chapter 5). The BMR of cystic fibrosis patients was similar to that of control subjects. Cystic fibrosis patients are also likely to be less active than healthy individuals, especially during disease exacerbations which would reduce the total daily energy expenditure (Chapter 5). Therefore, the inadequate intake of energy from the diet or increased energy requirements are not likely to contribute to a great extent to energy deficit and to impaired growth in the cystic fibrosis patients studied. The most likely cause is the reduced availability of energy from the gastrointestinal tract. The altered metabolic disposal of dietary lipid may also influence growth. The possible causes for the increased lipid losses in stools and altered metabolic disposal of dietary lipid will be discussed below.

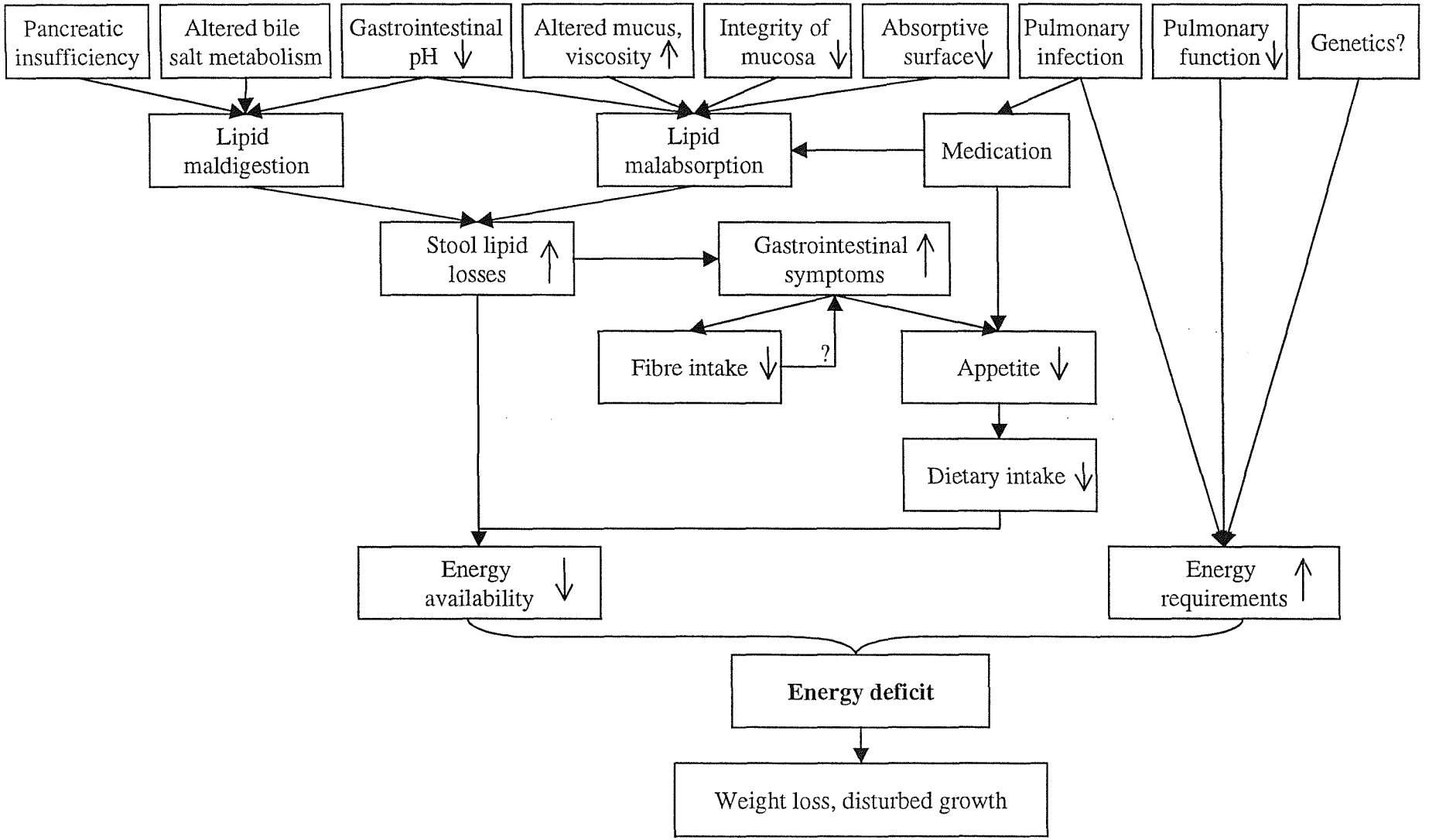


Figure 8.1. Factors causing energy deficit and disturbed growth and weight loss in cystic fibrosis.

8.2.1 Why are stool lipid losses increased in cystic fibrosis patients?

The findings of the present study and those of the others have shown that stool lipid losses are commonly increased in cystic fibrosis patients even when they receive PERT. By applying stable isotope techniques in the present study it was possible to differentiate excretion of dietary lipid in stools from endogenous and bacterial lipid excretions and also to differentiate between maldigested and malabsorbed lipid. Pancreatic enzyme preparations were omitted from the labelled meal in the present study to investigate the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid. Excessive lipid losses in stools in cystic fibrosis patients are often regarded as originating from pancreatic insufficiency and therefore maldigestion of dietary lipid. Therefore the clinical management of stool lipid losses has focused on PERT and no clear differentiation between maldigestion and malabsorption of dietary lipid has been made. This has been partially due to lack of specific and clinically applicable method that can be used to differentiate between maldigestion and malabsorption. Previous studies have mainly investigated total lipid losses in stools.
The main aim of this thesis was to characterise stool lipid losses in a more specific way by investigating the type of stool lipid (TAG and FA) and it was suggested that in addition to maldigestion cystic fibrosis patients may have malabsorption of lipid to a considerable extent. The possible reasons for maldigestion and malabsorption of lipids in cystic fibrosis patients are discussed below.

Why do cystic fibrosis patients have maldigestion of dietary lipid?

In healthy individuals dietary TAG is mainly hydrolysed by pancreatic lipase. Pancreatic lipase is activated in the presence of co-lipase and bile salts. This activation results in a conformational change of lipase molecule whereby hydrophobic site of the lipase is exposed and can interact with a lipid-water interface where TAG is hydrolysed to FA and MAG [Guerciolini 1997]. Therefore, both reduced or absent secretion of pancreatic lipase and co-lipase or reduced activity of lipase would result in a decreased digestion of dietary lipid and excessive TAG losses in stools. This is very likely in cystic fibrosis patients, because it has been estimated that about 85% of patients are pancreatic insufficient, which is manifested both as reduced and altered (more viscous) pancreatic secretions including reduced pancreatic lipase secretion [Gaskin *et al.* 1982]. In the present study the capacity of

the pancreas to secrete lipase was not measured, but all the patients were clinically treated as pancreatic insufficient and were following PERT. Ten of the patients were ΔF508 homozygotes, a genotype, which has been shown to consistently associate with pancreatic insufficiency [Wilschanski & Durie 1998]. The genotype of one patient was not known and the 12th patient had undergone pancreatectomy. It may therefore be assumed that all the patients studied were likely to be pancreatic insufficient. In addition to reduced secretion of lipase, pancreatic insufficiency causes reduced secretion of bicarbonate resulting in relatively acidic conditions of the luminal contents [Sturgess 1984]. Reduced luminal pH may inactivate pancreatic lipase therefore causing maldigestion. Therefore, both secretion of lipase by the pancreas and activity of lipase may be reduced (or absent) in cystic fibrosis patients, and are very likely causes for maldigestion of dietary lipid in the patients studied.

In addition to reduced lipase secretion or lipase activity, maldigestion of lipids may be due to a reduced bile salt concentration in the gastrointestinal lumen. Bile salts facilitate digestion by increasing the surface area of the lipid droplets (solubilisation) and therefore increasing the lipid-water interface where lipase, co-lipase complex hydrolyses TAG [Borgström 1985]. It may be that if the amount of bile salts in the gastrointestinal tract is insufficient to solubilise lipid, the access of lipase on TAG molecules would be reduced resulting in excessive losses of maldigested TAG in stools. Bile salt losses of the patients were not measured in the present study, but it has been shown previously that cystic fibrosis patients may have higher losses of bile salts in stools compared to control subjects (See Table 2.4). Increased bile salt losses may be caused by precipitation of bile salts due to acidic luminal environment [Regan *et al.* 1979] or due to mucosal cell defect or dysfunction of ileal transport process, which prevents bile salt reabsorption [Harries *et al.* 1979]. Due to persistent bile salt losses in stools, the total body bile salt pool may diminish resulting in a reduced bile salt concentration in the gastrointestinal tract, which would be insufficient to solubilise dietary lipid and therefore would inhibit digestion. Furthermore, bile salt secretion from the liver may be reduced in cystic fibrosis reducing the overall size of the bile salt pool [Weizman *et al.* 1986]. Following digestion, bile salts form mixed micelles with the lipid digestion products and therefore reduced bile salt concentration (insufficient solubilisation) could be viewed as resulting in increased FA losses in stools. However, this is not likely because if the concentration of bile salts in the lumen falls below critical micellar concentration, not only the formation of mixed micelles (bile salts and lipid digestion products) would be inhibited, but the digestion of lipids would also be inhibited due to

reduced solubilisation (reduced water-lipid interface). Regan *et al.* (1979) showed that when patients with pancreatic insufficiency (pancreatic enzyme secretion less than 10% of the normal secretion) were given a meal without enzyme preparations or enzyme preparations but no acidity regulators, less lipids were present in the micelles compared to a meal when enzyme preparations were given with an acidity regulator (cimetidine). This suggests that if there is sufficient concentration of bile salts for solubilisation of lipid and therefore for digestion of lipid, the mixed micelles will also be formed in the gastrointestinal tract. Therefore, it may be possible to improve digestion of dietary lipid in cystic fibrosis patients by administration of pancreatic enzyme preparations together with acidity regulator. Attempts have been made to alter the acidic gastrointestinal environment in cystic fibrosis patients by administering regulators of the gastric acidity or stimulators of the duodenal bicarbonate secretion together with enzyme preparations, but the reduction of stool lipid losses has often been modest [Gow *et al.* 1981; Cleghorn *et al.* 1988; Robinson & Sly 1990; Carroccio *et al.* 1992; Heijerman *et al.* 1993]. With reference to the study of Regan *et al.* (1979) administration of enzyme preparations with acidity regulators should improve digestion of lipids by two mechanisms firstly by providing exogenous lipase to compensate for absence of endogenous lipase and secondly by preventing precipitation of bile salts and improving the solubilisation of lipid and therefore digestion of lipid. However, the failure of pancreatic enzyme preparations administered together with acidity regulators to reduced lipid losses in stools may have been due to a defect in absorption of the digested lipids, which will be discussed below. In the present study pancreatic enzyme preparations were omitted from the labelled meal and the patients did not receive any regulators of gastrointestinal acidity either.

Relatively high losses of ^{13}C-label in TAG fraction was observed only in five of the 12 patients. What could explain the low label excretion in the TAG fraction in the remaining 7 patients? Did these patients, despite apparent pancreatic insufficiency, have residual pancreatic secretory capacity left? It has been shown previously that only a small amount, about 1% to 2%, of the residual pancreatic function is required to prevent maldigestion of lipids [Durie & Forstner 1989]. Therefore it is possible that some lipase was secreted by the pancreas in the patients studied, which would have been sufficient to digest TAG. However, even the patient who had had pancreactomy excreted only 54% of the administered ^{13}C dose in total stools and 31% in the TAG fraction. If the hydrolysis of dietary TAG was solely due to pancreatic lipase activity, more ^{13}C-label should have been excreted in stools in

this patient. It is well known that pancreatic lipase is not the only lipase that can digest dietary lipid in the gastrointestinal tract. Lingual and gastric lipase secretion has been shown in both healthy individuals [Hamosh *et al.* 1975; Moreau *et al.* 1988] and in cystic fibrosis patients [Fredrikzon & Bläckberg 1980; Abrams *et al.* 1984]. Both lipases are active in gastric juice [Hamosh *et al.* 1975; Bläckberg *et al.* 1977] and are passed to duodenum where they may remain active particularly in the relatively acidic conditions of cystic fibrosis gastrointestinal tract [Fredrikzon & Bläckberg 1980]. The gastric lipase activity may even be higher in cystic fibrosis patients compared to control subjects [Roulet *et al.* 1980]. Lingual and gastric lipases do not have specificity for short or long-chain FA [Gargouri *et al.* 1986] and therefore they could have hydrolysed the administered tripalmitin in the present study. In a previous study it was estimated that the lingual and gastric lipases may hydrolyse as much as 40% to 70% of dietary TAG in the absence of panceatic lipase [Abrams *et al.* 1984]. The residual lipolytic activity in the gastrointestinal tract could have explained the low label losses in stools in some of the cystic fibrosis patients observed in this study.

In conclusion, 5 of the 12 patients studied had high excretion of ^{13}C-label in stool TAG fraction suggesting maldigestion of dietary lipid. Insufficient hydrolysis of dietary TAG was likely to be caused by three principal factors: 1) insufficient secretion of lipase and co-lipase by the pancreas, 2) inactivation of lipase due to an acidic gastrointestinal pH resulting from reduced bicarbonate secretion and 3) poor solubilisation (reduced water-lipid interface) of dietary lipid due to an altered bile salt metabolism. Prior to entering the study all the patients were receiving PERT and the majority of the patients had a genotype which has been shown to associate with pancreatic insufficiency. Therefore it may be assumed that the patients did have pancreatic insufficiency. Bile salt metabolism was not determined in this thesis but it has previously been shown that cystic fibrosis patients commonly have increased bile salt losses in stools. However, a low label excretion in stool TAG fraction in 7 of the 12 patients is likely to reflect the residual capacity of the gastrointestinal tract to digest the administered TAG.

Why do cystic fibrosis patients have malabsorption of dietary lipid?

The current view is that digestion products of TAG (FA and MAG) pass from mixed micelles into absorptive enterocytes by passive diffusion [Tso 1985]. The first step in the

absorption of FA and MAG is diffusion through unstirred water layer, which is overlaying the microvilli of the enterocytes [Wilson *et al.* 1971]. Once in the enterocytes, FA are bound to fatty acid binding proteins, which ensures that an inward diffusion gradient is maintained for absorption [Ockner *et al.* 1972]. Therefore, malabsorption of lipids could result from inability of FA and MAG to pass through unstirred water layer to the enterocytes, dysfunction of enterocytes themselves including fatty acid binding protein or alterations in the integrity of the gastrointestinal mucosa affecting the diffusion of substances across the membrane. In the present study excretion of ^{13}C-label in stool FA fraction exceeded the upper limit of the 95% confidence interval for control subjects in 11 of the 12 cystic fibrosis patients suggesting that most of the patients studied would have malabsorption of lipids. What factors could have caused malabsorption of lipids in these patients?

For absorption to occur lipid digestion products need to cross through the unstirred water layer to the enterocytes. According to Wilson *et al.* (1971), the thickness of the unstirred water layer determines the rate of absorption. The basic gene defect in cystic fibrosis causes abnormalities of electrolyte transport in epithelial cells resulting in reduced secretion of fluids in the gastrointestinal lumen and reduced secretion of bicarbonate in the pancreas [Wilschanski & Durie 1998]. As a result the viscosity of the gastrointestinal contents is increased and mucus covering the intestinal epithelium is increased and composition altered [Wesley *et al.* 1983; Bouquet *et al.* 1988]. Although it is not known whether the thickness of the unstirred water layer is altered in cystic fibrosis, it is possible that alterations in the properties of the mucus may also affect the unstirred water layer. Thicker unstirred water layer could delay the uptake of FA by the mucosa, but might not affect the overall absorption of lipids. A more likely factor than the thickness of the unstirred water layer that could inhibit the absorption of lipids in cystic fibrosis patients is the altered mucus itself. Thick, viscous mucus in cystic fibrosis patients could prevent the access of lipids to the enterocytes by acting as a barrier for diffusion. If this was the case the absorptive surface area could be reduced, since the mucus may cover microvilli of the small intestine and only the tip of the villi may be available for absorption. In addition to lipase, pancreatic enzyme preparations contain amylase and protease, which could hydrolyse some of the glycoproteins in the mucus thereby possibly normalising the properties of the mucus in cystic fibrosis patients. Although, pancreatic enzymes were omitted from the labelled meal in the present study, it is not known what are the effects of pancreatic enzyme

preparations on the mucus and whether enzyme preparations could improve absorption of lipid by altering the mucus. A large range in the excretion of ^{13}C-label in the FA fraction (3% to 50% of the administered dose) was observed in the present study. Could it be that those patients who had lower FA losses in stools, also had less alterations in the mucus? Freye *et al.* (1964) showed (small intestinal biopsies) that cystic fibrosis patients with increased lipid losses in stools had thickened mucus, whilst no such thickening was observed in one patient who had normal lipid losses in stools. This would suggest that the extent to which mucus is altered might influence the extent to which lipids are absorbed. However, further research is required to investigate whether and in what way an altered mucus in cystic fibrosis patients may influence lipid absorption.

A reduced absorptive surface area of the gastrointestinal tract in cystic fibrosis patients could be due to factors other than altered mucus. Some cystic fibrosis patients require resection of ileum as an infant due to meconium ileus [Kopelman 1991]. It is possible that resection of a proportion of the small intestine as an infant may influence the development of small intestine and result in a reduced absorptive surface area as an adult. Three patients in the present study (Figure 6.7; numbers 2,3, and 4) had had resection due to meconium ileus as an infant. Two of these patients had high ^{13}C-label excretion in FA fraction and one patient had similar ^{13}C-label losses in total stools compared to control subjects. Some of the FA losses in stools in these patients could be explained by the intestinal resection as an infant. However, animal studies have shown a hypertrophy of villi and microvilli in nonresected area following a partial resection of small intestine as reviewed by Weser (1979). The effects of intestinal resection as an infant on the absorption of nutrients in later life remain unclear, but the remaining intestine may not be able to fully compensate for the lost absorptive capacity of the resected intestine. It is believed that the majority of FA is absorbed in jejunum [Borgström *et al.* 1963] in which case the resection of ileum may not affect the absorption of lipids to a large extent.

The absorption of lipids may also be influenced due to alterations in the structure of villi or integrity of the intestinal mucosa. One cystic fibrosis patient studied, who had coeliac disease in addition to cystic fibrosis was found to have a high ^{13}C-label excretion in total stools (78% of the administered dose) and in FA fraction (50% of the administered dose). It is well known that villous atrophy is associated with coeliac disease. Although, this patient was following a gluten-free diet, it is possible that villous atrophy might have been present and contributed to the malabsorption of lipids. Do cystic fibrosis patients generally have an

altered mucosal structure? It has been shown previously in a microscopic examination that small intestinal mucosa of cystic fibrosis patients is similar to that of control subjects [Freye *et al.* 1964]. However, the functionality of the enterocytes may be altered despite apparently normal histological presentation of the mucosa. It has been shown in malnourished patients (based on clinical examination, plasma albumin concentration and anthropometry) that despite normal villous structure of small intestine, the intestinal permeability was increased as assessed by lactulose:mannitol test [Welsh *et al.* 1998]. Increase in intestinal permeability has also been shown in cystic fibrosis patients by lactulose:rhamnose test [Penny *et al.* 1986] and by cellobiose test [Dalzell *et al.* 1990]. Although, the absorption of FA may not be directly evaluated by these tests, it may give an indication about the integrity of the gastrointestinal mucosa. Both lactulose and cellobiose are disaccharides, which are absorbed across tight junctions between adjacent enterocytes, whereas mannitol is monosaccharide and is absorbed by diffusion across the cells [Dalzell *et al.* 1990]. Increased permeability of disaccharides suggests that large molecules may leak through the mucosa in the gastrointestinal tract of cystic fibrosis patients. This may cause an alteration in the overall permeability of the mucosa or function of the enterocytes by changing the diffusion gradients, which is important for the absorption of FA. Reduced integrity of mucosa could even result in an outward diffusion of nutrients towards lumen, which would prevent further absorption of nutrients. Increased permeability of the intestinal mucosa may also result in increased susceptibility to infections. Infections may further deteriorate the intestinal integrity due to increased cell turnover and therefore increased nutrient requirements. Turnover of cells in the gastrointestinal mucosa is fast (2 to 6 days) and its integrity mainly depends on the production of new cells at a rate equal to that at which cells are lost [Mathers 1998]. Therefore even a short term deficit in the supply of nutrients to the mucosa may affect the mucosal integrity and result in villous atrophy. Nutrition through gastrointestinal tract is important in maintaining mucosal structure and function and lack of nutrients may result in decreased villous height, increased permeability and decreased immunity [Souba & Wilmore 1994]. Probably the single most important nutrient to the mucosa is glutamine (both from luminal and vascular sides) which is consumed by replicating cells and affects the structure and function of the cells. Lack of glutamine may cause villous atrophy as reviewed by Souba and Wilmore (1994). Vitamin A is also important for differentiation of cells and may have an important role for the mucosal function. Therefore, it may be possible that a reduced absorptive surface area, an increased

permeability and an inadequate nutrition of the gastrointestinal tract result in kind of a viscous cycle, which prevents the normal function of the gastrointestinal tract and normal absorption of lipids and other nutrients. In this case it may be necessary to provide nutrition to the gastrointestinal tract via the parenteral route, which would make it possible for the gastrointestinal mucosa to regain its integrity and therefore would improve the absorptive capacity of the gastrointestinal tract.

In conclusion, the most likely cause for malabsorption of dietary lipid in cystic fibrosis patients in the present study was a reduced absorptive surface of the small intestine due to various reasons such as altered mucus obstructing the access of FA to the enterocytes, small intestinal resection at infancy or presence of another disease (coeliac disease) affecting villous structure. It is also possible that the integrity of the mucosa is altered in cystic fibrosis due to increased permeability of mucosa or inadequate nutrition of the mucosa, which may affect the absorption of lipids.

8.2.2 What factors could explain altered metabolic disposal of dietary lipid in cystic fibrosis patients?

Although, it has been suggested that cystic fibrosis patients may preferentially use carbohydrates for energy production during postabsorptive state [Bowler *et al.* 1993], very little is known about the metabolism of absorbed lipid, especially during postprandial period in cystic fibrosis. It was shown in the present study that the cumulative ^{13}C-label excretion on breath over 10 hours was lower in the cystic fibrosis patients compared to the control subjects following administration of [1,1,1-^{13}C]tripalmitin labelled meal. The difference between the groups persisted even when the label excretion on breath was corrected for stool losses of the label indicating that the oxidation of the ingested ^{13}C-labelled lipid and therefore oxidation of dietary lipid was reduced in cystic fibrosis patients compared to the control subjects. The possible causes for the reduced oxidation of dietary lipid in the cystic fibrosis patients will be discussed below.

Is lipid oxidation system defective in cystic fibrosis?

Fatty acids derived from diet or from endogenous stores are transported to mitochondria and oxidised firstly by β-oxidation to derive Acetyl-CoA which is then oxidised in citric acid

cycle to generate CO_2 and energy [Murray *et al.* 1996]. Deficiency, altered function or reduced capacity of the enzymes and cofactors associated with the transport and oxidation of FA in β-oxidation and citric acid cycle could cause a reduced lipid oxidation. Both postabsorptive and postprandial net lipid oxidation were similar in the cystic fibrosis patients compared to the control subjects suggesting that the lipid oxidation system as such is not altered in cystic fibrosis. This may have been unexpected, because the basic defect in cystic fibrosis is related to the regulation of the transport of substances across membranes, which could potentially influence FA oxidation. It has been reported that cellular calcium levels may be increased and calcium movement to mitochondria may be altered in cystic fibrosis, which might cause changes within the respiratory system [Feigal *et al.* 1982; Von Ruecker *et al.* 1984]. However, the overall significance of the altered calcium metabolism for lipid oxidation is not understood. In the present study, the reduced oxidation of lipids was observed only during the postprandial period. It is unlikely that a defect in the oxidation system itself would have affected only the postprandial lipid oxidation and therefore other factors than a defect in the oxidative system are more likely to explain the lower oxidation of dietary lipid in cystic fibrosis patients.

Is postprandial lipid partitioning altered in cystic fibrosis patients compared to control subjects?

Both cystic fibrosis patients and control subjects consumed equal meals containing [1,1,1-^{13}C] tripalmitin in a proportion to their body weight (10 mg/kg body weight) at similar study conditions. Why was the ^{13}C-label excretion on breath in the cystic fibrosis patients lower compared to the control subjects? Could it be explained by an altered postprandial partitioning of the ingested lipid? Postprandial partitioning of dietary lipid is a very complex area regulated by factors including enzymes and hormones and a detailed discussion will be beyond this thesis, but the main issues that could influence the oxidation of dietary lipid are discussed below. Firstly, the lower excretion of ^{13}C-label on breath in cystic fibrosis patients could be due to a delayed gastric emptying and a small intestinal transit, which would delay the delivery of the dietary lipid to the enterocytes and therefore transport to the oxidative site. Although, similar gastric emptying has been found in cystic fibrosis patients compared to control subjects [Roulet *et al.* 1980; Smith *et al.* 1990], cystic fibrosis patients may have a prolonged small intestinal transit [Bali *et al.* 1983; Taylor *et al.*

1999]. However, in the present study the label recovery on breath was corrected for the stool losses of the label and therefore, expressing the label excretion on breath as a proportion of the absorbed dose excludes the effects of the gastric emptying and gastrointestinal transit on the label excretion on breath. Could the absorbed lipid be retained in the enterocytes? This is unlikely, because the absorption of FA and MAG in the enterocytes is regulated by inward diffusion gradient whereby absorbed FA and MAG are bound by fatty acid binding protein, re-esterified to TAG and packaged to chylomicrons for absorption to continue [Ocker *et al.* 1972; Shiau 1981]. Therefore, the capacity of the enterocytes to store the lipid digestion products is relatively small and the lower excretion of the ^{13}C-label on breath in the cystic fibrosis patients is not likely to be caused by retention of lipids in the enterocytes.

Chylomicrons transport dietary lipid from enterocytes to extrahepatic tissues where LPL catalyses the hydrolysis of TAG releasing FA, which are mainly taken up by the tissues [Griffiths *et al.* 1994; Frayn *et al.* 1995]. The enzymes LPL and HSL have a key role in the regulation of the uptake of FA by the tissues and the release of FA from the stored TAG [Frayn *et al.* 1995]. The postprandial chylomicron clearance and the functional capacity of the LPL have not been investigated in cystic fibrosis patients, but it is possible to speculate that if their function was altered in cystic fibrosis that could contribute to the lower excretion of ^{13}C-label on breath. A reduced activity of LPL could result in a delayed uptake of FA from chylomicrons and therefore in a delayed chylomicron clearance from the circulation. Consequently due to a delayed uptake of FA from the diet, the endogenous FA would be the principal source of FA oxidation explaining the lower excretion of ^{13}C-label on breath. The activity of LPL is mainly stimulated by insulin, which is secreted as a response to a rising glucose concentration in the circulation, following consumption of a mixed meal [Coppack *et al.* 1992]. Cystic fibrosis patients may have both reduced insulin secretion due to pancreatic insufficiency and insulin resistance [Dodge & Morrison 1992]. Therefore, a low insulin concentration during the postprandial period would down-regulate LPL activity causing a delayed chylomicron clearance, whilst insulin resistance would lead to hyperinsulinemia and therefore may increase LPL activity and uptake of FA by the tissues. Insulin stimulates the LPL activity especially in the adipose tissue but may stimulate less or even inhibit the LPL activity in the muscle [Coppack *et al.* 1992]. Insulin also stimulates the re-esterification of FA to form TAG in the adipose tissue and therefore it is likely that hyperinsulinaemia caused by insulin resistance could result in increased partitioning of FA

towards storage rather than towards oxidation in the cystic fibrosis patients. In previous studies, Hardin *et al.* (1999) and Ward *et al.* (1999) found no differences in the postabsorptive lipid or carbohydrate oxidation rates amongst patients with cystic fibrosis, cystic fibrosis related diabetes mellitus and control subjects. The prevalence of cystic fibrosis related diabetes mellitus is relatively high, about 25% in younger patients and increases to about 75% in older patients aged 30 [Lanng *et al.* 1995]. Altered insulin metabolism and glucose intolerance have known effects on lipid metabolism and could influence nutritional status also in cystic fibrosis patients. Therefore further research is required to compare lipid metabolism in cystic fibrosis patients with and without diabetes mellitus.

In the present study, the label recovery on breath in both the cystic fibrosis patients and the control subjects returned close to the baseline abundance by the end of the breath sample collection period. If the consumed labelled lipid was delayed in the circulation the expected outcome would have been a continuing label recovery on breath in the cystic fibrosis patients, whilst the label recovery of the label on breath in the control subjects started to return to the baseline. Therefore, it is unlikely that the lower ^{13}C-label excretion on breath could be explained by altered postprandial partitioning of lipids in cystic fibrosis patients studied. However, whether these mechanisms could explain the lower excretion of the ^{13}C-label on breath in cystic fibrosis patients remains unanswered until studies specifically designed to investigate the postprandial lipid metabolism including chylomicron clearance, LPL activity and insulin concentration are carried out.

Is altered metabolic disposal of lipids in cystic fibrosis patients associated with energy metabolism and nutritional status of the patients?

The factors that regulate the extent to which different dietary macronutrients are utilised for energy metabolism are poorly understood even in healthy individuals. Is lipid oxidation simply determined by energy requirements or does previous diet or current dietary macronutrient composition influence lipid oxidation? It has been suggested that due to their limited storage capacity, carbohydrates and proteins are preferentially oxidised after consumption of a mixed meal [Flatt *et al.* 1985; Abbott *et al.* 1988] and the energy requirements are met by the lipid oxidation and any excess is stored. Studies combining stable isotope techniques and traditional indirect calorimetry measurements have shown that

following consumption of a mixed meal, the endogenous lipid oxidation is suppressed, whilst the lipid from the diet is being oxidised [Jones *et al.* 1998; Bennoson *et al.* 1998]. In the present study, an increase in the ^{13}C-label excretion on breath was observed in the control subjects, but cystic fibrosis patients excreted very little label on breath following consumption of the ^{13}C-labelled meal. Although subjects in both groups consumed the same ^{13}C-labelled meal, the cystic fibrosis patients had higher lipid losses in stools. Despite correcting the ^{13}C-label excretion on breath for stool losses of the label, it is possible that the overall meal size was smaller in cystic fibrosis patients. Consequently, it may be that due to a lower lipid load, the cystic fibrosis patients oxidised endogenous lipid to satisfy the metabolic demand for energy, whilst the control subjects oxidised dietary lipid resulting in a lower excretion of ^{13}C-label on breath during the postprandial period in cystic fibrosis patients. However, no association was found between the extent of stool lipid or stool label excretion and oxidation of lipids in cystic fibrosis patients suggesting that oxidation of dietary lipid was reduced in all patients despite the extent of stool lipid losses.

Previous dietary intake may influence the subsequent substrate oxidation. A higher carbohydrate intake prior to the study may result in a relative increase in the subsequent carbohydrate oxidation [Bowler *et al.* 1993]. Although it is debatable whether higher lipid intake results in increased lipid oxidation [Flatt *et al.* 1985; Schutz *et al.* 1989; Griffiths *et al.* 1994; Gottrand *et al.* 1999]. Some evidence is available from a study using stable isotope techniques that an increase in lipid content of the meal increases oxidation of lipid derived from the diet [Bennoson *et al.* 1998]. In the present study the median intakes of lipid, carbohydrates and protein (g/d) from diet and supplementary feed were about 1.5 times higher in the cystic fibrosis patients compared to the control subjects (diet only). However, there was no difference in the proportional intake of macronutrients of the energy (% of energy) between the groups. Was the lower excretion of ^{13}C-label on breath in cystic fibrosis patients due to the higher habitual intake of carbohydrates compared to the control subjects? Why did the higher lipid intake not result in a higher lipid oxidation? Or does the habitual energy intake regulate the extent to which lipid is oxidised? The latter is the most likely option, because the habitual median intake of energy from diet and supplementary feed (kJ/day) was about 1.4 times higher in the cystic fibrosis patients compared to the control subjects (diet only). Also the energy intake per kg body weight was higher in the cystic fibrosis patients compared to the control subjects. The energy intake from the diet and supplementary feed also exceeded the estimated energy requirements (stool losses taken

into account) in 9 of the 12 patients. Therefore it may be possible that due to the additional energy derived from the supplementary feeding, these patients were overfed in comparison to their energy requirements. Due to a limited capacity to oxidise excessive energy intake, the overfeeding may result in the partitioning of dietary lipid towards storage rather that towards oxidation during immediate postprandial period.

Deficiency or marginal reserve of iron, manganese, magnesium, nicotinic acid and riboflavin, which are vitamins and minerals involved in the β-oxidation and citric acid cycle as co-factors [Murray *et al.* 1996], could potentially result in a reduced FA oxidation. Although vitamin and mineral status was not determined in the cystic fibrosis patients in the present study, the effects of nutrient deficiencies on lipid oxidation cannot be excluded. Cystic fibrosis patients may have deficiencies of lipid soluble vitamins A, D, E and K, possibly water-soluble vitamin B_{12} and zinc and iron as reviewed by Dodge (1985) and Peters and Rolles (1993). However, any alteration in the oxidation of fatty acids due to a limiting nutrient would equally affect both FA oxidation and carbohydrate oxidation, because both are converted to acetyl-CoA for oxidation in the citric acid cycle. However, no difference was found in net lipid or net carbohydrate oxidation rates between the cystic fibrosis patients and control subjects. Furthermore, cystic fibrosis patients receive daily supplements of vitamins A, E and D, which reduces the risk of deficiencies of these vitamins.

Studies in malnourished children (kwashiorkor) suggest that lipid oxidation may be reduced in malnutrition [Iputo *et al.* 1998] and subsequent re-feeding may result in an adaptation and therefore in an increased efficiency of energy utilisation [Kennedy *et al.* 1990]. All the patients in the present study were receiving supplementary feeding via a gastrostomy because patients had previously had or currently had disturbed growth or difficulties in weight maintenance. Therefore, the patients might have metabolically adapted to the previous inadequate energy availability. The metabolic effects of the malnourishment and re-feeding on substrate oxidation are not well known and have not been investigated in cystic fibrosis. However, the lower lipid oxidation during malnourishment may simply be caused by the specific fuel demands of vital tissues for survival, such as brains, which can only utilise glucose or ketone bodies. Therefore, lipids may not be oxidised as FA, but are formed to glucose or ketone bodies, which are then oxidised by these tissues. It is also likely that the overall energy requirements are reduced during malnourishment resulting in a reduction in the energy production and therefore also reduction in the oxidation of lipids.

The cystic fibrosis patients in the present study were eating and furthermore received supplementary feeding via a gastrostomy and therefore these patients are not comparable to chronically malnourished patients. There was no evidence of a slowed down energy metabolism (BMR) in these patients either (Chapter 5) which could have explained the reduced oxidation of dietary lipid. However, it has been shown in recovering malnourished infants [MacLean & Graham 1980] and men [Barac-Nieto *et al.* 1979] that an increase in body weight during feeding is largely contributed to an increase in fat mass. A tendency towards lower LBM mass was also seen in cystic fibrosis patients in the present study. Storage of energy as fat mass may be due to adaptation to the previous inadequate energy supply and may therefore be of benefit, since it may serve as an energy reserve for the possible re-occurrence of an inadequate food supply. However, it is more likely that a disproportional increase in the fat mass is due to overfeeding or fast feeding rate during rehabilitation. Due to a limited capacity to oxidise nutrients, the excessive intake of energy is stored. However, it remains unanswered whether patients recovering from malnourishment including cystic fibrosis patients are simply overfed and therefore store the excessive energy as fat mass or whether these patients actually have a failure in metabolism of dietary lipid.

In the present study there was a tendency for the cystic fibrosis patients to have less LBM than the control subjects. Since the energy requirements (BMR) are mainly determined by LBM [Cunningham 1980] one possibility that needs to be excluded is that the lower lipid oxidation in the cystic fibrosis patients was related to the lower LBM. However, the label excretion on breath was not associated with the LBM or BMR in either group studied. As discussed previously (See Chapter 5), there was no difference in the BMR between the cystic fibrosis patients and the control subjects and no difference was found in the net lipid oxidation per kg LBM between the two groups. Therefore, the body composition or BMR do not seem to explain the lower label recovery on breath in the cystic fibrosis patients.

In conclusion, although the effects of limiting nutrient, metabolic adaptation and body composition on substrate oxidation cannot be definitely excluded, the most likely explanation for the reduced oxidation of dietary lipid in cystic fibrosis patients was the energy intake in excess to the requirements. The metabolic capacity to oxidise macronutrients is limited and therefore the excessive habitual energy intake resulted in the metabolic partitioning of dietary lipid towards storage rather than towards oxidation

explaining the lower excretion of ^{13}C-label on breath in cystic fibrosis patients compared to control subjects. However, further studies are required to confirm whether reduced oxidation of dietary lipid occurs in all cystic fibrosis patients or whether it is a specific feature related to the cystic fibrosis patients receiving supplementary feeding.

8.3 Implications

Maldigestion and malabsorption of dietary lipid

Increased stool lipid losses due to maldigestion and malabsorption have several implications for the well-being and clinical management of cystic fibrosis patients. As already discussed, the increased lipid losses in stools reduce the availability of energy from the diet and therefore may cause energy deficit, which in turn may cause disturbed growth in children or weight loss in adults. Cystic fibrosis patients may have very variable lipid losses in stools. Therefore, patients with minor lipid losses in stools (total stool lipid less than 15 g/d) may be able to compensate the energy losses in stools by increasing the energy intake from the diet by increasing the frequency of meals (snacks). However, some patients may have very high lipid losses in stools (up to 55 g/d) and it is impossible to compensate for the losses of this magnitude by increasing dietary intake. Even increasing the energy intake by supplementary feeding via a gastrostomy or a nasogastric tube may be unsuccessful to increase weight gain and growth if stool lipid losses remain elevated. Therefore, the only possibility to increase energy availability via the gastrointestinal tract is to reduce lipid and energy losses in stools.

It is recognised that increased lipid losses in stools cause uncomfortable gastrointestinal symptoms such as abdominal pain and flatulence. Would it be possible to use the extent or severity of the gastrointestinal symptoms to assess the extent of lipid losses in stools? Bowel habit questionnaire or diary, such as used in this thesis (Appendix 3) could be used to characterise the frequency and severity of the gastrointestinal symptoms and frequency of bowel habit in cystic fibrosis patients. If the symptomology was related to the measured stool and stool lipid losses, bowel habit diary or questionnaire could be used in the clinical practice as a simple and non-invasive tool to characterise those patients who have problems with the gastrointestinal function. The cystic fibrosis patients studied generally reported no gastrointestinal symptoms or mild symptoms during the study period

(See 5.5). Only a small proportion of the patients reported moderate or severe symptoms. However, in terms of symptoms, the study group was selected, because those patients who did not feel well due to various reasons including gastrointestinal symptoms declined to participate to the study. Therefore, the information concerning the gastrointestinal symptoms will be limited to patients who were according to their own estimation relatively well at the time of the study. Did those patients who reported severe symptoms or high frequency of stools passed per day (2-5 times/d) also have increased lipid losses in stools? It was shown in this thesis that the wet stool weight was associated with lipid losses in stools (Figure 6.1), therefore if it would be possible to reliably estimate stool losses, without collecting the stools, the lipid losses in stools could be estimated (regression equation). No systematic associations were observed when the frequency and severity of the symptoms reported by the patients were compared to the measured stool and stool lipid losses. However, four patients who constantly reported high frequency of stools per day and loose stools also had increased total lipid losses in stools (12 to 55 g/d). Two of these patients had both maldigestion and malabsorption of lipid (Figure 6.7; patient no 1 and 7) and two patients had malabsorption of lipid (Figure 6.7; patients no 4 and 8). The two patients with malabsorption also reported more frequent abdominal pain and had higher stool losses (wet weight) per day than the other patients. It may be that those patients with severe malabsorption of lipid generally have increased frequency and amount of stools passed per day and more severe symptoms compared to patients with maldigestion or less severe malabsorption of lipids. However, two other patients who also had malabsorption (Figure 6.7; patient no 9 and 10) of lipids reported no symptoms or infrequent symptoms and normal appearance of stools. All the cystic fibrosis patients were diagnosed as infants and it is likely that most patients have had gastrointestinal symptoms and abnormal stools for all their lives. Therefore, it may be possible that cystic fibrosis patients have got accustomed to their symptoms, which could be the main reason for the lack of association between reported symptoms by the patients and measured lipid losses in stools. These patients may consider for example frequent abdominal pain as 'normal' and therefore did not report having abdominal pain or underestimated the intensity of the symptoms. Assessment of the gastrointestinal function by bowel habit diary or questionnaire would be fairly easily carried through at the clinical practice. Patients may consider it unpleasant to discuss about their bowel habit and a questionnaire would give privacy to the patient. However, the diary or questionnaire does not seem to give accurate information about the gastrointestinal

symptoms, which would have been related to the measured lipid losses in stools. Nevertheless, questionnaire or diary may be useful to identify those patients who have marked gastrointestinal dysfunction, especially due to malabsorption of lipids and facilitate creating a link between excessive stool losses and weight loss or disturbed growth. The most feasible factors characterising the gastrointestinal dysfunction seem to be frequency of stools passed, an estimate of size of the stools and possibly appearance of stools.

In addition to direct effects on energy availability and gastrointestinal symptoms, excessive lipid losses in stools may reduce the availability of essential fatty acids and lipid soluble vitamins from diet and increase losses of minerals such as calcium in stools. Essential fatty acids that are derived only from diet are required especially for growth and function of membranes [Gurr & Harwood 1991]. Therefore, in addition to more systemic effects, essential fatty acid deficiency could also affect the function of the gastrointestinal mucosa (alterations in permeability) further deteriorating the gastrointestinal function. According to Durie and Pencharz (1992) the essential fatty acid deficiency in cystic fibrosis patients is rare, but low plasma essential fatty acid concentration may be found especially in patients with pancreatic insufficiency. However, also similar plasma fatty acid concentrations in cystic fibrosis patients compared to control subjects have been found [Burdge *et al.* 1994]. The risk of essential fatty acid deficiency may be related to the extent of lipid losses in stools and it is possible that before symptoms for clinical diagnosis of essential fatty acid deficiency occur, some alterations in the gastrointestinal mucosa or other membranes may occur affecting the membrane fluidity. It has been reported that cystic fibrosis patients may have excessive losses of vitamin A in stools [Ahmed *et al.* 1990], which may be related to increased lipid losses in stools or may be due to a specific defect in vitamin A absorption unrelated to lipid absorption. Also reduced blood vitamin E concentrations, which have been found to associate with lipid losses in stools have been documented in cystic fibrosis [Congden *et al.* 1981]. Although lipid soluble vitamin deficiencies may be avoided by regular vitamin supplementation and monitoring of vitamin status, increased lipid losses in stools may contribute to increased losses of lipid soluble vitamins. Lipid soluble vitamins are passed to epithelial cells in mixed micelles and therefore the reduced digestion and absorption of lipids caused by various reasons such as precipitation of bile salts (due to acidic pH) may also reduce the digestion of lipid soluble vitamins (e.g. retinol ester and tocopherol ester) and the micelle formation resulting in loss of the vitamins in stools. Although, maldigestion and malabsorption of lipids is generally not

thought to influence absorption of minerals and trace elements, excessive FA losses in stools may contribute to their loss. Free fatty acids in the gastrointestinal tract may form FA salts with minerals such as calcium, magnesium and zinc, which therefore would be lost in stools. Although bone metabolism and its regulation is a very complicated system, excessive losses of especially calcium and vitamin D could influence the bone formation and accumulation of adult bone mass. Zinc has various functions in body and is also required for growth. It has been shown that zinc supplementation in patients with disturbed growth results in induced growth [Halsted & Smith 1970; Golden & Golden 1981]. Low plasma zinc levels [Halsted & Smith 1970] and zinc deficiency, which were related to disturbed growth [Dodge & Yassa 1978] have been also reported in cystic fibrosis.

Malabsorption of dietary lipid has not generally been acknowledged as a cause for excessive lipid losses in stools in cystic fibrosis. In controlling excessive stool lipid losses patients follow PERT. However, the dose of enzyme preparations taken might rise above the recommended because self-regulation of the dose to inappropriately address lipid losses in stools caused by malabsorption. No advantages will be achieved by increasing the dose of enzyme preparations in patients who mainly have lipid malabsorption. Furthermore, excessive enzyme doses might have harmful effects on the gastrointestinal tract since high-dose, high-strength lipase usage has been associated with the occurrence of fibrosing colonopathy [Smyth *et al.* 1994; Mac Sweeney *et al.* 1995]. Malabsorption of lipids in cystic fibrosis patients is of concern because the current ways of management of the malabsorption are very limited. In the present study, the patient with extremely high both total lipid (55 g/d) and total label losses (78% of the administered dose) in stools was found to malabsorb dietary lipid. The growth of this patient was disturbed and puberty delayed and he was frequently admitted to hospital to improve his nutritional status. The possible mechanisms for malabsorption of lipids were discussed earlier and it was evident that for example satisfactory nutrition of the gastrointestinal tract is important for the integrity of mucosa and sufficient absorptive surface area is required for efficient absorption to occur. At least intermittent total parenteral nutrition may be required to induce growth in patients with excessive malabsorption of lipids.

Altered oxidation of dietary lipid

Excretion of ^{13}C-label on breath following administration of a ^{13}C-labelled meal was lower in cystic fibrosis patients compared to control subjects suggesting a reduced oxidation of dietary lipid in cystic fibrosis. This finding is likely to be related to the specific characteristics of the patients studied, because a lower oxidation has not been observed previously in cystic fibrosis patients following administration of [1,1,1-^{13}C] tripalmitin [Murphy *et al.* 1998]. The lower oxidation of dietary lipid during the postprandial period may be due to various factors as was discussed earlier in this chapter, but was most likely caused by overfeeding of the patients (gastrostomy feeding). The metabolic capacity to oxidise dietary lipid may be limited and therefore the energy intake in excess to the requirements may have led to the metabolic partitioning of the dietary lipid towards non-oxidative disposal (storage) rather than towards oxidation. Energy intake in excess to the requirements is likely to cause a disproportional gain of fat mass and LBM. The patients studied here also tended to have a lower LBM compared to the control subjects. Therefore, frequent follow-up when feeding patients via a gastrostomy or nasogastric tube is required to optimise the energy intake to meet the requirements including the requirements for growth. Further research is needed to determine the optimal nutrient composition of diet for growth of stature and LBM rather than fat mass.

The reduced oxidation of dietary lipid in cystic fibrosis patients may also have implications for the use and interpretation of breath tests in diagnosis of gastrointestinal handling of dietary lipid. Only by correcting the label excretion on breath for the stool losses of the label it was possible to detect the lower label excretion on breath that was independent of the digestion and absorption of dietary lipid in the present study. Furthermore, no association was found between the label excretion in stools and on breath suggesting that the label excretion on breath does not reflect the gastrointestinal handling of the administered substrate alone. The assumption of the lipid breath tests is that only one step in the metabolism of the tracer is rate-limiting. Therefore, to detect maldigestion of lipids a ^{13}C-labelled TAG is orally consumed by patients and the cumulative label excretion on breath is measured which is believed to reflect digestion of the administered substrate in the gastrointestinal tract [Schoeller *et al.* 1977; Swart & Van Den Berg 1998; Weaver *et al.* 1998]. The most commonly used substrate has recently been mixed TAG (1,3 distearoyl, 2[^{13}C]octanoyl glycerol). This breath test is based on the assumption that the rate-limiting

step is hydrolysis of two stearic acids by pancreatic lipase and the resulting ^{13}C-labelled MAG (^{13}C octanoic acid in *sn-2* position) or ^{13}C-labelled octanoic acid, is uniformly absorbed and oxidised. It is likely that the oxidation of octanoic acid (medium chain FA) differs from that of tripalmitin, which was investigated in the present study. However, prior to using lipid breath tests in a clinical setting to assess the digestion and/or absorption of dietary lipid in cystic fibrosis patients, it is important to confirm that the label excretion on breath following administration of ^{13}C-labelled lipids is determined only by the gastrointestinal handling of the lipid. For interpretation of the results of the breath tests, it should be considered that cystic fibrosis patients may have lipid malabsorption in addition to lipid maldigestion and furthermore a low label recovery on breath may be due to a reduced oxidation of the administered lipid. Therefore, it is possible that the studies that previously have evaluated lipid metabolism in cystic fibrosis patients using trioctanoin [Watkins *et al.* 1977; Murphy *et al.* 1990; McClean *et al.* 1993] or mixed TAG [Amarri *et al.* 1997; Kalivianakis *et al.* 1999] have firstly failed to differentiate between maldigestion and malabsorption of dietary lipid and secondly have failed to determine whether the lower label recovery on breath in cystic fibrosis patients could be caused by a lower oxidation of the administered lipid. However, it has been shown that the label recovery on breath in cystic fibrosis patients increases when the ^{13}C-labelled meal is administered with pancreatic enzyme preparations compared to the administration of the meal without the enzyme preparations [Amarri *et al.* 1997; Murphy *et al.* 1990; McClean *et al.* 1993] suggesting that the label recovery on breath is at least partially determined by the gastrointestinal handling of the administered substrate. Providing that the extent to which the assumptions of breath tests are appropriate will be confirmed by direct analysis of stool losses of the label, the breath tests may prove a useful, non-invasive tool to be used in clinical practise to assess the gastrointestinal handling of dietary lipid. The common practice is to regulate the study conditions for breath tests such as limitation of physical activity and an overnight fasting before the test. However, in order to confirm that the metabolic state of the patient does not affect the test results, it may be necessary also to regulate the previous dietary intake in terms of absolute and/or proportional energy and macronutrient intake as it may have been that the lower oxidation of tripalmitin in the present study was due to previous excessive energy intake compared to energy requirements.

Proposed model for nutritional management of cystic fibrosis patients

Some cystic fibrosis patients have disturbed growth despite current treatment practices, which however may be inadequate due to a lack of understanding of the gastrointestinal handling of nutrients and metabolism of the absorbed nutrients. Furthermore, the disease characteristics of cystic fibrosis are expressed in several ways such as pancreatic exocrine insufficiency, altered bile salt metabolism and altered properties of gastrointestinal mucosa. The gastrointestinal handling and metabolic disposal of dietary lipid in cystic fibrosis patients receiving supplementary feeding via a gastrostomy has specifically been addressed in this thesis. A model for the management of those cystic fibrosis patients who present weight loss or disturbed growth is proposed and is depicted in Figure 8.2.

The model proposes that patients who present weight loss or disturbed growth should be assessed for gastrointestinal dysfunction, mainly for stool lipid losses. Clinical assessment may be performed according to the practice normally adopted by the cystic fibrosis clinic. The clinical assessment may involve actual measurement of total lipid losses in stools or be based on gastrointestinal symptomology supported by bowel habit questionnaire or diary.

If the clinical assessment does not suggest excessive lipid losses in stools, the weight loss or disturbed growth may be due to inadequate energy intake and therefore prompts estimation of energy and nutrient intakes in reference with requirements. Energy and nutrient intake may be assessed by food diary or food frequency questionnaire and energy requirement according to Ramsey *et al.* (1992). Intervention to increase an otherwise inadequate intake may involve dietary supplements and enteral feeding through nasogastric tube or gastrostomy and possibly a careful increase in fibre intake to reduce gastrointestinal symptoms. A reduced dietary fibre intake in cystic fibrosis has been related to increased symptoms [Gavin *et al.* 1997]. The current practices of dietary management in cystic fibrosis have been addressed elsewhere [Littlewood & MacDonald 1987; Ramsey *et al.* 1992] and will not be further discussed here. Sufficient dietary intake with reference to the requirements including growth should be supported and possibly increased to compensate for excessive energy losses in stools, which might have been overlooked in the clinical assessment. If dietary intervention does not result in weight gain or improved growth, assessment of the gastrointestinal function in more depth is necessitated.

Patients who present with increased lipid excretion in stools receive PERT as part of their clinical management. Therefore the initial evaluation should involve assessment of

adherence to recommended PERT usage and other medication including gastrointestinal acidity regulators. The observed weight loss or disturbed growth may be due to increased stool losses but also due to a reduced intake therefore it is of importance to assess dietary energy and nutrient intake concurrently with the assessment of the gastrointestinal function. The failure to improve the observed weight loss, disturbed growth and excessive lipid losses in stools by optimising PERT proposes further investigation of the gastrointestinal dysfunction. The approach developed in this thesis to differentiate between maldigestion and malabsorption of lipids using ^{13}C-labelled tripalmitin can be of benefit in characterising the underlying causes of excess stool lipid losses. Having characterised whether lipid losses in stools are due to maldigestion, malabsorption or both enables addressing the source of increased stool lipid. In case of maldigestion, it may be possible to further adjust current PERT or change the preparation or dosing schedule to reduce lipid losses in stools. It may be possible to improve digestion of lipids by bile salt or taurine supplementation or by altering the luminal pH in order to improve the solubilisation of ingested lipid. Malabsorption in cystic fibrosis may originate from several causes and methods for treatment are limited, however further investigation for causes of malabsorption should be considered. No advantages will be achieved by increasing enzyme preparations when excessive lipid losses in stools are due to malabsorption. It may be possible to reduce the intake of enzyme preparations and therefore lower the risk of fibrosing colonopathy. In case of mucosal abnormalities or insufficient absorptive surface area due to intestinal resection it may be necessary to provide nutrition via alternative route other than gastrointestinal tract (parenteral nutrition) at least intermittently in order to improve the integrity of mucosa. Although it is not known whether different FA are absorbed to different extent in cystic fibrosis, an alteration of dietary lipid composition by providing unsaturated FA or shorter chain FA may improve absorption. A limiting nutrient may influence the integrity of mucosa and therefore assessing vitamin and mineral status may be of benefit in identifying causes of malabsorption. Lastly it may be necessary to develop novel therapies to correct malabsorption, such as changing the characteristics of altered gastrointestinal mucus.

Re-evaluation and follow-up of the gastrointestinal function, dietary intake and growth are an essential part of the nutritional management of cystic fibrosis patients. The anticipated outcome of the interventions is an increased energy availability, which improves and sustains weight maintenance and growth and together with respiratory infection control, the overall well-being of the patients. The model may be used to facilitate the diagnosis of

causes, and management for weight loss and disturbed growth in cystic fibrosis but further research and validation is required to confirm the feasibility of stool TAG and FA losses in differentiation between lipid maldigestion and malabsorption. The most applicable clinical approach would be to analyse stool TAG and FA concentrations from a single spot stool sample to differentiate between maldigestion and malabsorption, but further validation is required to confirm the sensitivity and specificity of this approach. In absence of facilities for investigation the metabolism of ^{13}C-labelled tripalmitin it is of benefit to recognise malabsorption as a possible cause for steatorrhoea and to consider investigation of causes and management of malabsorption in cystic fibrosis patients.

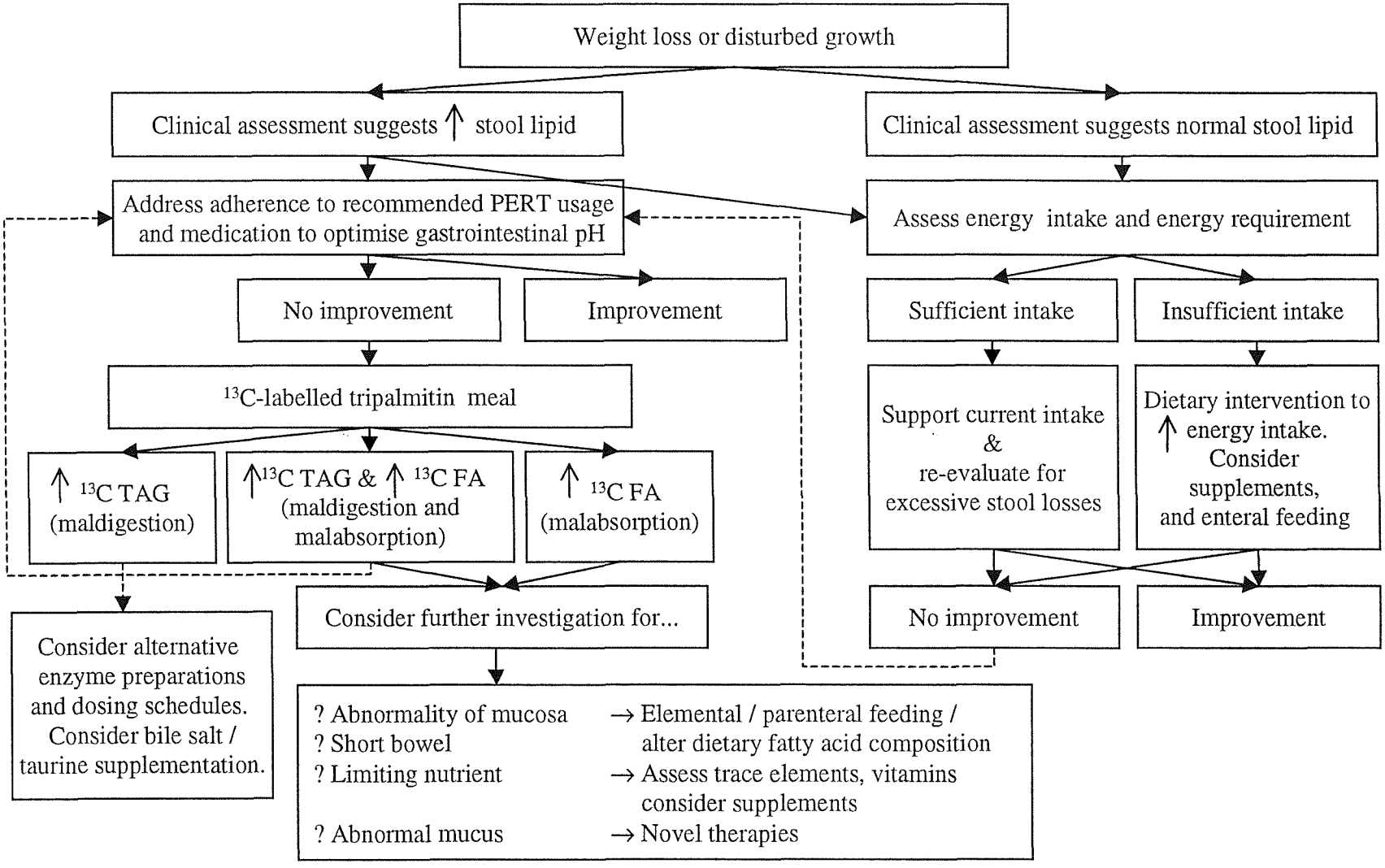


Figure 8.2. A model for assessment and management of cystic fibrosis patients with weight loss or disturbed growth.

8.4 Future studies

Although, the research presented in this thesis has increased the understanding of the digestion, absorption and metabolic disposal of dietary lipid in cystic fibrosis patients, it is clear that further research is required to clinically better manage these patients. It would also be advantageous to expand the experience accumulated from cystic fibrosis to other diseases with gastrointestinal presentation such as Crohn's disease, coeliac disease and chronic pancreatitis. Selected specific studies that would increase the understanding of the mechanisms underlying the altered lipid metabolism in cystic fibrosis patients are suggested below.

Digestion and absorption of dietary lipid

Although it is known what factors may influence the gastrointestinal handling of dietary lipid, it is not known what are the factors that ultimately determine the extent to which dietary lipid is digested and subsequently absorbed. This could be studied by comparing luminal factors, such as enzyme activities, bile salt concentration and pH in aspirates collected from small intestine to the extent to which lipid is digested (TAG content) and absorbed (FA and MAG content) following consumption of a meal with known lipid content. Using a meal containing ^{13}C-labelled TAG would make it possible to directly trace the extent to which the administered ^{13}C TAG has been digested and absorbed by the time meal has reached the terminal ileum. This direct approach would make it possible to (1) confirm whether cystic fibrosis patients have malabsorption of lipid in addition to maldigestion of lipid and (2) what factors principally influence the extent to which lipid is digested and absorbed by cystic fibrosis patients. Furthermore, if stools were collected it would be possible to (3) confirm what are the effects of bacteria on the composition of maldigested and malabsorbed lipid in the colon by comparing the excretion of ^{13}C-label in TAG and FA fractions in stools and terminal ileum. Excretion of lipid in stools could also be used to further evaluate whether (4) stool TAG and FA concentrations could be simply used to define the extent of maldigested and malabsorbed dietary lipid. Completing the study with and without PERT would make it possible to evaluate what (5) factors determine the efficacy of PERT and therefore enable development of new therapies to enhance lipid digestion and absorption in cystic fibrosis patients.

Metabolism of absorbed lipid

It was shown for the first time in this thesis that the metabolic disposal of dietary lipid may be altered in cystic fibrosis patients. However, this observation may have been related to the specific patient group studied (gastrostomy fed cystic fibrosis patients) or to the administered substrate, tripalmitin. Therefore it is important firstly to confirm whether the oxidation of dietary lipid is reduced in all cystic fibrosis patients or only in those patients receiving supplementary feeding. Further studies are also required using a variety of ^{13}C-labelled lipids to investigate whether other fatty acids are oxidised to the same extent as palmitic acid in cystic fibrosis patients. Mixed TAG (1,3 distearoyl, 2[^{13}C]octanoyl glycerol) has been the most common substrate used recently in the breath tests to evaluate the digestive capacity of the gastrointestinal tract. Considering the findings of the present study, prior using the mixed TAG breath test or other breath tests in a wider clinical practice, it is important to determine whether the label excretion on breath is solely determined by the digestion of the labelled substrate or whether absorption or oxidation are also altered. Therefore it is required to compare the excretion of label on breath corrected for stool losses of the label in cystic fibrosis patients and control subjects. The lower oxidation of dietary lipid in cystic fibrosis patients may also be due to altered posptrandial partitioning of the administered labelled lipid resulting in a reduced availability of the fatty acids to the oxidative site. The appearance of chylomicrons to and clearance from the circulation, insulin concentrations and uptake of fatty acids by the tissues would be required to determine what factors influence the metabolic disposal of dietary lipid in cystic fibrosis patients. These studies would require collection of blood specimens following administration of a meal preferably labelled with ^{13}C.

8.5 Conclusions

The digestion, absorption and metabolic disposal of dietary lipid in cystic fibrosis patients receiving supplementary gastrostomy feeding and in control subjects were investigated by combining stable isotope technology with traditional balance techniques and indirect calorimetry measurements.

Stool lipid losses were elevated in cystic fibrosis patients compared to control subjects and varied from similar losses as in the control subjects to up to 78% losses measured by excretion of ^{13}C-label in stools following administration of the labelled meal containing [1,1,1-^{13}C] tripalmitin. Pancreatic enzyme preparations were omitted from the labelled meal to investigate the residual capacity of the gastrointestinal tract to digest and absorb dietary lipid. Malabsorption was indicated in most (11/12) cystic fibrosis patients with excess stool ^{13}C-label losses, in some cases FA accounted for the majority of the label in stools. Most patients appeared to have little evidence of maldigestion even when the pancreatic enzyme preparations were omitted with the labelled meal and only five patients exhibited pronounced label losses as TAG in stool. These results imply that some cystic fibrosis patients may have a considerable residual capacity to digest (lingual and gastric lipase) and absorb the majority of dietary lipid. Furthermore, continuing excessive lipid losses in stools in some patients was largely attributed to malabsorption rather than maldigestion of lipid. These findings highlight the differences between cystic fibrosis patients in the extent to which they can digest and absorb dietary lipid and the need to target the management according to the causes of excessive lipid losses in stools. Comparison of unlabelled and labelled data demonstrated that it is necessary to use labelled lipid to trace the metabolism of dietary lipids in cystic fibrosis patients. Lipid maldigestion and malabsorption may be caused by several factors such as insufficient pancreatic lipase secretion or inactivation due to acidity within the gastrointestinal tract, altered bile salt metabolism or mucosal alterations. Increasing the intake of pancreatic enzyme preparations has no advantage in patients who mainly malabsorb dietary lipid. For the appropriate treatment of excessive lipid losses in stools and for the development of new therapies it is of benefit to determine whether excess stool lipid losses are due to maldigestion or malabsorption. At the moment the possibility of malabsorption as a cause of excessive lipid losses in stools is not sufficiently acknowledged and it is evident that the ways of treating malabsorption are poorly understood. Continuing losses of dietary lipid in stools contribute to the

development of energy deficit and may impair the growth and nutritional status of the patients. The factors that determine the overall capacity of the gastrointestinal tract to digest and absorb dietary lipid are poorly understood and further studies are required to investigate the significance of each individual factor contributing to lipid digestion and absorption in cystic fibrosis. Further studies are also required to confirm the possible effects of the colonic function on the form of stool lipid. The potential use of stool TAG and FA concentrations in determining the extent of lipid maldigestion and malabsorption should be investigated further, as it would be a feasible tool to use in clinical practice.

The postprandial recovery of the ^{13}C-label on breath was lower in cystic fibrosis patients compared to control subjects. The difference between the groups remained even when the label excretion on breath was corrected for the stool losses of the label suggesting that the oxidation of dietary lipid was reduced in the cystic fibrosis group. No difference was found in either postabsorptive or postprandial net lipid oxidation between the groups implying that the metabolic capacity of the cystic fibrosis patients to oxidise dietary lipid was altered not the oxidation process per se. These results suggest that the metabolism of absorbed dietary lipid was altered in the cystic fibrosis patients and was due to a reduced oxidation of dietary lipid. The reasons for the reduced oxidation of dietary lipid are unclear but may be related to the clinical characteristics of the patients receiving supplementary feeding such as body composition, current overfeeding due to supplementary gastrostomy feeding or postprandial events related to the meal consumption. The reduced oxidation of dietary lipid may influence the growth and body composition of the patients contributing to the accumulation of adipose tissue instead of LBM and increase of stature and may also have implications for the use of breath tests in defining the capacity of the gastrointestinal tract to digest and absorb dietary lipid. Further studies are required to investigate whether the oxidation of dietary lipid is reduced in all cystic fibrosis patients or only in those receiving supplementary feeding. The postprandial metabolic partitioning of dietary lipid, including insulin response and chylomicron clearance, has not been investigated in cystic fibrosis and would be of importance in determining the underlying causes of reduced oxidation of dietary lipid.

Appendix 1. Chemicals and laboratory materials

Cambridge Isotope Laboratories Inc., Andover, MA, USA

Carbon dioxide reference standard

Central Laboratory Supplies, Basingstoke, UK

Screw topped tubes (10 ml) with lids

Elemental Microanalysis Ltd. Okehampton, UK

Chromosorb

Tin capsules

Fisher Scientific, Loughborough, UK

Chloroform

Diethyl ether

Ethanol

Glacial acetic acid

Hexane

Hydrochloric acid (HCl)

Methanol

Propanol-2-ol

Sulphuric acid (H_2SO_4)

Toluene

Hewlett Packard, Bracknell, UK

GC minivials

GC minivial inserts

GC minivial lids

International Atomic Energy Agency, Vienna, Austria

Reference sucrose standard

Masstrace, Woburn, USA

[1,1,1-^{13}C] tripalmitin (99 atom % excess)

Merck Ltd., Poole, UK

Filter paper (⌀ 240 mm)

TLC plates, silica gel 60 aluminium sheets

Sigma-Aldrich Company Ltd., Poole, UK

Butylated hydroxytoluene (BHT)

Calcium chloride ($CaCl_2$)

Dipotassium carbonate (K_2CO_3)

Fluorescein natrium salt

Heneicosanoic acid

Monoerucin

Oleic acid

Palmitic acid

Potassium bicarbonate ($KHCO_3$)

Rhodamine B

Stearic acid

Tricosanoic acid methyl ester

Triheptadecanoin

SMA Nutrition, Maidenhead, UK

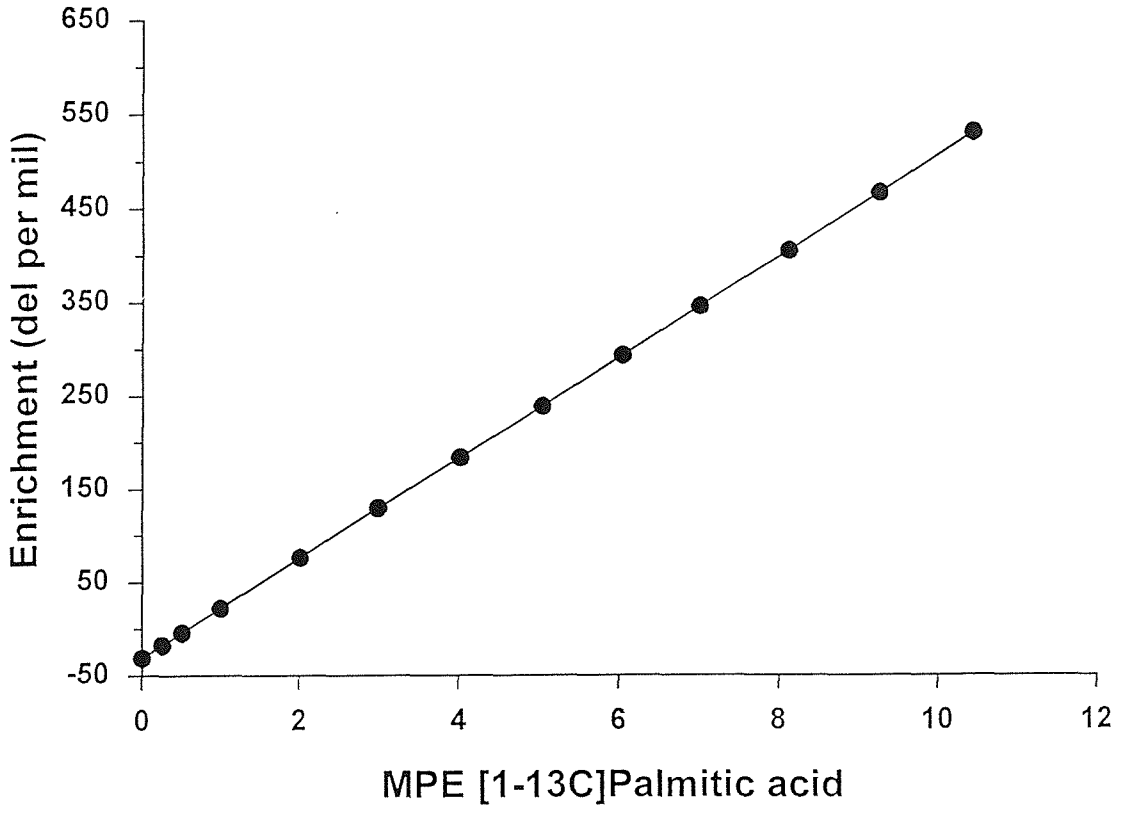
SMA Wysoy: soy protein infant formula

Appendix 2. Standard curve for calculation of mass of ^{13}C enriched palmitic acid in lipid fractions

Standard curve shows an increase in the measured enrichment as labelled palmitic acid was added to the unlabelled palmitic acid from -31.23 δ units for baseline enrichment of unlabelled fatty acid to 530 δ units when 10% of total palmitic acid was present in the labelled form [Stolinski *et al.* 1997]. MPE = moles % excess compared to baseline value.

y = 53.80x - 31.57

r = 0.999



Appendix 3. Bowel habit diary

BOWEL HABIT DIARY

Name:_____

Date of starting the diary:_____

Please mark the occurrence of symptoms and bowel movements in each day by circling your answer. Please look at the *example* below.

Date:	**EXAMPLE DAY**
Upper stomach ache	None Mild Moderate Severe
Lower stomach ache	None Mild Moderate Severe
'Wind' or bloating	None Mild Moderate Severe
Nausea (feeling sick)	None Mild Moderate Severe
Vomiting	None Mild Moderate Severe
Heartburn	None Mild Moderate Severe
Pain when passing stools	None Mild Moderate Severe
Number of stools passed per day	0 1 2 3 4 5 6 7 8
Consistency of stools	Normal Hard Loose Diarrhoea
Colour of stools	Brown Pale Black Red (bloodi n stool)

Date:	DAY 1
Upper stomach ache	None Mild Moderate Severe
Lower stomach ache	None Mild Moderate Severe
'Wind' or bloating	None Mild Moderate Severe
Nausea (feeling sick)	None Mild Moderate Severe
Vomiting	None Mild Moderate Severe
Heartburn	None Mild Moderate Severe
Pain when passing stools	None Mild Moderate Severe
Number of stools passed per day	0 1 2 3 4 5 6 7 8
Consistency of stools	Normal Hard Loose Diarrhoea
Colour of stools	Brown Pale Black Red (blood in stool)

Date:	DAY 2
Upper stomach ache	None Mild Moderate Severe
Lower stomach ache	None Mild Moderate Severe
'Wind' or bloating	None Mild Moderate Severe
Nausea (feeling sick)	None Mild Moderate Severe
Vomiting	None Mild Moderate Severe
Heartburn	None Mild Moderate Severe
Pain when passing stools	None Mild Moderate Severe
Number of stools passed per day	0 1 2 3 4 5 6 7 8
Consistency of stools	Normal Hard Loose Diarrhoea
Colour of stools	Brown Pale Black Red (blood in stool)

Date:	DAY 3
Upper stomach ache	None Mild Moderate Severe
Lower stomach ache	None Mild Moderate Severe
'Wind' or bloating	None Mild Moderate Severe
Nausea (feeling sick)	None Mild Moderate Severe
Vomiting	None Mild Moderate Severe
Heartburn	None Mild Moderate Severe
Pain when passing stools	None Mild Moderate Severe
Number of stools passed per day	0 1 2 3 4 5 6 7 8
Consistency of stools	Normal Hard Loose Diarrhoea
Colour of stools	Brown Pale Black Red (blood in stool)

Date:	DAY 4
Upper stomach ache	None Mild Moderate Severe
Lower stomach ache	None Mild Moderate Severe
'Wind' or bloating	None Mild Moderate Severe
Nausea (feeling sick)	None Mild Moderate Severe
Vomiting	None Mild Moderate Severe
Heartburn	None Mild Moderate Severe
Pain when passing stools	None Mild Moderate Severe
Number of stools passed per day	0 1 2 3 4 5 6 7 8
Consistency of stools	Normal Hard Loose Diarrhoea
Colour of stools	Brown Pale Black Red (blood in stool)

Date:	DAY 5
Upper stomach ache	None Mild Moderate Severe
Lower stomach ache	None Mild Moderate Severe
'Wind' or bloating	None Mild Moderate Severe
Nausea (feeling sick)	None Mild Moderate Severe
Vomiting	None Mild Moderate Severe
Heartburn	None Mild Moderate Severe
Pain when passing stools	None Mild Moderate Severe
Number of stools passed per day	0 1 2 3 4 5 6 7 8
Consistency of stools	Normal Hard Loose Diarrhoea
Colour of stools	Brown Pale Black Red (blood in stool)

Appendix 4. The ^{13}C-labelled meal

^{13}C-labelled emulsion drink

Ingredients:

22 g	Double cream
3.5 g	Olive oil
3 g	Sunflower oil
12 g	Casein (Sanatogen Originals, Glycerophosphated Casein Mix, Fisons, Loughborough, UK)
9 g	Glucose (Glucose, Dextrose powder, Thornton & Ross Ltd, Huddersfield, UK)
4.5 g	Beet sugar
10 g	Flavour (Chocolate/strawberry Nesquick, Nestle, UK)
70 ml	Bottled water
10 mg/kg bwt	[1,1,1-^{13}C] tripalmitin (Carboxyl 1,1,1-^{13}C tripalmitin, 99 APE, Masstrace Inc,Woburn, USA)
~ 100 ml	Water for rinsing

Preparation:

1) Heat the blender in hot water from kettle.
2) Heat water to 90°C in a beaker (on hot plate or in microwave oven). Heat also water for rinsing.
3) Melt tripalmitin, cream and oils in a mug kept in a pan of water at 85°C.
4) Add 90°C water to glucose and sucrose in mixing bowl.
5) Add casein gradually to sugarwater mixing with blender. This should be done with mixing bowl immersed in a bowl of hot water from kettle.
6) Add the mixture from step 5) to melted fats (85°C) and mix with blender for 5 minutes. This should be done with mug immersed in a bowl of hot water from kettle.
7) Add flavour.
8) Rinse blender with 50 ml hot water.
9) Give test drink for subject.
10) Rinse mug with 50 ml warm water. (Both the flavoured test drink and rinsings from the blender and mug should be consumed by the subject.)
11) Give breakfast for subject.

Breakfast

45 g	White bread, thick cut
10 g	Butter
100 g	Sugar free orange flavoured squash

Nutrient composition:

	Emulsion drink	Breakfast	Total
Energy:	1210 kJ	754.0 kJ	1960 kJ
Fat:	19.7 g	9.0 g	28.7 g (54 % energy)
CHO:	21.6 g	22.2 g	43.8 g (37 % energy)
Protein:	11.4 g	3.8 g	15.2 g (13 % energy)

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