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Evaluation of ^{13}C Mixed Triglyceride Breath Testing and Fecal Elastase 1 assays for the assessment of pancreatic function in babies with cystic fibrosis

Including an assessment of healthy babies to establish the range of normal for the ^{13}C Breath test

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PUBLICATIONS

This thesis aimed to evaluate the clinical utility of both the noninvasive, nonradioactive ^{13}C-mixed triglyceride (MTG) breath test with NDIRS technique and fecal elastase-1 (FE1) measurements in comparison with the 'gold standard' 72-h fecal fat assessment in infants with cystic fibrosis (CF), as well as the evaluation whether reference values for the adequacy of fat digestion and subsequent fat absorption, set in the non-dispersive infrared spectrometry (NDIRS) system software IRIS® proposed for children and adults using ^{13}C MTG breath test, are also applicable for healthy infants during their first two years of life. It resulted in the following publications (chronological order):

- Kent DS, Remer T, Blumenthal C, Gaskin KJ. ^{13}C mixed triglyceride breath testing using infrared spectrometry: comparison of two devices in early infancy. Eur J Clin Nutr. 2016 Aug; 70(8):959-62.
- Kent DS, Remer T, Blumenthal C, Hunt S, Simonds S, Egert S, Gaskin KJ. ^{13}C-Mixed Triglyceride Breath Test and Fecal Elastase as an Indirect Pancreatic Function Test in Cystic Fibrosis Infants. J Pediatric Gastroenterol Nutr. 2018 May; 66(5):811-815.
- Kent DS, Remer T, Blumenthal C, Hunt S, Simonds S, Egert S, Gaskin KJ. Evaluation of the ^{13}C-Mixed Triglyceride Breath Test as an indirect pancreatic function test in healthy babies during the first two years of life.
  Conference presentation at the North American Cystic Fibrosis Conference in Denver October 18th-20th 2018.

SUMMARY

Evaluation of ^{13}C Mixed Triglyceride Breath Testing and Fecal Elastase 1 assays for the assessment of pancreatic function in babies with cystic fibrosis (Including an assessment of healthy babies to establish the range of normal for the ^{13}C Breath test)

Background and aims The 'gold standard' test for the indirect determination of pancreatic function status in infants with cystic fibrosis (CF), the 72-hour fecal fat excretion test, is likely to become obsolete in the near future. Alternative indirect pancreatic function tests with sufficient sensitivity and specificity to determine pancreatic phenotype need further evaluation in infants with CF and healthy controls. The **first aim** of this thesis was the evaluation of the clinical utility of both the non-invasive, non-radioactive ^{13}C-mixed triglyceride (MTG) breath test and fecal elastase-1 (FE1) in comparison with the 72-hour fecal fat assessment in infants with CF. The **second aim** of this thesis was the longitudinal assessment of the ^{13}C MTG breath test to measure pancreatic function in healthy babies less than two years of age.

Methods and patients ^{13}C MTG breath test with non-dispersive infrared spectrometry (NDIRS) and the monoclonal and polyclonal FE1 assessment in stool was compared with the 72-hour fecal fat assessment in 24 infants with CF and ^{13}C MTG breath testing was also performed longitudinally in a group of 53 babies during their first two years of life with at least three consecutive breath tests. Babies were divided into 4 consecutive age groups: first ^{13}C breath test on babies less than 5 months of age, second breath test between 6-9 months of age, third breath test between 9-12 months and fourth breath test between 9-24 months and with a different test meal (of yoghurt).

Results Sensitivity rates between 82-100% for CF patients with pancreatic insufficiency assessed by both the ^{13}C MTG breath test and the FE1 tests proved to be high and promising. However, the ^{13}C MTG breath test (31-38%), as well as both FE1 tests assessed by the monoclonal (46-54%) and the polyclonal (45%) ELISA-kits showed unacceptably low sensitivity rates for the detection of pancreatic sufficient CF patients in the current study. In healthy babies a percentage of between 39% (9-24 months old) to 56% (9-12 months old) of individual results were found lower compared to the minimum reference point in all age groups. Intra-individual variation of the $^{13}CO_2$ response, in which no rate-limiting variation in pancreatic exocrine function was expected, was also assessed and showed that out of the 53 longitudinally assessed babies with three to four consecutive MTG breath tests, only 6 babies (3%) had consistent results above the minimum ^{13}C cut-off point during the three to four breath test assessments with NDIRS technique.

Conclusion The ^{13}C MTG breath test with NDIRS technique, as well as both FE1 tests, are not alternatives to the fecal fat balance test for the evaluation of pancreatic function in CF infants. NDIRS technique proved also to be too insensitive to determine pancreatic function status in healthy babies within the first two years of life and therefore the fecal fat balance test remains the gold standard test in assessing CF patient's pancreatic function in the first two years of life.

ZUSAMMENFASSUNG

Evaluierung des gemischten ^{13}C-Triglycerid (MTG) Atemtests und der Pankreas-Elastase 1 Untersuchung zur Emittlung der Pankreasfunktion an Babys mit Cystischer Fibrose (CF). (Sowie der Untersuchung mittels ^{13}C MTG Atemtest an gesunden Babys zur Erfassung von normalen Kontrollwerten).

Hintergrund und Ziele Es ist wahrscheinlich, dass die Goldstandardmessung für die indirekte Ermittlung der Pankreasfunktion an Babys mit CF, der 72-stündige Fettausscheidungstest im Stuhl, in nächster Zeit nicht mehr durchgeführt werden wird. Alternative indirekte Pankreasfunktionstests mit zureichender Sensitivität und Spezifität zur Ermittlung des pankreatischen Phänotypes benötigen weitere Evaluierungen bei Neugeborenen mit CF und bei gesunden Babys. Das **erste Ziel** ist daher die Evaluierung des ^{13}C MTG Atemtests und der Pankreas-Elastase 1 Untersuchung im Vergleich zum 72-stündigen Fettausscheidungstests im Stuhl von Babys mit CF. Das **zweite Ziel** ist die Langzeituntersuchung von gesunden Babies in deren ersten zwei Lebensjahren mit dem ^{13}C MTG Atemtest zur Erfassung von Kontrollwerten.

Methoden und Probanden Der ^{13}C MTG Atemtest mittels nicht streuender Infrarotspectroskopie (NDIRS) Technik sowie der monoclonale und polyclonale Pankreas-Elastase 1 Test werden mit dem 72-stündigen Fettausscheidungstest an 24 Neugeborenen mit CF verglichen. Des Weiteren wird der ^{13}C MTG Atemtest longitudinal in einer Gruppe von 53 Babys im Zeitraum derer ersten zwei Lebensjahre mittels mindestens drei aufeinanderfolgender Atemtests erfasst. Die Babys werden nach Alter und Testmalzeit in folgende Gruppen unterteilt: Der erste Atemtest wird an Babies unter fünf Monaten durchgeführt, der zweite Atemtest an Babies zwischen dem sechsten und neunten Monat, der dritte Atemtest an Babies zwischen dem neunten und zwölften Monat und der vierte Atemtest an Babies zwischen dem neunten und dem vierundzwanzigsten Monat, mit der von den anderen drei Gruppen abweichenden Testmahlzeit Joghurt.

Ergebnisse Es konnten hohe Sensitivitätsraten zwischen 82-100% bei Patienten mit CF mit pankreasinsuffizientem Phänotype sowohl mit dem ^{13}C MTG Atemtest als auch mit den beiden Pankreas-Elastase 1 Messungen erzielt werden. In der Gruppe der CF Patienten mit suffizienter Pankreasfunktion konnten mit dem ^{13}C MTG Atemtest nur Sensitivitätsraten von 31-38%, von 46-54% mit dem monoclonalen und 45% mit dem polyclonalen Pankreas-Elastase 1 Test erzielt werden. Individuelle Ergebnisse an gesunden Babies zeigen, dass eine Prozentzahl von 39% (der neun bis 24 Monate alten Babies mit der Testmahlzeit Joghurt) bis zu 56% (in der Gruppe der neun bis zwölf Monate alten Säuglinge) in allen Altersgruppen unter dem minimalen Referenzwert für eine normale Pankreasfunktion lagen. Individuelle Messungen der einzelnen Babys, bei denen man konstante Messwerte vermutet hätte, zeigen dass aus der Gruppe der 53 Babys nur sechs Babys über dem gesamten Messverlauf der drei bis vier Atemtests mittels NDIRS Technik, konstante Atemtestergebnisse über dem minimalen Referenzwert erzielen konnten.

Schlussfolgerung Weder der ^{13}C MTG Atemtest mittels NDIRS Technik, noch die beiden Pankreas-Elastase 1 Untersuchungen stellen Alternativen zum 72-stündigen Stuhlfettausscheidungstest zur Ermittlung der Pankreasfunktion bei CF Säuglingen dar. Es hat sich gezeigt, dass die NDIRS Technik nicht sensibel genug ist, um die Pankreasfunktion gesunder Babys während derer ersten zwei Lebensjahre gegenüber erkrankten zu trennen. In den ersten zwei Lebensjahren bleibt somit die Technik des 72-stündigen Fettausscheidungstests die “Gold-Standard”-Methode zur Ermittlung der Pankreasfunktion von CF Patienten.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

BSSL	Bile salt stimulated lipase
CCK	Cholecystokinin
CF	Cystic Fibrosis
CFA	Coefficient of fat absorption
CFTR	Cystic fibrosis conductance regulator
CI	Confidence Interval
CPDR	^{13}C cumulative percentage dose recovered
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FE1	Fecal elastase 1
G-BA	Federal Joint Committee (Gemeinsamer Bundesausschuss)
IRT	Immunoreactive trypsin
LCT	Long Chain Triglyceride
MCT	Medium Chain Triglyceride
MS	Mass spectrometry
MTG	Mixed triglyceride
NBS	Newborn screening
NBT-PABA	N-benzoyl-L-tyrosyl-p-aminobenzoic acid
NDIRS	Non-dispersive isotope selective infrared spectrometry
NSW	New South Wales (State of Australia)
PABA	P-aminobenzoic acid

PAP	Pancreatitis associated protein
PDB	PeeDee Belemnite
PERT	Pancreatic enzyme replacement therapy
PI	Pancreatic Insufficient
PPV	Positive predictive value
PS	Pancreatic sufficient
PST	Direct Exocrine Pancreatic function testing
SN	Safety net

1 INTRODUCTION

1.1 Overview and History of Cystic Fibrosis

Cystic Fibrosis (CF) is the most common autosomal recessive genetic disease seen in the Caucasian population affecting 1 in 2,500 to 1 in 4,000 live births. The average incidence affecting European countries is reported to be 1:2000. The highest incidence in Europe is found in Ireland with 1:1800, in Germany the incidence is reported to be 1:3300 and the lowest incidence is found in Finland with 1:25000 (12). In other ethnic groups the frequency is much lower including Hispanics (1 in 8000 to 9500), African Americans (1 in 15300) and Asian Americans (1 in 32100) (107). Around 1 in 25 people of European descent is a carrier of a CF mutation.

The first descriptions of CF, as referenced by Fanconi and colleagues (42), were found in the middle Ages. At that time, it was believed that, when an infant tasted salty when kissed it would die rapidly. Those children were considered 'hexed'. In the early 1930s CF was considered to be "celiac syndrome" with the additional feature of bronchiectasis (42). However, in 1938 an autopsy study demonstrated cystic fibrosis of the pancreas leading to its recognition as a disorder different to celiac disease (6). In all CF patients at death extensive lesions of pancreatic exocrine glandular tissue were found commensurate with poor production of exocrine pancreatic secretions, and the occurrence of bronchiectasis. (6). In the 1950s evidence emerged that CF was associated with anomalies of sweat, electrolyte and fluid transport with CF patients having 4-10 times higher sodium and chloride concentration in their sweat compared to controls (33). In the same decade a standardized procedure for the measurement of sweat electrolyte concentrations, known as the Gibson-Cooke method, was established (56). This test remains the gold standard for the diagnosis of CF (45).

Other important findings included the discovery of impaired pancreatic HCO_3^- secretion and the subsequent demonstration that this impairment was related to both an underlying primary defect of HCO_3^- secretion and a destruction of the pancreas by fibrosis (48, 61). Poor anion secretion was further associated with impaired pancreatic fluid secretion and ductal protein aggregation in CF patients (52, 90). A later study demonstrated on the same group of patients that pancreatic chloride fluid secretion was also impaired (85) and this poor anion secretion is likely causative of impaired pancreatic fluid secretion.

Subsequently in the 1980s Quinton and colleagues (114) discovered a deficit in epithelial chloride transport in the CF sweat duct, and Knowles and colleagues reported further an elevated potential difference across the respiratory epithelium in CF patients compared with controls (82). In combination with the description of the epithelial electrolyte transport defects the

major finding leading to substantial progress in the understanding of CF was the discovery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in 1989. CFTR plays a key role in fluid and electrolyte secretion in the intestine, pancreas, sweat gland secretory coil, bile ducts and the vas deferens, and in sweat gland duct and airway epithelia, it participates in fluid and electrolyte absorption (122). Dysfunction of CFTR chloride-channel disrupts trans-epithelial ion transport and the function of these epithelial ioned organs. This leads to the wide-ranging manifestations of the disorder, which can include severe airway disease (respiratory failure), pancreatic failure, meconium ileus, liver disease, male infertility, and elevated sweat salt levels. Loss of CFTR channel function, being the consequence of CFTR gene mutations, results in a reduced volume of more acidic secretion. It has been suggested (138) that such a situation leads to the precipitation of thick, viscous, highly concentrated protein-containing secretions in the pancreas and it is assumed that similar phenomena occur in the lung, liver and reproductive tract, leading to obstruction and organ damage.

Pulmonary disease remains the leading cause of morbidity and mortality in patients with CF. Lung disease results from clogging of the airways due to mucus build-up, decreased mucociliar clearance and resulting inflammation. Lung function is believed to be normal in newborns with CF (84). Unlike healthy children, however, children with CF develop bacterial infections early in life. Individuals with CF demonstrate characteristic bacterial flora in their sputum, including S *aureus* and P *aeruginosa.* Recent evidence found colonization can occur in CF patients less than 6 month of age up to adult age (21). Initially bacterial infections appear to clear with vigorous antibiotic therapy, but later permanent colonization of the airways is established. A characteristic progressive loss of lung function beginning in the teenage years averaging between 1% and 4% per year may be observed in cystic fibrosis patients (21).

Infection and the often associated persistent neutrophil inflammatory response, induces production of neutrophil and bacterial enzymes: proteases and phospholipases and other inflammatory mediators, which ultimately destroy the airway wall. Furthermore, there is an increase in volume of glands and secretory cells in the epithelium: these secretions contribute to airway obstruction and Bronchiectasis ensues. With time, a varying degree of emphysema develops and the emphysematous bullae at the periphery of the lung predispose to pneumothorax (131). Aggressive antibiotic therapy and lung clearance treatment remains the basis of care for CF lung disease.

The major gastrointestinal abnormality seen in CF patients is exocrine pancreatic insufficiency. Between 80-90% of patients experience insufficient endogenous pancreatic enzyme excretion leading to fat maldigestion and malabsorption and require oral

supplementation of pancreatic enzymes to maintain normal growth. These patients are termed pancreatic insufficient (PI), and if not treated with pancreatic enzymes they are likely to experience malnutrition with linear growth failure (147). The remaining 10-20% of CF patients are pancreatic sufficient (PS) i.e. they retain adequate pancreatic function to permit normal digestion without exogenous enzyme therapy (52). Thus, the assessment of exocrine pancreatic function should be a mandatory procedure at the time of diagnosis, to determine whether the patient should be given pancreatic enzyme replacement therapy (PERT). However, many hospitals do not test the pancreatic function and give PERT to every newly diagnosed patient with CF, which means unnecessary medication in patients with PS.

In this dissertation the use of the ^{13}C breath test as a diagnostic tool to assess fat malabsorption and its ability to accurately determine pancreatic phenotype in infant patients with CF as well as healthy infants will be investigated. Furthermore, ^{13}C breath test results will be compared with the gold standard measurement the 3-day fecal fat balance test. The dissertation will also evaluate the use of fecal elastase 1 (FE1), which although validated in older children has yet to be evaluated in newborn CF patients.

The introduction will further discuss exocrine pancreatic disease, the pathophysiology and the genetics, newborn screening in CF for pancreatic disease and the range of pancreatic function tests.

1.2 Cystic fibrosis transmembrane conductance regulator (CFTR) and description of CFTR dysfunction related to abnormalities

The Cystic fibrosis transmembrane conductance regulator (CFTR) gene was first described in 1989 (117). This gene, which is located on the long arm of chromosome 7 (83), encodes a 1480-amino acid long transmembrane protein (39), that functions as the cyclic AMP-dependent chloride channel (138). The CFTR protein is predicted to consist of five domains: two membrane-spanning domains each composed of six hydrophobic putative transmembrane segments; two nucleotide (ATP-) binding domains; and a unique hydrophilic regulatory domain (117, 148). Any mutation of the protein interferes with chloride transport. In functional terms, the membrane-spanning domains form the CFTR Cl^- channel pore. Mutation of specific residues within the first membrane-spanning domain can alter the anion selectivity of the channel (7). The nucleotide-binding domains regulate the channel gating through binding and hydrolysis of ATP and opening and closing of the CFTR Cl^- channel is tightly controlled by the balance of kinase and phosphatase activity within the cell and by cellular ATP levels (122). The regulatory domain determines channel activity, as phosphorylation of the regulatory domain, usually by cAMP-dependent protein kinase, is required for the channel to open (148). Conversely protein phosphatases dephosphorylate the regulatory domain to return the channel to its quiescent state.

More than 2000 mutations of the CFTR gene have been identified (30). CF patients will be either homozygous for the same mutation of CFTR on both alleles or compound heterozygotes with two different mutations. The most common mutation is ΔF508, a three base-pair deletion in exon 10 resulting in the loss of a single phenylalanine residue at position 508 of the predicted CFTR polypeptide, and it affects 68% of CF chromosomes, i.e. the latter translates into 50% being ΔF508 homozygotes with a single copy of ΔF508 on both alleles, worldwide (80, 132). Across Europe and neighboring areas of Asia, there is a northwest to southeast gradient in relative frequency of ΔF508, with the highest frequency in Denmark 86.7% (120) and the lowest in Turkey 32% (104).

1.3 Classes of CFTR mutations

A classification of CFTR mutations by using the putative mechanisms by which they disrupt CFTR function was first proposed by Tsui (132). Tsui divided the CF mutants into three classes according to their properties. This classification of CFTR mutations has been further refined over an IV class model (148) and to a recent V class model as following (152):

Class I) Reflects defective protein production, associated with missing CFTR messenger ribonucleic acid, involves splice site abnormalities, frameshifts, due to insertions or deletions, or nonsense mutations. Such proteins are often unstable, would be expected to be degraded relatively rapidly or have little or no function.

Class II) Represents defective protein processing of the CFTR gene, associated with failure to traffic to the correct cellular location. The majority of CF alleles, including the most common mutation ΔF508, are in this category. Following its production, the partially glycosylated mutant protein is degraded, and cannot be detected at the cell surface.

Class III) Involves defective regulation of the CFTR protein. Although CFTR inserts correctly into the apical membrane, mutations in the nucleotide-binding domains can be seen. The resulting decrease in net Cl^- channel activity is likely responsible for the detective epithelial Cl^- permeability in patients with this mutation.

Class IV) CFTR inserts normally into the apical membrane but mutants have altered channel properties, resulting in a reduction in the amount of channel function.

Class V) Abnormal splicing or defective processing of CFTR without alteration of the genomic coding sequence, leading to a reduced synthesis of normally functioning CFTR protein.

Some authors have considered a VI class of CFTR mutations with controversial descriptions. Zielenski (154) described class VI CFTR mutations with decreased stability of the CFTR protein. The CFTR protein in class VI, is present at the apical membrane; and it is functional but unstable, associated with a severe CF presentation (154). Vankeerberghen et al. (135) however, described a class VI CFTR mutation which resulted in defective regulation of other channels, and is associated with pancreatic sufficiency.

There is a clear correlation between the loss of chloride transport and the severity of CF phenotype: the most severe loss of function mutations in CFTR are associated with the most severe forms of CF (132). CFTR mutation classes I, II, and III, are associated with substantial declines in CFTR expression or function and are associated with pancreatic insufficiency. Whereas classes IV and V are associated with residual epithelial cell CFTR function and typically associated with a milder pancreatic phenotype, PS (4, 81). In general class IV and V or “mild” mutations are dominant over severe mutations, so a patient with mild/severe genotype has a mild presentation phenotypically, related to less severe CFTR dysfunction.

1.4 Exocrine pancreatic disease in CF

1.4.1 Pathology

Pathologic lesions in the pancreas and the clinical manifestations of pancreatic deficiency first attracted attention and gave the disease its name, 'fibrocystic disease of the pancreas' (6). It has been concluded that despite its name, 'fibrocystic disease of the pancreas' CF is not primarily a disease of the pancreas, but a generalized condition in which this organ is virtually always, involved (34).

Impaired pancreatic HCO_3^- and fluid secretion in CF patients compared to non-CF controls during stimulation of the pancreas with intravenous secretin and cholecystokinin was first described by Hadorn and colleagues (61, 62). However, uncertainty remained whether the impaired HCO_3^- and fluid secretion were related to the underlying primary defect or to destruction of the exocrine pancreas. The study of Gaskin et al. showed that at any level of pancreatic enzyme secretion or acinar function, $HCO3^-$ secretion were significantly lower in CF patients compared to non-CF controls, indicating a primary defect in pancreatic $HCO3^-$ secretion in CF (48). Furthermore, in a subsequent study impaired chloride secretion was also found and likely anion secretion as a whole was impaired and is contributed to by an underlying primary defect in CFTR as well as fibro-fatty degeneration of the gland itself (48).

Low bicarbonate secretion and poor water flow are factors which may produce protein hyper-concentration precipitation, and obstruction in the exocrine pancreatic ducts (86). Digestive pro-enzymes are retained in pancreatic ducts, which, prematurely activated, could lead to tissue destructions and fibrosis (116). Duct obstruction is accompanied by destruction of acinar tissue and acinar atrophy, dense fibrosis, and cyst formation, and eventually extensive fatty replacement of the pancreas (31). The intensity of the obstruction and organ damage determines the progression of the disease. A study (31) with computer tomography and sonography on three CF patients revealed a wide spectrum of severity of clinical findings and morphologic changes in the pancreas. In one patient with pancreatic sufficiency only minimal pathologic lesions were found, while patients with pancreatic insufficiency showed marked fibrosis, fatty replacement, calcification and cyst formation in the pancreas. Complete loss of CFTR function, due to severe class I, II, III mutations on both alleles, appears to induce rapid pancreatic atrophy through obstruction of secreted proteins within the lumina of acini and small ducts (4). Post-mortem studies of the CF pancreas from preterm infants and neonates have demonstrated that this process begins in utero, which can explain the rapid progression of pancreatic failure in early infancy (39, 66). It has been stated that up to the stage where total or

mean total fat replacement of the pancreas occurred, there is no correlation between histologic findings and pancreatic function as judged by pancreatic function tests (31).

1.4.2 Original pancreatic function studies

One manifestation seen in CF patients on presentation, and the most frequently used criterion for the confirmation of exocrine pancreatic insufficiency, is a history of oily bowel movements and steatorrhea (47). Steatorrhea, the passage of loose, pale, bulky, foul-smelling feces, is a result of increased fecal loses of fat and nitrogen in the stool due to maldigestion in CF patients with pancreatic exocrine insufficiency. It occurs when fecal fat excretion is greater than 7% in patients older > 6 months, or 10% for infants < 6 months of ingested fats measured over a 72-hour period (70, 143), or 2g/d in exclusively breastfed infants (143) (full description in Section ‘Fecal fat Balance Test’). Prior to the availability of duodenal fluid analysis technique, Daniel Darrow made a CF diagnosis in the mid 1930’s on the evidence of pancreatic dysfunction as determined by fat and nitrogen balance studies (124).

Early duodenal fluid analysis studies revealed absent pancreatic trypsin, lipase and amylase activity from the duodenal fluid of patients with CF, and the intravenous injection of secretin did not result in an increase in fluid or alteration in enzyme activity (96). At that time the absence or marked diminution of tryptic activity in the duodenal aspirate in childhood was considered pathognomonic of CF, and conversely, the presence of tryptic activity was considered sufficient evidence to discard the CF diagnosis (123).

1.4.3 First studies where normal fat absorption demonstrated

Early pancreatic function studies with duodenal intubation with or without intravenous secretion stimulation measured enzyme concentration in duodenal aspirate. Gibbs et al (55) in 1950 were the first to demonstrate a CF patient with normal stool fat and a normal trypsin concentration in duodenal aspirate and described the patient as having “incomplete pancreatic deficiency”. Subsequently in 1955 di Sant’Agnese (34) reported 9 patients with ‘fibrocystic disease of the pancreas’ in whom the pancreatic function appeared to be ‘normal’ in 3 patients. The pancreatic function was evaluated by duodenal assay, which showed tryptic activity within ‘normal’ limits, and intestinal absorption of fat and nitrogen were found at efficient levels between 97 and 98 per cent respectively. Pancreatic function was evaluated by determination of the proteolytic (‘tryptic’) activity of the duodenal content described in viscosimetric units per milliliter. Of the 6 other patients, pancreatic function was partially present in 3 patients, and in the remaining 3 pancreatic function appealed to be ‘normal’ at first but decreasing to low levels during the assay. It was stated that tryptic activity found in ‘normal’ concentration in duodenal juice does not negate the diagnosis of CF. Findings of duodenal fluids with ‘normal’ or ‘near-normal’ proteolytic activity in CF patients lead others to estimate that 10% to 15% of all CF patients had minimal or no discernable evidence of pancreatic enzyme deficiency (123). However, the reliability of measuring the tryptic activity in duodenal fluid done was questioned. Although not every patient had concomitant fecal fat studies in those who did most with zero activity had fat malabsorption, and those with normal activity normal absorption. Of concern was that some with zero activity had normal fecal fats and conversely others with normal or near normal trypsin activity, fat malabsorption. Still others with intermediate values between zero and normal had various degrees of fat malabsorption or normal absorption.

Possible reasons for these anomalies were the failure to aspirate all duodenal fluid to enable estimation of the total amount of fluid and enzyme secreted and the failure to perform lipase assays, which in retrospect was an oversight considering they were correlating an enzyme with fecal fats. Other problems were the unavailability of cholecystokinin (CCK) and the validity and reproducibility of the assays used. Regarding these issues validated enzyme (both trypsin and lipase) assays had not been developed until 10-15 years later after these original studies. Recognizing some of these difficulties, studies were then performed by stimulation of the pancreas with both secretin and pancreozymin (124). Moreover, the viscosity of duodenal aspirate is high in CF and volumes aspirated were often small and varied considerably between patients rendering much of the data uninterpretable. Hadorn et al in the mid to late 60’s introduced the pancreatic stimulation technique by having a gastric balloon to block the pylorus after the duodenal tube had been inserted and a second balloon at the end of the duodenal tube

to block secretions hence lost further down the intestine (61, 62). Their subsequent intubation studies enabled them to recognize that CF patients had a HCO_3^- secretion deficit but they were unable to prove that thus was related to destruction of the pancreatic tissue, with a primary defect related to the underlying disease or both. Moreover, while there was a much better correlation of enzyme output with fecal fats, they still had some patients with near zero lipase output who had normal fat absorption and some who had lipase output in the same range as those with normal absorption, yet they had fat malabsorption. Of interest they often found a discrepancy between lipase and trypsin with lipase significantly lower than expected given a normal fecal fat and recognizable trypsin output. Further there was no proof that the balloon technique prevented incomplete collections particularly in the presence of the very viscous fluids of the CF patients.

In the subsequent decade two adult gastroenterology units developed a quantitative pancreatic stimulation test using a radioactive marker perfusion technique (57, 89). The investigators were able to prove that the amount of radioactive marker perfused, the amount aspirated was the inverse proportion of the amount lost in the stools thus validated the technique. The Hospital of Sick Children in Toronto had one of the largest CF clinics worldwide with over 500 patients in the late 70's. Based on 3-day fecal fat determinations, over 15% of these CF patients had normal fat absorption. They adapted the marker perfusion pancreatic stimulation test using non-radioactive and non-absorbable markers bromosulfophthalein and later gentamicin (48, 49, 50, 51, 85, 143). In CF patients and controls marker recovery in duodenal aspirates was very similar near 70% and of considerable importance electrolyte and fluid secretion was 2-3 times greater in controls and two to ten times greater in CF patients compared to those reported by Hadorn et al (61). The HCO_3^- secretion studies demonstrated that irrespective of the level of enzyme secretion (from <12 to 100% of enzyme normal values) CF patients had very significantly reduced HCO_3- secretion identifying it was a primary deficit related to the underlying genetic disease (49).

Another important step to improve assay sensitivity was the discovery of Morgan and Borgstrom (103) of the effect of colipase. Pure mammalian pancreatic lipase was completely inhibited by bile acid concentrations above the critical micellar concentrations and colipase relived this inhibition. New very sensitive assays for lipase and colipase were developed after the discovery of the effect of colipase. The assays were simple titrimetric assays and a patient's endogenous colipase secretion was represented by the lipase activity assayed in the presence of bile acids. Of note the true lipase activity (called maximal lipase activity) was only obtained when the assay was saturated with exogenous colipase. Lipase and colipase secretion rates were calculated in normal young adults and expressed as units of lipase and colipase secreted

per hour and per kilogram of body weight for standardization purposes, In CF patients from < 3 months up to 18 years of age secretion rates were calculated and expressed as a % of the average young normal adult values.

1.5 Genotype/Phenotype relations – PI and PS

Following a review (132) which proposed that class I, II or III mutations were associated with PI and classes IV and V with PS in children (beyond early childhood) and adults the concept developed that mutations I-III were “severe” and classes IV and V were “mild”, genotype/phenotype relations were further explored. High sweat chloride concentrations were associated with “severe” mutations and with “mild” mutations sweat chlorides were still abnormal (>60mmoles/L) but in general significantly lower than in the “severe” group (152). In an extensive study PI was associated with “severe” mutations and PS with “mild” mutations confirming the previous proposal (132) and although pancreatic anion (Cl^- and HCO_3^-) secretion was compromised in all was significantly lower in the “severe” group (4). Generally, the pancreatic insufficient phenotype is characterized by an early onset of the disease, a high incidence of meconium ileus, high sweat chloride levels, steatorrhea, poor nutritional status, failure to thrive, male infertility, high rate of P aeruginosa colonization and higher mortality (24, 81, 100). Of interest however, even among patients carrying the same severe mutations substantial variability in the extent of pulmonary involvement has been reported (81). In contrast the pancreatic sufficient phenotype is associated with milder lung disease (52), lower rates of intestinal obstruction (143), a higher BMI on average 2.6 points higher (69), lower mortality and an approximately 20-year longer lifespan compared to PI patients (20). Mild class IV or V CFTR gene mutations, which result in some residual ductular function, appear to protect the exocrine pancreas from complete destruction at an early age (4). At a later age however, approximately 20% of patients carrying at least one class IV or V mutation may develop progressive pancreatic damage as a consequence of chronic pancreatitis (4). Of importance heterogeneity of acinar reserve and ductular dysfunction among patients carrying class IV and V mutations have been described. Possible explanations for this heterogeneity in between the group of PS patients are environmental factors and genetic modifiers that are known to alter the course of the disorder (29). Additionally, specific mutant alleles belonging to classes IV and V may exhibit varying degrees of chloride channel dysfunction, which in turn may influence disease progression.

1.6 Fat absorption in CF breast-fed, bottle-fed and older ages

The James Fairfax Institute of Paediatric Nutrition (JFI) within the Department of Gastroenterology at the Children's Hospital at Westmead (CHW), Australia, is a world leader in the assessment of pancreatic function particularly in infants with CF diagnosed by newborn screening and the evolution of pancreatic function with age. NSW has been one of the largest neonatal screening programs for CF since 1981. The Gastroenterology Research Unit, under the direction of Professor Kevin Gaskin, James Fairfax Professor of Paediatric Nutrition, and Head of Gastroenterology is acknowledged worldwide for its expertise and pioneering work in infants with CF. Only two centers worldwide, one is the JFI at the CHW, and another center in Verona, have performed 3-day fecal fat balance studies over 30 years on at least 600 patients total during this interval in a newborn screened (NBS) population. In the early studies of Gaskin et al (50) on patients with steatorrhea and patients with normal fat absorption the authors proved that colipase and lipase secretion rates as low as 1 and 2 percent of normal values respectively, prevent patients from having malabsorption. In the study of Waters et al. (143) of CF PI infants diagnosed by NBS colipase and lipase secretion rates were less than 1% and 2% respectively of young normal adult controls similar to the study of older CF children (50). Another study by Massie et al. (98) from the same author group on ΔF508 heterozygotes infants confirmed that most infants had colipase secretion rates in the normal adult range (assessed in reference 49) despite being only up to 6 weeks of age when standardizing pancreatic colipase secretion per kilogram of body weight per hour. Although quantitative pancreatic stimulation tests for ethical reasons have never been performed in normal infants, it is likely that colipase secretion rates were similar to infants assessed by Massie (98) with single ΔF508 heterozygotes with no other CF related mutation and therefore have well developed pancreatic enzyme secretion even at this very young age.

In the study of Gaskin and colleagues (143) on CF infants using direct pancreatic function tests and fecal fat balance tests actual daily fecal fat output in a group of exclusively breast-fed infants and a separate group of bottle-fed infants was compared. Fecal fat data in the exclusively breast-fed infant group varied from 0.1 to 15 g/d. All PI patients had fecal fat outputs greater than 2.0 g/d. Bottle-fed infants were considered PI if they had fat excretion rates $\geq$10% and PS if their fecal fat was <10% (143). Pancreatic stimulation tests were performed on 19 of these babies, 14 babies with colipase secretion > 1% of normal (range: 2% to 77%) had fecal fats <10%, and 5 babies with colipase secretion < 1% fat malabsorption. Based on these results patients <6 month of age with fecal fat output >2 g/d in breast-fed infants or >10% in bottle-fed infants or fecal fat >7% of oral fat intake in older children are referred to as PI and those with normal fat absorption as PS (26,143). PS infants rarely have any of the symptomatology of PI

patients or signs of suggestive malabsorption at the time of diagnosis by neonatal screening. However, the occurrence of frequent loose or watery stools in breast-fed PS infants, similar to their PI counterparts, is not uncommon. Clinically it is virtually impossible at this early age to designate the pancreatic phenotype of PS patients thus appropriate investigation is needed.

1.7 Newborn screening in CF

Following the discovery in New Zealand that immunoreactive trypsin (IRT) was elevated in the dried blood spots card in CF infants; the investigators established a national neonatal screening program (28). Collaboration was developed over the next 2 years with the New South Wales (NSW) State NBS laboratory. Screening for CF was commenced in NSW, Australia, in July 1981 using a two-step IRT technique with IRT being assayed from dried blood spots in the first week of life and if elevated being repeated on a second blood spot at approximately 6 weeks of age (150). If both tests demonstrated elevated values the patient was then referred to one of the 3 CF centers in NSW and underwent sweat chloride testing (150). The use of IRT assays as a screening method for the detection of CF in newborns had been questioned. A US task force stated that IRT assays hadn't been validated for newborns with CF with 'normal' pancreatic function and because approximately 10% of CF patients are PS, would possibly lead to a 10% obligatory false-negative diagnosis (2). The study of Waters and colleagues (143) did some further investigations and proved that IRT- assays can identify PS patients as well as PI patients with CF. They screened newborns using IRT assays in NSW between 1981 and 1987 and their findings showed no significantly different mean IRT values for 38 patients with PS compared to 23 patients with PI. IRT proved to be a sensitive and specific test for CF in babies aged 4 to 7 weeks (at repeated blood samples).

Following the discovery of CFTR the screening strategy was changed in 1993 and an elevated 4-day IRT is currently followed by a mutation analysis performed on the same blood spot. The study by Wilcken et al (151) compared the efficiency of the two different protocol approaches, IRT alone versus IRT/DNA approach, and concluded an improved sensitivity in unexpected cases, 89% for the IRT protocol and 98% for the IRT/DNA test and an improved specificity with the IRT/DNA protocol. This IRT/DNA strategy is considered suitable in populations of mainly northern European ethnic origin as at least one ΔF508 mutation is present in up to 95% of CF patients, but the use of the IRT/DNA strategy in a multi-ethnic community will not identify patients with rare mutations specific to some ethnic origins. When preliminary DNA analysis in CF patients is considered negative or not fully informative, increased sweat chloride concentration remains an important diagnostic marker.

At that point in time other regions of the world including most of North American-, British- and European CF centers did not accept NBS for CF. They were skeptical because of the lack of randomized control trials in terms of the beneficial outcome and prognosis of NBS despite studies using historical controls and assessing outcomes over prolonged intervals over 10-20 years with the demonstration of the maintenance of normal growth and nutrition, significantly better pulmonary function and less mortality (to 25 years post screening) with screening (32, 99,

144). In a randomized clinical trial over a 13-year period, researchers in the Wisconsin CF Neonatal Screening Study group compared patients identified by screening (early diagnosis) with control patients (standardized diagnosis – clinical symptoms and signs and confirmatory sweat chloride). The screened group of patients demonstrated significantly greater growth during the 13-year period and overall enhanced long-term nutritional status (44). However, in regard to the outcome of the pulmonary function testing their findings indicated no difference between the screened and non-screened patient groups (43). Deficiencies of this study were the failure to stratify for PS and one of their clinics was unsegregated from older CF patients. Both factors confounded with the outcomes of pulmonary function testing namely the result that there is no difference detected in any measure of pulmonary dysfunction between the two groups of screened and therefore early diagnosis compared to the group of later, standardized diagnosis. Despite the outcome of the Wisconsin CF Neonatal Screening Study group, other studies showed further advantage for early vs delayed diagnosis in CF (59, 153) and NBS gained further worldwide acceptance. Most European countries developed national or regional screening programs for CF in the last decade. NBS commenced in Germany in 2005. Since 2008, the nationwide introduction of NBS for CF has been under discussion in the Federal Joint Committee in Germany. Meanwhile, in the neighboring countries - France, the Netherlands, Austria, Poland and Switzerland - such programs have already been in successful operation for years (106). Concerns about DNA testing as part of CF NBS, including detection of heterozygotes, clinically equivocal forms, legislative and ethical issues, as well as limited coverage of ethnic diversity; has led to consideration of alternative purely biochemical protocols in Germany in favor of the well performing IRT/DNA protocols. In 2008, two regional NBS centers launched individual studies evaluating CF screening protocols with IRT as first and pancreatitis associated protein (PAP) as second tier analysis. The CF NBS center in Dresden, which has experience with CF NBS since the 1980s, started using IRT/PAP according to the original protocol. The NBS center Heidelberg started with a modified IRT/PAP protocol, which is since then compared internally to a standard IRT/DNA protocol (using the most common four mutations in Southwest Germany) run in parallel. In contrast to other NBS centers evaluating PAP based protocols both German NBS centers implemented an IRT-dependent safety net (SN) into the protocol. Accordingly, CF-NBS was also rated positive when IRT was 99.9th centile. In 2013 a third German NBS center (Greifswald) has offered CF screening using the IRT/PAP protocol with SN as done in Dresden. Since 2008, the Federal Joint Committee (Gemeinsamer Bundesausschuss, G-BA) is responsible for the decision whether to include CF as part of the regular NBS program in Germany. In 2014, the G-BA published a first draft directive on CF NBS in Germany (71). It was recommended that CF NBS with a third-tier analysis namely IRT/PAP/DNA protocol should be implemented. The studies to date had shown that PAP may

provide advantages in the German screening program which routinely collects samples between 36 and 72 hours of age. The inclusion of PAP shows sufficient sensitivity and specificity. However, the committee has realized that a lower positive predictive value (PPV) is obtained in screening protocols using IRT/PAP solely when compared to other existing CF screening protocols. Therefore, third tier analysis will make use of a DNA panel with 31 CFTR-mutations (41) and was implemented on 1st September 2016 as part of CF NBS in Germany.

1.8 PS leading to PI or persistent PS

In any large CF population of children > 3 years of age 80 to 90% of patients are PI with malabsorption and 10 to 20% are PS with adequate absorption (53). However, in newborn screened populations, in the first two months of life, 60 to 70% are found PI and 30-40% PS (26, 53, 143). A number of follow up studies in CF patients demonstrated a decline in pancreatic function over time (26, 52, 53, 143). These results reflect the higher preservation of acinar function in CF patients in early life, with a deterioration of function over time. Approximately 50% of the PS patients become PI by the end of childhood and that explains the lower proportion of PS patients at an older age (52).

The pancreatic phenotypes PI and PS appear directly related to genotypes with most PI patients being homozygotes or compound heterozygotes for two “severe” CFTR classes I, II or III gene mutations (4, 40, 132, 152). Of importance, the study of Cipolli et al (26) demonstrated that at the time of neonatal diagnosis 34 of 231 infants with two ‘severe’ PI associated mutations (ΔF508 homozygotes, ΔF508 /x and x/x) were PS. This is an unusual finding in older CF patients, but previously Gaskin et al. (50) have demonstrated that this subgroup loses their pancreatic function with age. Walkowiak and colleagues (138) have indicated a similar phenomenon in a small group of 27 infants with Class I or II mutations, noting all 8 with PS at diagnosis had become PI within the first year of life. Cipolli et al (26) described that all 20 PS patients that transitioned from PS to PI had two “severe” class I, II or III CFTR mutations, whereas those with persistent PS had at least one class IV or V “mild” mutation. They also reported that the age of onset of PI varied considerably, ranging from 2 months to 11 years of age (26).

These findings clearly indicate that one should not assign the PI or PS phenotype to CF infants based on the results of phenotype/genotype studies in older patients. All PS patients with at least one severe or unknown CFTR mutation should be longitudinally assessed for the progression of pancreatic dysfunction.

1.9 Reason behind screening for PI + PS

CF patients require careful nutrition management to avoid nutrition deficiencies, which include fat-and water-soluble vitamins, minerals, essential fatty acids, and trace elements. Deficiency states of a specific nutrient can occur in CF patients due to decreased intake, maldigestion, increased losses, and increased needs, oxidative stress, and possible metabolic abnormalities at the cellular level (1, 107). Untreated PI patients have an average fecal fat loss of 40% of fat intake, but it may exceed 50% and can extend up to 80% (51). The nutritional consequences of these high losses are evident in patients from non-screened populations at presentation and include failure to thrive, poor weight gain, evidence of protein energy malnutrition, including wasting and stunting, symptomatic deficiencies of fat-soluble vitamins (A, D, E, K), and salt depletion in patients living in hotter climates as a result of excess salt loss in sweat.

The findings of a comparison study of the nutritional status of PI and PS infants at diagnosis indicated compromised nutritional status as evidence by poor weight, depressed body fat stores, and low albumin levels in PI infants, but not in PS infants (26). CF patients with insufficient pancreatic enzyme excretion have malabsorption and require supplementation of pancreatic enzymes to maintain normal growth. Once the diagnosis of PI is established, most patients can be readily treated with PERT, the mainstay of managing CF PI patients for the last fifty years. However CF PS patients do not need pancreatic enzyme replacement therapy. They have sufficient pancreatic function, normal stools, good growth and nutrition, are well on unrestricted diets without addition of pancreatic extract (47). Of importance however, the functional capacity of the exocrine pancreas in PS patients varies widely, from values just above the threshold for developing PI to values that are within the reference range for healthy controls. PS patients also have decreased volume of bicarbonate-rich secretion (48) but continue to produce enough pancreatic enzymes to avoid steatorrhea (50). Giving PERT to PS infants as currently occurs in many clinics is an unnecessary expense also there is some evidence that at least in the short-term, exogenous pancreatic supplements lead to inhibition of endogenous pancreatic secretion (141). It was pointed out that a chronic pharmacologic inhibition of pancreatic secretion by exogenous pancreatic enzyme supplements could be additive and potentially transform a PS patient to one who is PI and needs PERT in the long run (15). Therefore, pancreatic function should be tested at CF diagnosis and subsequently monitored in PS patients to delineate the changeover from PS to PI (26, 143).

1.10 Pancreatic enzyme replacement therapy

All oral pancreatic enzyme products in current use are porcine origin (76). The external coat of the whole capsule is usually gelatin and this coat undergoes acid dissolution when the capsule hits the stomach and the microspheres are released into the stomach. The microspheres which contain porcine pancreatic enzymes have an enteric coating that is acid resistant and this coating only dissolves once the PH reaches above 5.5-6 in the duodenal or upper jejunal environment. However, in CF patients reduced pancreatic and duodenal bicarbonate secretion may fail to neutralize gastric acid and thereby prevent or delay dissolution of the enteric coating until the microspheres have passed the major absorptive surface area in the duodenum and jejunum. Even if the coating dissolves, the activity of most pancreatic enzymes, particularly pancreatic lipase/colipase, is greatly impaired in an acidic intraluminal environment. This could be the reason why in some CF patient's lipid digestion and absorption cannot be normalized despite high doses of PERT. Despite recent improvements in the pharmacokinetics of these supplements, many patients continue to experience a certain degree of steatorrhea, however it has been shown that an increase in the dose of pancreatic enzymes does not completely correct malabsorption. In addition, the use of high-strength pancreatic enzyme supplements in high doses (lipase doses of over 20000 iu/kg/day) to increase lipolysis can decrease compliance, increase costs and the risk of complications such as perianal irritation and colonic structures (46, 125). The recommended dose of pancreatic oral enzyme supplementation is 1000-2000 lipase units/kg body weight per meal, and this is used as a standard dose in the CHW CF unit (approximately 5000 units/kg/day).

The pancreatic function of patients with CF should be assessed before giving PERT. In the US 85% of CF patients never had an assessment for their pancreatic function status. Of the CF patients who are cared for at US CF Foundation-accredited centers 93% take exogenous pancreatic enzyme supplements on a routine basis, despite probably only 85% of patients in the United States are PI i.e. needing to take PERT (15). Wiedeman et al (149) described a similar situation in Germany with only 7 % of CF patients not taking PERT and 20.3% of patients receiving >10,000 lipase units per kg/day.

1.11 Diagnosis of PI and PS: Pancreatic function tests

The importance of accurately determining pancreatic function status in CF patients has already been mentioned, i.e. enzyme therapy for PI patients and avoidance in PS patients. There are several possible investigations available to examine the adequacy of fat absorption. These include direct measurement of pancreatic enzyme activity following duodenal intubation and stimulation with intravenous CCK and secretin and indirect tests including stool examination for fat globules, 3-day fecal fat absorption, measurements of fecal enzymes such as elastase or chymotrypsin and ^{13}C breath tests.

Direct and indirect pancreatic function tests are discussed in the following sections.

1.11.1 Direct Exocrine Pancreatic function tests in CF (PST)

For accurate, quantitative assessment of exocrine pancreatic function, determination of the total volume, the content of electrolytes Na^+, K^+, Cl^-, and HCO_3^- and enzymes secreted during a stated period of time, before and after a standardized secretory stimulus of the gland with secretin and pancreozymin and the use of non-absorbable marker perfusion technique, to offer an ability to quantify recovery of pancreatic secretions, is necessary (54). The latter technique permits accurate quantification of fluid output as described above on page 21 (54, 83).

The method of duodenal intubation has been described briefly as the following procedure 48, 143). After an overnight fast and sedation of the patient a double lumen is passed and positioned with the tip of lumen at the ligament of Treitz with several openings for aspiration of duodenal fluid and the second lumen opening at the ampulla of Vater for perfusion of the marker solution. The initial collection period is 20 minutes. At the end of the initial period CCK and secretin are infused intravenous simultaneously over 3x 20-minute periods during which duodenal fluid is aspirated continuously over the same 3x 20-minute period into conical flasks. Immediately after collection volume, bicarbonate concentration, pH, and lipase activity are measured and enzymatic activities of trypsin, chymotrypsin, carboxypeptidase A, and amylase are determined after storage of the juice at -80 °C. As above described the direct quantitative exocrine pancreatic function test, namely duodenal intubation, with intravenous stimulation of the pancreas with CCK and secretin, and with the marker perfusion technique correlates exceptionally well with fat malabsorption and absorption. It is considered the traditional “gold standard” measurement of assessing pancreatic function status with the highest sensitivity and specificity compared with all other pancreatic function tests (20, 50).

However, disadvantages of this invasive direct test are the need to use sedation for the patients (62) and radiation exposure for correct placement of the duodenal tube. Also, direct pancreatic function testing is shown to be complex and requires specialized skills to administer the test, which is limited in most centers (47, 57, 89, 143). The clinical practice of direct pancreatic stimulation tests is described to be expensive, time consuming, and uncomfortable (13, 20, 139) and CF patients have become increasingly reluctant to undergo intubation studies. As a result, direct exocrine pancreatic function intubation tests are no longer in routine use at CF centers.

1.11.2 Indirect exocrine pancreatic function tests in CF

Indirect pancreatic function tests are used routinely in clinical practice because they are non-invasive, simple and less expensive than direct tests (139). The most important clinical criterion for an indirect pancreatic function test is a sufficient sensitivity for the detection of mild and moderate forms of pancreatic impairment, since severe forms are hardly a challenge with the available tests (94).

1.11.2.1 Fecal fat Balance Test pros/cons

A fecal fat balance study is the 'gold standard method' among the indirect exocrine pancreatic function tests (20). Van de Kamer (133) described the method for demonstrating fat absorption and stated that intestinal motility is variable and therefore it is advisable to determine the fat balance on several consecutive days. Traditionally this method is a quantitative measurement of fat excretion over a fixed time, usually a minimum of 3 days, and an estimate of dietary intake of fat over the same period. Of note, the van de Kamer technique only measures long chain triglycerides. If infants receiving medium chain triglyceride (MCT)-containing formula. the technique of Jeejeebhoy for measuring MCT is required (72), otherwise serious under-estimation by the van de Kamer technique would occur.

At the CHW fecal collection is undertaken in every newly diagnosed patient with CF, and in those CF patients with PS suspected of having a decline in exocrine pancreatic function. A 5-day food record is kept and analyzed for the estimation of fat intake. Of importance is the accurate description of the food intake and the quantity as an incorrect diet history leads to incorrect estimates of fecal fat malabsorption. Fat absorption is measured by the coefficient of fat absorption (CFA). CFA is determined by recording ingested fat, measuring excreted fat, and making a ratio

$$\text{CFA}\ (\%) = \frac{\text{Fat intake (g)} - \text{ fecal fat losses (g)}}{\text{Fat intake (g)}}\ x\ 100$$

The stool fat excretion is age dependent. In adults, adolescents, school age, preschool children, and infants older than 6 month the coefficient of fat absorption should exceed 93% and in infants younger than 6 month a cut-off level of 90% is suggested (70, 143). A CFA below these percentages is an indication of pancreatic insufficiency (PI) and above these percentages as pancreatic sufficiency (PS). As dietary intake of exclusively breast-fed infants cannot be monitored, fecal fat excretion rates >2 g/d denote PI (26, 143).

When performed correctly, the 72-hour stool collection test is very reproducible and useful for determining pancreatic function (45). In the two units worldwide, who have performed simultaneous PST and fat balance studies (49, 50, 143) there have been only rare instances where fecal fat estimation did not correctly predict the pancreatic phenotype as defined by the gold standard, PST. It has been concluded that fecal fat estimation provides reliable and accurate data as evidenced by the comparison with PST data and follow-up data of individual patients in assessing the response to PERT (49, 50). However, this test is less than ideal and problems with accuracy can arise if inadequate food records are kept, fecal fat samples fail to be collected, or are simply discarded. For diagnostic purposes this test should be performed when patients are off PERT (45). Fecal samples can be smelly, handling of large quantities of feces makes this test unpleasant for patients and laboratory staff, and the risk of contamination and infection is involved (90). Despite its clinical demand, there has been a serious push from Australian laboratories to discontinue this test. In fact, in one Australian state, no laboratories offer the test and there is limited availability elsewhere (95).

1.11.2.2 N-benzoyl-L-tyrosyl-p-aminobenzoic acid (NBT-PABA) test pros/cons

The N-benzoyl-L-tyrosyl-p-aminobenzoic acid (NBT-PABA) test was mostly used in the late 1970s and early 1980s (13, 110). NBT-PABA, a synthetic tripeptide, is administered orally with a test meal. NBT-PABA cannot be absorbed from the small intestine. However, in the presence of a functional exocrine pancreas NBT-PABA is cleaved by chymotrypsin releasing p-aminobenzoic acid (PABA), which is rapidly absorbed from the gut, conjugated in the liver, and excreted in the urine. Therefore, the concentration of PABA in blood or urine after oral application of NBT-PABA can be used as a measure of pancreatic function. In PI patient's oral application of PABA is used to check the PABA individual absorption capacity and to demonstrate that low PABA concentrations in serum or urine after oral application of NBT-PABA are a result of the low or missing chymotrypsin activity (102). In a comparison study of CF PI patients, controls and a group of healthy infants, PABA recovery rates were assessed and PABA was concluded useful in the diagnosis of pancreatic insufficiency in children over 5 months (119). However, recovery values in healthy infants and the older controls differed significantly and some values of the healthy infant group overlapped with the CF PI patients. Comparison studies against the secretin-pancreozymin test reported average sensitivity in severe pancreatic dysfunction of 71%, whereas the average sensitivity for mild to moderate pancreatic impairment of the pancreas was only 46% (108). In another study NBT-PABA was compared to the secretin-pancreozymin test and the fecal fat test and it was stated that there is no diagnostically useful distinct cut-off point with the NBT-PABA test detectable between mild and severe pancreatic dysfunction (90).

In conclusion NBT-PABA is a simple, non-invasive (77), indirect pancreatic function test that requires only minimal technical resources and personnel and can be useful in patients with pancreatic insufficiency (10, 102). However, the test is not necessarily as sensitive or as specific compared with direct intubation tests in pancreatic insufficient patients (10) and failed to be useful in patients with mild or moderate pancreatic dysfunction (108). Another disadvantage of the test is the potential of carcinogenesis with PABA's use and its withdrawal from the market by the US Food and Drug Administration (FDA).

1.11.2.3 Stool chymotrypsin pros/cons

Chymotrypsin has been shown to be a very stable enzyme that can be detected in stools. Stool chymotrypsin is representative of duodenal chymotrypsin activity as it is considered to be resistant to trypsin degradation in the gut (58). In contrast to FE1, chymotrypsin is quantified by measuring enzyme activity. Typically, three random stool samples are collected. Pancreatic enzyme supplementation can cause false-normal results and therefore should be stopped 5 days prior to the stool collection (13). Oher diseases such as diarrhea, malabsorption, or hepatitis can influence the results. Fecal chymotrypsin levels are reduced in pancreatic insufficiency and cut-off values for the diagnosis of pancreatic insufficiency range between 3 and 6 units of chymotrypsin activity per gram of stool (8, 60, 91, 93). In comparison studies using direct exocrine pancreatic function tests as a reference, sensitivity of the chymotrypsin test of patients with mild pancreatic impairment has been described to range between 16% to 65% (78, 91, 93, 94), around 50% in patients with moderate pancreatic impairment (93, 108), and around 75 to 91% in patients with severe pancreatic disorder (91, 93, 94, 108). The specificity of the chymotrypsin test has been reported to range between 49% and 95% (78, 91, 93, 94, 108) depending on the composition of the patient group and the selection of the control subjects studied.

Attractive features of this test are the low cost, easy handling and the convenience for the patient (13). Random stool samples can be mailed from outpatients to diagnostic centers because of the stability of chymotrypsin over a number of days at room temperature (78). However, most importantly, the specificity and sensitivity of this test are only acceptable in severe pancreatic insufficiency and most cases of mild forms of pancreatic impairment are missed (108).

1.11.2.4 Immunoreactive trypsin (IRT) pros/cons

In 1979 it was demonstrated that blood immunoreactive trypsin (IRT) concentrations are elevated in newborn babies with CF, and that IRT could be assayed in dried blood samples collected routinely for other neonatal screening tests (28). Studies proved further that the test could distinguish between patients with CF and age-matched controls, even when the CF patient had sufficient pancreatic function (143, 150). It was concluded that IRT assays performed in the neonatal period is an excellent screening test for the detection of CF with a satisfactory sensitivity and specificity and it became the basis for neonatal screening worldwide (150).

However, the Australian study of Waters et al. (143) on CF patients in the neonatal period showed no significantly different mean IRT values for 38 patients with PS compared to 23 patients with PI. This study indicated that IRT assays are not useful as a pancreatic function test to distinguish CF phenotype. Using a specific radioimmunoassay Durie et al (36) recognized that the vast majority of the circulating IRT elicited by an Elisa immunoassay technique was in fact circulating trypsinogen. This was elevated in both PI and PS CF infants but above 7 years only remained so in those with PS (37).

1.11.2.5 Fecal Elastase 1 (FE1) test pros/cons

Measurements of pancreatic enzymes in spot stool samples has gained wide-spread acceptance in Europe and is rapidly becoming a standard of care for patients with cystic fibrosis in the United States (20, 76). The use of FE1 as a measurement for indirect pancreatic function testing has become very popular and has been entrenched in protocols (11, 13, 18, 19, 20, 27, 76, 112,121, 138, 139, 140, 142). Elastase is one of the 20+ enzymes secreted by pancreatic acinar cells (115). Elastase 1, an anionic protease, belonging to the family of serine proteases, is a human specific enzyme that is mainly bound to bile salts during the intestinal passage, and is not - by contrast with other pancreatic enzymes - degraded during intestinal transport through the gut, whereas it is five to six fold enriched in human feces compared with pancreatic juice (18, 21, 35, 93). Since the early 1990s, a highly sensitive enzyme-linked immunosorbent assay (ELISA) for human fecal and duodenal elastase 1 determination using two specific monoclonal antibodies has been commercially available (20, 130). According to the FE1 analysis kit (test kit from ScheBo, Biotech, Giessen, Germany) values for adults and children after the first month of life >200µg/g are considered normal exocrine pancreatic function and levels <200µg/g are an indicator for exocrine pancreatic insufficiency.

To evaluate a novel pancreatic function test comparison with the gold standard pancreatic function test, PST should be considered. Studies comparing results of the FE1 determinations with those of the secretin-pancreozymin test (or analogous tests such as the secretin-caerulein test) and fecal fat balance tests in patients with CF, chronic pancreatitis, patients with non-pancreatic diseases and controls reported FE1 sensitivities for patients with pathological PST tests with steatorrhea between 82% and 100% and in patients with impaired PST without steatorrhea between, 25% and 100% (91, 93, 126, 127, 137). Specificities of FE1 were described between 55% and 98% (91, 93, 94, 126, 127, 137).

Studies have been conducted in children and adults with CF, but studies extensively in infants with CF are limited. A pilot study of Wallis and colleagues (142) on three meconium samples of infants with CF homozygous for the ΔF508 mutation, showed zero activity of FE1 in all three newborns. Longitudinal testing over a subsequent period of 3 weeks proved FE1 concentration remained consistently zero. The authors stated that this pilot study highlights the potential role that this assay may play as a screen test in neonates. To address the latter issue reference values for healthy preterm and full-term infants have been determined (25, 27, 87, 109). It has been demonstrated that during the first days of life in meconium, samples were significantly lower than levels determined later on. In full-term newborns the second sample taken by day 3-4 was normal. In premature infants, the lower the gestational age of the infant, the longer it took FE1 to reach normal levels, but even in the premature infants born at 28 weeks

of gestation or less, FE1 reached normal values by 2 weeks of age (87). These finding are in line with other studies reporting normal FE1 values from the second week onwards (25, 109). However, Kalnins et al. (76) pointed out that it is impossible to help define the normal range and lower limits by looking at the mean values for fecal elastase in premature and full-term infants. They also stated that if 200µg/g stool is used as the cut-off to define pancreatic insufficiency, infants may be prescribed enzyme therapy prematurely, and interference from enzyme therapy may unnecessarily hinder the establishment of normal infant feeding at the critical time.

In summary FE1 test has to be used with caution. On the one hand FE1 reflects exocrine pancreatic status because it is well correlated with duodenal lipase, amylase, trypsin, and bicarbonates (27). It is a sensitive, specific, and relatively inexpensive, non-invasive test (35). Only a small, random sample is needed, making it more acceptable to patients and care providers than timed collections of stools. This protein is stable through a wide range of pH and temperature, up to one week at room temperature, making it ideal to collect at home, transport to central laboratory, and analyses electively (20). The human monoclonal elastase antibody does not cross-react with porcine elastase; thus, the test can be performed while patients are taking exogenous PERT (13, 18). However, on the other hand, CF is a heterogenous disorder, and the rare milder mutations associated with PS are not necessarily detected by FE1 testing (35, 138, 139). FE1 should only be measured on formed stool (20). The possibility of obtaining a false positive outcome is likely when the stool is diluted. An occurrence of loose or watery stools in breast-fed infants is not uncommon. A dilution effect can also happen as a result of factors such as infectious diarrhea, severe enteropathies, short gut, or stool from an ileostomy, which applies to infants as well as older patients or in patients with severe intestinal enteropathy due to lack of stimulation to the pancreas (76, 78). All in all, FE1 tests have a high sensitivity among indirect tests, and superseded other indirect pancreatic function tests for routine use (9, 139).

1.11.2.5.a Comparison of Monoclonal and Polyclonal ELISAs for FE1 concentrations

Two commercial enzyme-linked immunosorbent assays (ELISAs) are currently available; one uses monoclonal antibodies directed against fecal elastase (ScheBo, Biotech, Giessen, Germany) while the other is a polyclonal based assay (BioServ AG, Rostock, Germany) (76). The difference between the mono-/and the polyclonal ELISAs is the reaction with porcine elastase. The monoclonal antibody does not cross-react with porcine elastase, so it can be used to measure endogenous production of FE1 in fecal samples while CF patients are receiving PERT (76). One limitation of the polyclonal test consists of the interruption of the enzyme substitution therapy prior to the test in order to avoid a cross-reaction with porcine proteins (18). So far, the polyclonal test is considered less reliable, because reference values for fecal elastase measured by the polyclonal antibody test are yet to be established (45). According to the manufacturer's instructions (Bioserv Diagnostics, Rostock, Germany) values >200µg elastase/g feces indicate normal exocrine pancreatic function; values between 100-200µg elastase/g feces indicate moderate exocrine pancreatic function and values <100µg elastase/g feces indicate exocrine pancreatic insufficiency.

Data on comparison of mono- and polyclonal tests are limited. In a comparison study of the monoclonal and the polyclonal ELISA with direct pancreatic function tests and fecal fat analysis in patients with severe pancreatic impairment the sensitivity for both tests was described to be 83% and in patients with mild to moderate pancreatic impairment sensitivity for both tests were only 40%, which was concluded unacceptable (63). The specificity of the polyclonal ELISA was described 95% compared with 80% with the monoclonal assay (97). Results of two further studies showed both monoclonal and polyclonal ELISA kits are equally suitable for evaluating exocrine pancreatic function both in children and adults (101, 115). Another study in adults compared the mono-/and the polyclonal assays and demonstrated significantly lower results used by the monoclonal assay compared to the polyclonal one. According to their findings the polyclonal elastase assay tends to overestimate elastase in human stool compared to the monoclonal elastase assay (113). It has been reported that there is concern that some patients with CF may be misclassified due to the overestimation of the polyclonal kit, so that some patients with CF may be categorized as PI by the monoclonal methodology but as PS by the polyclonal test (16). This concern has been confirmed by one study that compared both mono- and polyclonal ELISA kits against the fecal fat balance test in CF patients and their findings indicated that patients with a CFA ≥88% were classified PI by the monoclonal assay but would have been misclassified as PS by the polyclonal assay. They also reported three subjects with a CFA ≤93% which would have been misclassified as PI by both assays (18). The polyclonal test kit requires further validation in appropriate populations before it can be accepted for routine

clinical testing. There are theoretical reasons why a polyclonal assay may not be equivalent to a monoclonal assay. Variables could include a different standard curve depending on factors such as the purity of the elastase, differences in antibody binding and epitope identification, and the differences in assay conditions such as incubation time and the nature of the second antibody (18).

This dissertation will compare the results of the monoclonal and polyclonal FE1 assays and will evaluate the use of both kits to assess pancreatic function status in infants diagnosed with cystic fibrosis compared to fecal fat balance test results.

1.11.2.6 ^{13}C Breath test

There are three carbon isotopes in nature: ^{12}C, ^{13}C and ^{14}C. These are three varieties of the same chemical element, carbon, whose nuclei contain the same number of protons (six), but a different number of neutrons (six, seven and eight, respectively). Almost 99% of the atmospheric CO_2 contains the less heavy carbon, ^{12}C. A small part, 1.1% of CO_2 is somewhat heavier, since it contains ^{13}C. Finally, there is also in a very small proportion, a type of CO_2 that contains ^{14}C in the atmosphere, which is radioactive and unstable. Breath tests using compounds labelled with the stable isotope ^{13}C have replaced radioactive ^{14}C breath tests, which offer ways in which the fate of ingested lipids can be studied safely. All plants belong to two large groups, C3 and C4, depending on how their photosynthesis process is materialized. Most plants (85%) follow the C3 photosynthesis pathway, and the remaining 15% of the plants are of type C4. Naturally ^{13}C labelled material is produced by C4 plants. Owing to different photosynthetic pathways the carbon isotope fraction in these plants is less than in C3 plants resulting in slightly higher ^{13}C abundance (1.10% v 1.08%) (128). The choice of the ^{13}C labelled substrate determines whether the ^{13}C-breath test investigates transport, digestion, absorption, oxidation processes or enzymatic activities. Different substances can be labelled to allow the evaluation of pancreatic function: ^{13}C-triolein, cholesteryl-^{13}C-octanoat, distearyl-^{13}C-octanoyl-glycerol (mixed triglycerides), ^{13}C-trioctanoin, ^{13}C-glyceryl-tripalmitate/^{13}C-glyceryltrioleate mix, ^{13}C-hiolein, ^{13}C-protein, ^{13}C-starch (13, 23). Sensitivity and specificity rates of ^{13}C breath tests with different substrates show a high degree of variability. A study which used ^{13}C-labelled starch as a substrate demonstrated a sensitivity of around 70% compared to PST (92). This low sensitivity can be explained by the starch digestion by amylase produced in the salivary glands and the fact that malabsorbed starch undergoes colonic fermentation to $^{13}CO_2$. Furthermore, pancreatic amylase secretion becomes insufficient only in advanced stages of pancreatic disease (23). The best investigated triglyceride breath test is the ^{13}C mixed triglyceride (MTG) breath test with sensitivity rates reported to range between 92-100% for severe exocrine pancreatic insufficiency and 46-62% for mild pancreatic impairment compared to PST (94). The labelled substrate in this breath test consists of a triglyceride with two molecules of stearic acid at 1-sn and 3-sn position, and ^{13}C-octanoic acid in the 2-sn position. The rate limiting step in its digestion is the hydrolysis of the two stearyl groups by pancreatic lipase.

There are different general factors that can influence ^{13}C breath test results. All factors which influence the endogenous carbon dioxide production or excretion can affect ^{13}C- breath test results, e.g. food ingestion, physical activity, thyroid dysfunction, or respiratory diseases (23). Kalivianakis and colleagues (75) assessed the ^{13}C MTG breath test in healthy adults and studied the a) inter-/and intra-individual variation in the $^{13}CO_2$ response, b) the effect of two different test

meals, c) the effect of an additional meal during the test, d) the effect of physical exercise during the test. Their results showed that a) repeatability of the MTG test in adults is low, b) a solid and a liquid test meal, containing similar amounts of fats, give similar cumulative $^{13}CO_2$ responses, c) stringent prolonged fasting during the test is unnecessary, and d) standardization of physical activity seems preferable, since unequivocal effects of moderate exercise on the $^{13}CO_2$ response were observed in the individuals studied (75). A study on CF patients and controls confirmed that exercise gave lower test results, on average 85% of the percentage dose recovered (PDR) value at rest (129). Another confounding factor for ^{13}C breath tests might be the amount, quality, or content of the test meals used in different breath test protocols, which might alter gastrointestinal functions, especially motility. Therefore, standardization of test meals would be required, but many breath test agreements on standard protocol are still pending (23). The study of Van Dijk-van Aalst pointed out that because of different composition of the test meals used it is difficult to compare reference values between different age groups. A further factor that can influence breath test results is the use of ^{13}C-enriched food during the day preceding the test. It has been reported that consumption of ^{13}C-enriched carbohydrate during daytime can lead to declining $^{13}CO_2$ enrichment in breath on the following day in the post absorptive state (88). Confirming this fact in another study the $^{13}CO_2$ excretion during the test showed a decline instead of a rise after the test meal, leading to a negative cumulative production of $^{13}CO_2$. The baseline $^{13}CO_2$ excretion in that patient was also described to be high, which was traced to nocturnal use of ^{13}C-enriched tube feeding. This inappropriate low-test result for the ^{13}C MTG breath test was explained by assuming that baseline $^{13}CO_2$ enrichment was not stable during the test but declined at a rate greater than the rate of increase in $^{13}CO_2$ production from oxidation of malabsorbed ^{13}C octanoate from MTG (129). In conclusion all patients having breath tests or breast-feeding mothers should be advised to restrict ^{13}C-enriched food (e.g. cornflakes, corn-chips, maze, custard, soft drinks, supplementary feeding products) on the day before the breath test is planned to be conducted and during the breath test. One further fact of note is that tracer recovery in breath is never complete as a substantial amount of tracer remains in the carbon pool in the body (23). Therefore, a breath test can only be considered a semi-quantitative diagnostic tool.

1.11.2.6a ^{13}C Breath test as a method to assess pancreatic function

The use of isotope ratio discriminating breath testing for measuring pancreatic function in adults was first described by Ghoos et al in 1981 (54), and subsequently in children 1982 (145). Studies comparing the appearance rate of the ^{13}C tracer with the PST and fecal fat balance test in adult patients with pancreatic disease, steatorrhea of non-pancreatic origin, patients with gastrointestinal diseases of non-pancreatic origin, and controls showed good correlation (r = 0.89) between the $^{13}CO_2$ production and the lipase secretion rate assessed with PST. Sensitivity and specificity of the $^{13}CO_2$ output was reported to be 0.89% and 0.81% respectively compared to PST in one study (136) and another study confirmed that the ^{13}C MTG breath test very sensitively reflects exocrine pancreatic insufficiency (92-100%), but has limited sensitivity for the detection of mild cases with pancreatic impairment (46-62%), and a limited specificity of 69-85% compared to PST (94).

In adult PI CF patient's distinction could be made between patients and healthy controls, with no overlap in CO_2 recovery. When these patients were given a test meal without PERT, no recovery of ^{13}C was reported, however when given PERT with the test meal, recovery of ^{13}C normalized (118). The ^{13}C breath tests have proved useful in older CF patients but studies in children and especially neonates are limited. A pilot study on CF PI children taking PERT and controls showed that the ^{13}C MTG breath test can discriminate between PI and healthy controls, even if the CF patients are on PERT (68). Another study on CF PI patients and controls confirmed that the ^{13}C MTG breath test can distinguish between CF children with PI and controls when a test meal was given without PERT and showed values of ^{13}C recovery within the normal healthy control range, when these PI children used PERT with their test meals (5).

Until recently the $^{13}CO_2/^{12}CO_2$ isotope ratio of ^{13}C breath test has been measured with the gold standard method, isotope ratio mass spectrometry (MS), which represents a very sensitive but rather complex and expensive method (13). The MTG breath test using the technique of MS has proven to be useful in measuring intraluminal duodenal lipolysis in adults (75,136), children (5, 68,134) and formula fed infants (197,134). A new technique using non-dispersive isotope selective infrared spectrometry (NDIRS) has been recently developed for more widespread clinical use. The NDIRS technique opened up a lower priced analytical alternative with adequate precision compared to the technique of MS (13). The NDIR spectrometer is small, weighs less, is easy to install and does not require special gas supply (14). Both breath test analyzers - IRIS and FANci2 - used in this dissertation, use the new technique of NDIRS and the same methodology. Reference values set in the NDIRS system software IRIS® for the determination of pancreatic sufficiency and pancreatic insufficiency are based on studies using the technique of MS in children and adults.

There are advantages and disadvantages with ^{13}C breath tests. ^{13}C breath tests appear to be attractive and convenient to patients and investigators because only breath samples have to be collected and the simplicity and non-invasiveness of the ^{13}C breath test makes it very applicable in a clinical setting (23). Further advantages can be seen in the fact that the digestion of the substrate used is exclusively dependent on pancreatic lipase (74, 75). However, a general problem and disadvantage of all the breath tests for the diagnosis of pancreatic insufficiency are the various steps involved besides the duodenal activity of lipolytic pancreatic enzymes. These steps are: gastric emptying of the tracer, mucosal absorption, hepatic circulation, metabolism, endogenous CO_2 production, and pulmonary excretion. All these different factors might contribute to the reduction of the sensitivity and specificity of breath tests (94). Further disadvantages are that the equipment is not widely available, the high costs of the equipment and substrate, and the long test periods with many breath samples to be collected during the study (22). When ^{13}C-labeled MTG is used in bottle-fed infants the problem that ^{13}C-labeled MTG substrate might adhere to the wall of the bottle used to administer liquid test meals might also occur, in which case the actual intake of the ^{13}C-labeled MTG is lower than the value used in the calculation and the expired percentage of $^{13}CO_2$ would be underestimated (134).

All in all, fecal fat balance tests are so far the only reliable indirect pancreatic function test compared with the gold standard PST. However, ^{13}C MTG breath test proved useful in older patients with CF but has as yet to be evaluated in neonates. To ascertain whether these tests are sufficiently sensitive to distinguish those infants who malabsorb fat from those that do not, comparison of tests needs to be evaluated in neonates.

1.12 Summary

Infants diagnosed with cystic fibrosis need further evaluation to determine their pancreatic function status. CF patients exhibiting normal fat digestion and absorption are designated PS and those CF patients identified as having malabsorption are designated PI. Only PI patients will require PERT to improve nutrient digestion and absorption. Depending on the age, between 10 to 40% of CF patients will be PS at diagnosis (26, 52 143) and 25-40% of PS patients will lose their pancreatic function over the first 10 years of life (26, 53).

In order to distinguish between the two phenotypes of CF a variety of direct and indirect pancreatic function tests are available. The gold standard test, which correlates exceptionally well with fat malabsorption and absorption, with the highest sensitivity and specificity compared with all other pancreatic function tests, is the direct quantitative exocrine pancreatic function test, namely duodenal intubation with intravenous stimulation of the pancreas (26, 49, 50, 57, 143). However, this direct test is invasive and is no longer routinely in use in CF centers worldwide. The gold standard of the indirect pancreatic function tests is considered the fecal fat balance test. Fecal fat estimation almost always predicts pancreatic phenotype correctly as determined by comparison with direct tests (50, 143). Fecal fat estimation involves a record of fat intake, which needs to be kept for at least 72 hours and a stool collection period over the same time. In bottle-fed infants up to 6 month of age, fecal fat <10% of fat intake is consistent with normal absorption and in exclusively breast-fed infants, fecal fat excretion levels <2 g/day are considered normal (26, 143). Due to the odious nature of fecal collection, handling and analysis, many laboratories worldwide are reluctant to perform this test (36). Currently in one state of Australia the test is unavailable and other Australian wide laboratories are considering terminating this service (95).

More recently other indirect pancreatic function tests including ^{13}C breath tests and FE1 have been developed. The MTG breath test measures intraduodenal lipolysis and has been used in adults (136), children (68), and infants (97). The appearance rate of this carbon tracer has been shown to correlate well with pancreatic lipase secretion (136). This test has proven useful in CF patients with severe pancreatic insufficiency compared to controls. ^{13}C breath testing has been used to evaluate the development of fat digestion in newborn infants (97), but as yet studies on CO_2 recovery in PI and PS CF children have not been validated in newborns. FE1 is a pancreas specific protease that is not degraded during intestinal transport and is up to 6-fold concentrated in feces (18, 20, 35, 93). An enzyme linked immunosorbent assay (ELISA) has been developed enabling quantification of FE1 levels on a random 100mg stool sample. Most healthy children older than 2 weeks of age have FE1 levels above 400µg/g stool (76).

Comparison studies of FE1 with the direct pancreatic function tests and fecal fat balance tests showed sensitivity rates between 82 and 100% in patients with severe pancreatic insufficiency but in patients with mild or moderate pancreatic impairment sensitivity rates described varied between 25 and 100% (18, 20, 35, 93). The problem arises in those patients with milder pancreatic disease, who based on PST or fecal fat balance tests have normal fat absorption and are PS. Many of the studies on FE1 have inadequate numbers for PS patients with milder pancreatic disease. A predictive value of FE1 in this group of CF patients is poor and FE1 levels <50µg/g stool are not always indicative of PI (35, 93).

Reliance on FE1 alone would result in misclassification of PS patients as PI, with unnecessary prescription of PERT. Furthermore, FE1 levels are influenced by stool volumes, with diarrhea resulting in low FE1 levels due to a dilution effect (9). This fact is particular important in the newborn screening program as breast-fed infants have watery stools and may display erroneously low FE1 levels leading again to misclassification of pancreatic status. Studies on FE1 that have included neonates are very limited thus reliable reference levels remain as yet undefined (76).

Both ^{13}C breath test and FE1 allow discrimination between CF children with PI and controls. However, the sensitivity of these tests needs to be evaluated further in neonates. Direct pancreatic stimulation tests and fecal fat balance studies allow identification of neonates with low pancreatic function, but who do not need PERT, noting the PS phenotype may have lipase/colipase secretion from 1% up to 100% of average normal values (50).

^{13}C breath testing and FE1 need to be further evaluated in this population group to see if there is sufficient sensitivity to allow identification of infants with low pancreatic function, but who do not require PERT. Non-invasive tests are needed to define pancreatic function status thus justifying the use of PERT and preventing patients from unnecessary treatment.

1.13 Hypothesis and main aims

We hypothesize that ^{13}C MTG Breath test and/or the FE1 test will be sufficiently sensitive to distinguish pancreatic sufficient from pancreatic insufficient newborns with cystic fibrosis

Main aims of the thesis

1) To evaluate the pancreatic function status in a group of consecutive breast-/ and bottle-fed CF infants newly diagnosed by neonatal screening using both the ^{13}C MTG breath test with NDIRS technique and FE1 measurements in comparison with the 'gold standard' 72-h fecal fat balance test to determine their capability of accurately identifying pancreatic phenotype in these infants.

2) To evaluate if reference values to assess the adequacy of fat digestion and subsequent fat absorption set in the NDIRS system software IRIS® provided for children and adults are also applicable for infants diagnosed with CF and for a group of healthy babies, including exclusively breast-fed and formula-fed infants, of less than 2 years of age using NDIRS technique with two different devices: IRIS and FANci2.

2 SUBJECTS AND METHODS

2.1 Study population

2.1.1 CF-babies

The study group included 28 full-term infants, 11 girls and 17 boys, ranging in age from 1-13 months (21 < 4 months of age), identified as having cystic fibrosis by the NSW State neonatal screening program. Study participants were consecutive patients whose parents consented for their infant to participate in the study.

2.1.2 Control-babies

67 full-term infants were recruited from family, friends and staff of the CF clinic or infants who had undergone minor surgical procedures at the Children's Hospital Westmead, Australia between September 2010 and July 2012.

A total of 55 infants, 38 exclusively breast-fed and 17 exclusively formula-fed, of less than 5 months of age, mean age 103±13 days (range 77 – 148), without any symptoms or signs of malabsorption were part of the initial investigation with the ^{13}C MTG breath test with NDIRS technique. All infants were thriving on the same or increased weight and height percentiles, were tested negative on newborn screening for cystic fibrosis and had none of the known features of the rare congenital pancreatic hypoplasia disorders Schwachman-Diamond syndrome, Pearson's marrow-pancreas syndrome, or Johanson-Blizzard syndrome nor the rare conditions of congenital villous atrophy and Abeta- or Hypobetalipoproteinemia, and none have presented with subsequent failure to thrive.

A subgroup of 53 babies were longitudinally assessed with the ^{13}C MTG breath test and NDIRS technique during babies first two years of life with at least three consecutive breath tests. Babies were divided into 4 consecutive age groups: first ^{13}C breath test on babies less than 5 months of age, second breath test between 6-9 months of age, third breath test between 9-12 months and fourth breath test between 9-24 months and with a different test meal (of yoghurt).

2.2 Methods

All 28 CF patients identified by neonatal screening had a fecal fat balance study, ^{13}C MTG breath test and FE1 measurements by both the monoclonal and polyclonal tests performed. PERT (if already commenced) was stopped at least 8 hours before the start of the ^{13}C MTG breath test and 1 day before the fecal fat and FE1 assessment. The predictive ability of the ^{13}C MTG breath test and the mono-/and polyclonal FE1 assay to identify infants with pancreatic sufficiency and insufficiency was assessed relative to the definitive fecal fat assessment.

2.2.1 Fecal Fat Analysis

Stool samples were collected from all CF newborns over a 72-hour period, pooled and frozen at -20°C until analysis. Additionally, for formula-fed infants a record of the volume of formula ingested over this period were kept.

After being thawed, faeces samples were weighed and well homogenized. None of the infants received Medium Chain Triglyceride (MCT) based formulas and thus fecal fat excretion was determined according to the method of Van de Kamer et al for measuring Long Chain Triglycerides (LCT). This method is based on the principle that fatty acids and fat can be extracted almost quantitatively with petroleum ether from an acidic, alcoholic solution of about 60% ethanol, saturated with NaCl containing a small amount of amyl alcohol (125). The effectiveness of fat absorption was measured by the coefficient of fat absorption [(fecal fat (g) / fat intake (g)) (100)].

Pancreatic sufficiency was defined in infants less than 6 months of age as fecal fat excretion < 10% of fat intake in formula-fed infants and < 2 g/day in breast-fed infants according to Waters et al. (143) and in babies > 6 months of age, as fat excretion ≤ 7% of oral fat intake.

2.2.2 ^{13}C Mixed Triglyceride (MTG) Breath Test

^{13}C MTG breath test for the determination of pancreatic lipase activity was performed in all infants diagnosed with CF and controls. The MTG, 1,3-distearyl, -2-[13C-carboxyl] octanol glycerol, contains a ^{13}C medium chain fatty acid in the C2 position and long chain fatty acids in the C1 and C3 positions. After ingestion, the MTG is hydrolysed by pancreatic lipases, releasing ^{13}C octanoate. This is absorbed across the intestinal mucosa and transported to the liver. After entering metabolic pathways, the ^{13}C-octanoate is oxidised giving rise to $^{13}CO_2$ which is expired in the breath. Measurement of the enrichment of $^{13}CO_2$ in the infant's breath reflects the degree of lipolysis and subsequent fat absorption.

2.2.2.1 Test meal

The test substrate of 1,3-distearyl, -2-[13C-carboxyl] octanol glycerol used to measure fat digestion, was obtained from Wagner Analytical Systems, Bremen, Germany, and was 99% ^{13}C-enriched. Breast-feeding mothers and mothers of babies with additional food intake were instructed to avoid naturally ^{13}C-enriched foods (e.g. corn or corn products, custard, pineapple, cane sugar, soft drinks) for at least the day prior to the study. Test meals for the MTG breath test should be designed to have minimal effect on basal breath CO_2 enrichment. Infants of less than 6 months of age received 200mg MTG test substrate mixed in 5ml of expressed breast milk, infant formula or mixed in apple gel on a spoon followed by a normal feed of breast milk or formula. In older babies (> 6 months of age) MTG was mixed in two teaspoons of apple-gel or yoghurt followed by a meal consisting of C3 ingredients, with no interference of the oxidation of the ^{13}C-labeled substrate. Care was taken to note any regurgitation of the mixture or subsequent vomiting of the feed. The MTG breath test commenced after a fasting period of at least 2 hours for babies < 4 months of age and 4 to 8 hours in babies > 4 months.

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2.2.2.2 Breath sampling

Duplicate breath samples (of 150ml each) were collected prior to administration of the test meal and at 30-minute intervals thereafter over the study period of 5 hours.

Expired infant's breath was collected by using an anaesthetic mask, connected via a one-way flap valve to an aluminized plastic bag. Breath bags were closed with a plastic cork immediately at the end of exhalation and stored for analysis. Some of the older babies that were part of the longitudinal assessment in healthy babies were already able to fill the breath bags by

blowing directly into the bags via a straw. Breath samples were analysed promptly on the same day of the test.

2.2.2.3 Non-dispersive Infrared Spectrometry

^{13}C enrichment was measured using the technique of Non-Dispersive Infrared Spectroscopy (NDIRS) with breath test devices from two different manufacturers.

The study commenced on an Isotope -selective Non-dispersive Infrared Spectrometer, IRIS (Wagner Analytical Systems, Bremen, Germany) and was later continued on a FANci2 (Fischer ANalysen Instrumente GmbH, Leipzig, Germany). With the IRIS device the $^{13}CO_2/^{12}CO_2$ ratio is determined with an accuracy of ±0.4% (64, 65).

Enrichment of the breath samples was compared with the international limestone standard, PeeDee Belemnite (PDB), and the relative difference between the sample and PDB was expressed in delta (δ) per mil (%). The NDIRS system software IRIS® provides the resultant data from a group of healthy individuals with normal fat absorption, using a ^{13}C MTG breath test procedure. This reference data includes the mean, maximal and minimum results for ^{13}C cumulative dose recovered and $\%^{13}C$ dose/h. Breath test results were analysed and expressed as cumulative percentage dose of ^{13}C recovered (cPDR) over the 5-hour test period ($\%^{13}C$ cPDR) and the percentage of ^{13}C recovered dose of the initial amount of ^{13}C administered per hour ($\%^{13}C$ dose/h). Carbon dioxide production was calculated according to weight, height, age and sex.

2.2.3 Fecal Elastase Assessment

2.2.3.1 Monoclonal Faecal Elastase Assay

Monoclonal FE1 levels were determined in all CF infants using a commercially available enzyme linked immunosorbent assay (ELISA) kit (ScheBo, Biotech, Giessen, Germany). Two 50-100mg stool samples were taken from the pooled 72-hour stool sample; the first sample from the first day of the 72-hour stool collection and the second sample from the homogenised 72-hour combined stool sample. These samples were analysed according to the manufacturer's instruction. Briefly 1ml of diluted extraction buffer per 10mg stool was added to both stool samples. After homogenization, the supernatant was diluted with washing solution and pipetted into wells on an antibody-coated microplate, along with standards, positive and negative controls. After incubation and washing, a biotinylated second anti-human pancreatic elastase monoclonal antibody was dispensed into the wells, which were again incubated and washed. Streptavidin peroxidase conjugate was added and incubated; wells were washed; substrate was added; and the enzymatic reaction was stopped 20 min later. The absorbance of each well was read at 405nm on a microplate reader. Results were expressed as µg/g of stool. Using the assay, the limit of detection was 15 µg/g.

According to the manufacturer's instructions, values >200µg elastase/g stool indicate normal exocrine pancreatic function and values <200µg elastase/g stool indicate exocrine pancreatic insufficiency. These reference values refer to adults and children after the first month of life (www.schebo.com).

Beharry et al (9) assessed pancreatic function status in children with pancreatic disease and suggested FE1 concentrations of 100 µg/g stool to be a good cut-off point to distinguish between PI and PS status with excellent sensitivity and specificity. This cut-off point has also been discussed by the study of Loser et al (93) and will therefore also be evaluated in this thesis.

2.2.3.2 Polyclonal Fecal Elastase Assay

Polyclonal FE1 levels were determined using a commercially available polyclonal ELISA kit (Bioserv Diagnostics, Rostock, Germany). The Pancreatic Elastase ELISA from BIOSERV Diagnostics is a solid-phase enzyme immunoassay based on a double-sandwich technique applying two polyclonal antibodies recognizing several different epitopes on defined species- and organ-specific human pancreatic elastase peptide sequences.

The ELISA microplate is coated with antibodies directed against human pancreatic elastase binding the pancreatic elastase contained in the patient samples or in the standards respectively.

Diluted extraction buffer was added to two 30-100mg samples of the pooled 72-hour stool sample, 1ml diluted extraction buffer per 10mg feces. The first sample was taken from day 1 of the 72-hour stool sample and the second sample from the combined 72-hour homogenised stool. After homogenization by means of a vortex mixer, the supernatant was diluted with washing solution and pipetted into wells on an antibody-coated microplate, along with standards, and positive and negative controls. After incubation and washing, a biotinylated second anti-human pancreatic elastase antibody was dispensed into the wells, which were again incubated and washed. Streptavidin peroxidase conjugate was added and incubated; the wells were washed; substrate was added, and the enzymatic reaction was stopped 20 min later. The absorbance of each well was read photometrically on a microplate reader at 450nm.

According to manufacturer's instructions, values >200 µg elastase/g faeces indicate normal exocrine pancreatic function; values between 100-200 µg elastase/g faeces indicate moderate exocrine pancreatic insufficiency and values <100 µg elastase/g faeces indicate severe exocrine pancreatic insufficiency (www.bioserv-diagnostics.com).

2.3 Ethics

The Ethic Committee at The Children's Hospital at Westmead approved the study protocol and informed consent was obtained from parents of all infants before the start of the tests. Assessment and analysis of data were carried out according to privacy laws.

2.4 Statistical analysis

Sensitivity and specificity of the ^{13}C breath test and the FE1 assays were computed based on the pancreatic disease status as defined by the fecal fat analysis. Results are presented as mean, standard deviation, and range. The $P < 0.05$ level of significance was used. To compare cPDR results between the two different NDIRS devices paired samples t test was used and independent t test to compare cPDR results between pancreatic sufficient and insufficient CF infants and breast-fed and formula-fed healthy infants. To compare peak ^{13}C excretion rates between the two groups of breast-fed and formula-fed infants, Mann-Whitney U test was used. Bland and Altman analysis were performed to compare the cumulative dose of $\%^{13}C$ recovery for the two different NDIRS devices: IRIS and FANci2. All statistical analyses were carried out using SPSS version 20.00, SPSS Inc, LEAD Technologies, Illinois, USA.

3 RESULTS

3.1 General characteristics of CF patients

The study group consisted of 28 infants diagnosed with CF. However, 4 CF babies had total stool weight for the 72-h fecal fat balance assessment that did not exceed 10g. It was therefore questionable if the total amount of stool weight had been collected and as a result those 4 fecal fat balance test results had to be excluded from further analysis. General data of the 24 CF infants is presented in Table 1.

Table 1 General characteristics of CF patients

Number of CF-babies	24
Age at stool collection (days)	110 ± 103 (23-383)
Age at breath test (days)	112 ± 96 (26-397)
Sex (male/female)	14 / 10
Breast/formula-fed babies	12 / 12
Height (cm)	59.9 ± 8.0 (48.0-76.7)
Weight (kg)	5.8 ± 2.0 (3.1-10.3)

3.2 72-hour fecal fat balance test results

The mean total stool weight for the 72-h stool collection period was 73.8 ± 45.6 g. Mean fat excretion in the feces was determined to be 3.1 ± 2.9 g per day (ranged from < 0.1 – 9.4 g/d). According to Waters et al. pancreatic sufficiency is defined as a fecal fat excretion < 2.0 g per day (143). To be able to compare formula-fed and breast-fed baby results with fecal fat balance test results, formula-fed results were converted from percentage of fat excretion into g/d.

Out of 24 CF babies, 13 babies were determined pancreatic sufficient (fecal fat excretion ranged from < 0.1 - 1.9 g/d, with a 95% Confidence Interval (CI) between 0.44 – 1.13 g/d) and 11 infants' pancreatic insufficient (fecal fat excretion ranged from 2.9 - 9.4 g/d, with a 95% CI of 4.74 – 6.99 g/d).

3.3 ^{13}C MTG breath test results

Mean basal delta results of the 24 CF babies were -26.7 ± 3.8‰.

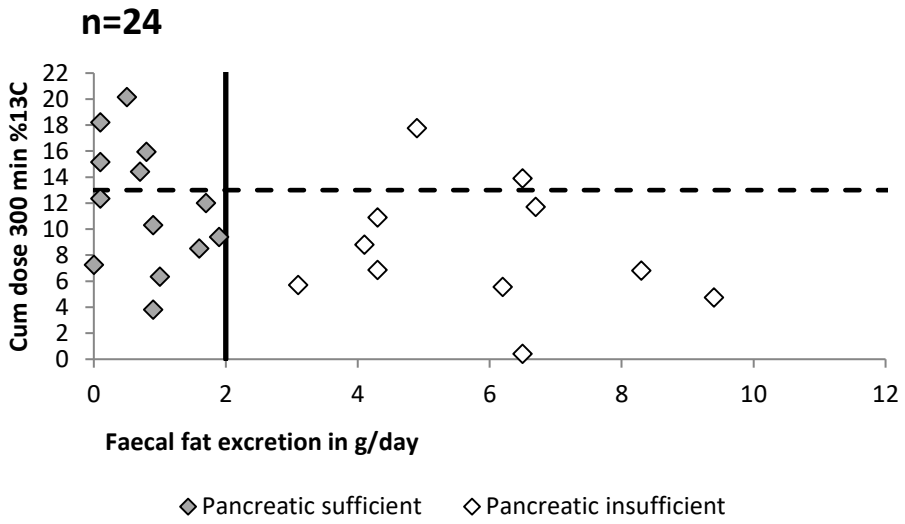
The database of the IRIS® system provides in its software the resultant data from a group of healthy individuals. These data include the mean, maximal, and minimum values for ^{13}C cumulative dose recovered values (cPDR) from a group of healthy subjects with normal fat

absorption following the test procedure using the ^{13}C MTG breath test. In this study $\%^{13}C$ cumulative dose is used for the determination of pancreatic function status. There are two options of assessing the pancreatic function status according to the reference values provided by the IRIS® system software. The first option to determine pancreatic function status is based on the $\%^{13}C$ cPDR value after 5 hours. The minimum reference value $\%^{13}C$ cPDR value provided by the IRIS® system software is 13. Therefore, cPDR results of 5 hours ≥ 13, is the cut-off point for PS. According to this classification 17 CF babies were determined PI (95% CI of 6.19 – 9.27 $\%^{13}C$) and 7 CF babies PS (95% CI of 14.8 – 18.2 %13C). The second option to determine the pancreatic function status is the comparison of the individual values of the ^{13}C cPDR values over the whole 5-hour study period. With this option CF babies are classified to be PS if ≥ 80% of ^{13}C cumulative dose results are above the minimum $\%^{13}C$ cumulative dose values provided by the group of healthy individuals of the IRIS® system software over the 5-hour period. Babies who do not meet the cut-off ≥ 80% of $\%^{13}C$ cumulative dose results are classified to be PI. According to this classification 20 babies were determined pancreatic insufficient and 4 CF babies pancreatic sufficient.

3.4 Comparison of the ^{13}C MTG breath test versus the fecal fat balance test

In Figure 1) ^{13}C MTG breath test results are plotted against the fecal fat balance test results. Sensitivity rates for option 1 for PI are 82%, for PS only 38%, and specificity rates are 38% for PI and 82% for PS. Sensitivity rates for option 2 are 100% for PI, however only 31% for PS and specificity rates are 31% for PI and 100% for PS.

Figure 1 Fecal fat excretion results compared to ^{13}C MTG breath test results in 24 CF infants



Dotted black line: $\%^{13}C$ cumulative dose recovered after 300 minutes cut-off point of 13%.
Solid black line: fecal fat excretion cut-off point of 2 g/day.

3.5 Fecal elastase 1 results

3.5.1 According to monoclonal ELISA- assessments

Spot fecal samples of 24 CF babies were analysed in duplicate with the monoclonal Elisa-kit. According to the manufacturer's determination criteria FE1 concentrations <200µg/g stool reflect pancreatic insufficiency; 18 out of 24 CF babies had FE1 concentrations below 200 µg/g stool (95% CI of 0.82 – 34.21 µg/g). FE1 concentrations >200 reflect pancreatic sufficiency and were determined in 6 out of 24 CF babies (95% CI of 500 – 500). According to the study of Beharry et al. (9) FE1 concentration of 100µg/g stool assessed with the monoclonal Elisa-kit provided excellent sensitivity and specificity to distinguish between the PI and PS status of patients with pancreatic disease. This cut-off point has also been discussed by the study of Loeser et al. (93). Therefore, we also analysed our results with a cut-off point of 100µg/g stool. 17 out of 24 CF babies had FE1 concentrations below 100µg/g (16 had FE1 concentrations below 50ug/g and one baby had FE1 concentrations between 50-100 µg/g) reflecting pancreatic insufficiency and 7 out of 24 CF babies with FE1 concentrations >100µg/g were designated with pancreatic sufficiency.

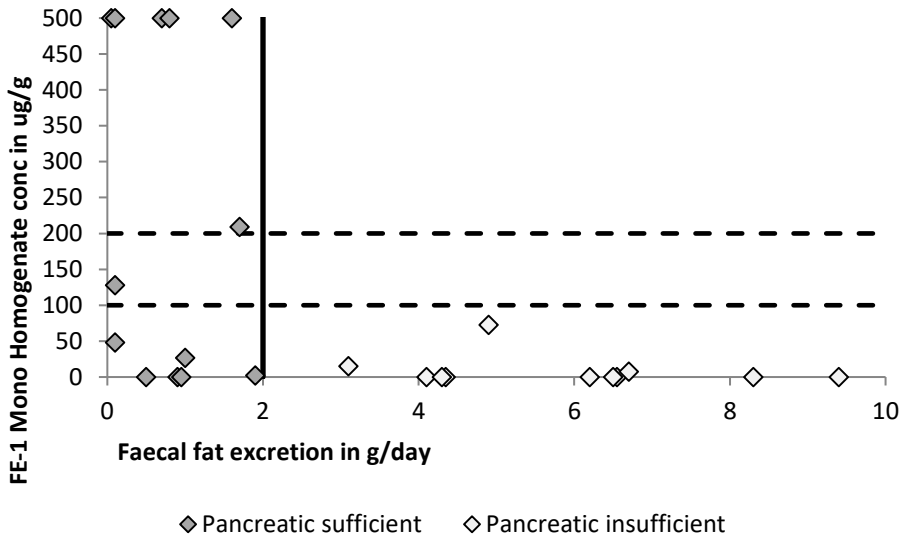
3.5.2 According to polyclonal ELISA- assessments

Analysis of the spot fecal samples with the polyclonal Elisa-kit revealed 16 out of 24 CF babies had FE1 concentrations <100µg elastase/g stool (95% CI of 27.89 – 47.11µg/g), indicating PI, 3 out of 24 CF babies had FE1 concentrations >200µg elastase/g stool, indicating PS, 4 out of 24 CF babies had FE1 concentrations between 100-200µg elastase/g stool, indicating moderate pancreatic insufficiency = PS. The 95% CI for PS was between 132.3 – 295.2µg/g). One duplicate sample gave contradicting results: sample A indicated PS (FE1 = 275µg/g stool) and sample B indicated PI (FE1 concentration = 47µg/g stool), this baby was designated PS on fecal fat balance test and was excluded for the sensitivity analysis compared to the FFB test. We also evaluated FE1 secretion rates with the mono- and the polyclonal kit per day, which lead to good sensitivity rates for PI, but even lower sensitivity rates for PS (39% with the monoclonal kit, 30% with the polyclonal kit).

3.6 Comparison of the fecal fat balance test and FE1 analysis

In Figure 2 FE1 Monoclonal results are plotted against the fecal fat balance test results. Sensitivity rates according to the manufacturer's cut-off values for PI are 100%, for PS 46% and specificity rates are 46% for PI and 100% for PS. According to the cut-off value used by Beharry (9) and Loeser (93) one would find sensitivity rates of 100% for PI and 54% for PS, and specificity rates of 54% for PI and 100% for PS.

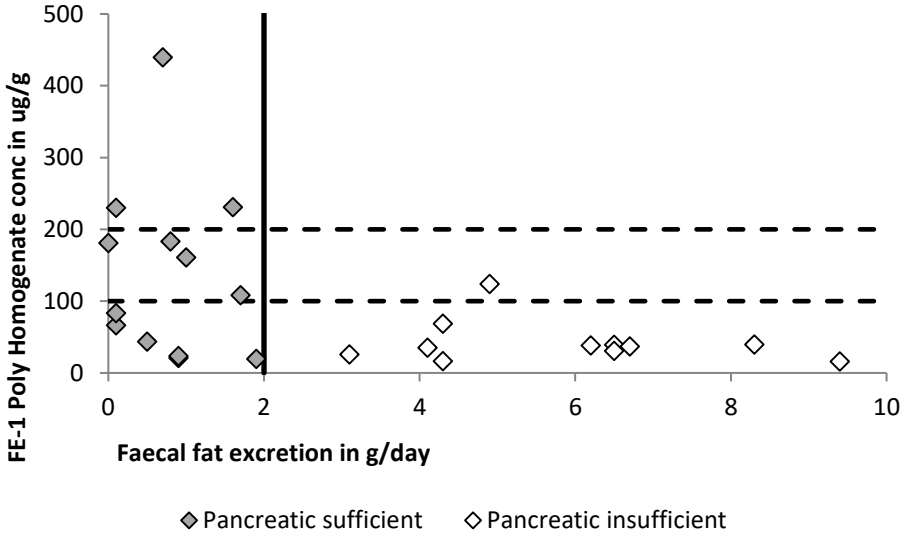
Figure 2 Fecal fat excretion results compared to fecal elastase 1 results assessed with the monoclonal Elisa kit in 24 CF infants



Dotted black lines: fecal elastase cut-off points of 100 µg/g stool and 200µg/g stool. Solid black line: fecal fat excretion cut-off point of 2 g/day.

In Figure 3 FE1 Polyclonal results are plotted against the fecal fat balance test results. Sensitivity rates for PI are 91%, for PS 46% and specificity rates are 46% for PI and 91% for PS.

Figure 3 Fecal fat excretion results compared to fecal elastase 1 results assessed by the polyclonal Elisa kit in 24 CF infants



Dotted black lines: fecal elastase cut-off points of 100µg/g stool and 200µg/g stool. Solid black line: fecal fat excretion cut-off point of 2 g/day.

3.7 Control-baby part

3.7.1 Demographic data

One breath test result (formula-fed baby) out of the 55 participating infants had to be excluded from the analysis, because the infant vomited soon after ingestion of the test meal. The demographic data of the 54 participating infants for the initial assessment are shown in Table 2.

Table 2 Demographic data

	Breast-/Formula-fed infants	**Breast-fed infants**	**Formula-fed infants**
Number of participants	54	38	16
Gender (male/female)	27/27	18/20	9/7
Age (days)	103 ± 13 (77-148)	102 ± 10 (78-119)	105 ± 17 (77-148)
Weight (kg)	6.1 ± 0.9 (4.5-8.7)	6.1 ± 0.9 (4.7-8.7)	6.1 ± 0.9 (4.5-8.0)
Height (cm)	60.7 ± 3.4 (54-68)	60.8 ± 3.5 (54-68)	60.3 ± 3.0 (56-68)

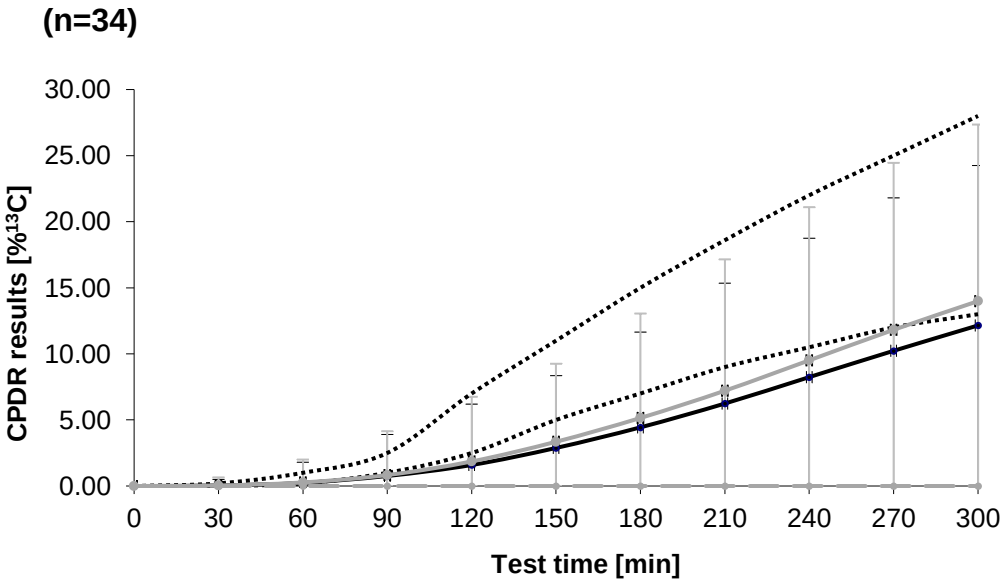
All participating infants were less than 5 months of age. Both groups of breast-fed and formula-fed infants were of similar age and gender distribution and had near identical mean and range for height and weight. No statistical differences were found comparing the demographic data of the infants in the two different groups. Out of the 54 infants, breath test analysis was exclusively assessed with the IRIS device on 5 infants, with FANci2 on 15 infants, and 34 infants were analyzed with both NDIRS devices. The following results include firstly a cPDR analysis for both NDIRS instruments, secondly the analysis of breast-fed versus formula-fed infants, and thirdly using $\%^{13}C$ dose/hour analysis for the same parameters.

3.7.2 CPDR results over the 5-hour test period in healthy infants

Comparison of both NDIRS devices: IRIS versus FANci2

To be able to compare cPDR results of breast-/and formula-fed infants over time, with the reference range set in the NDIRS system software IRIS®, results of the 34 infants who had ^{13}C MTG breath tests analysed on both NDIRS devices are plotted in Fig 4.

Figure 4 Mean %^{13}C cPDR results of 34 infants assessed on both NDIRS devices compared to the reference range set in the NDIRS system software IRIS®.

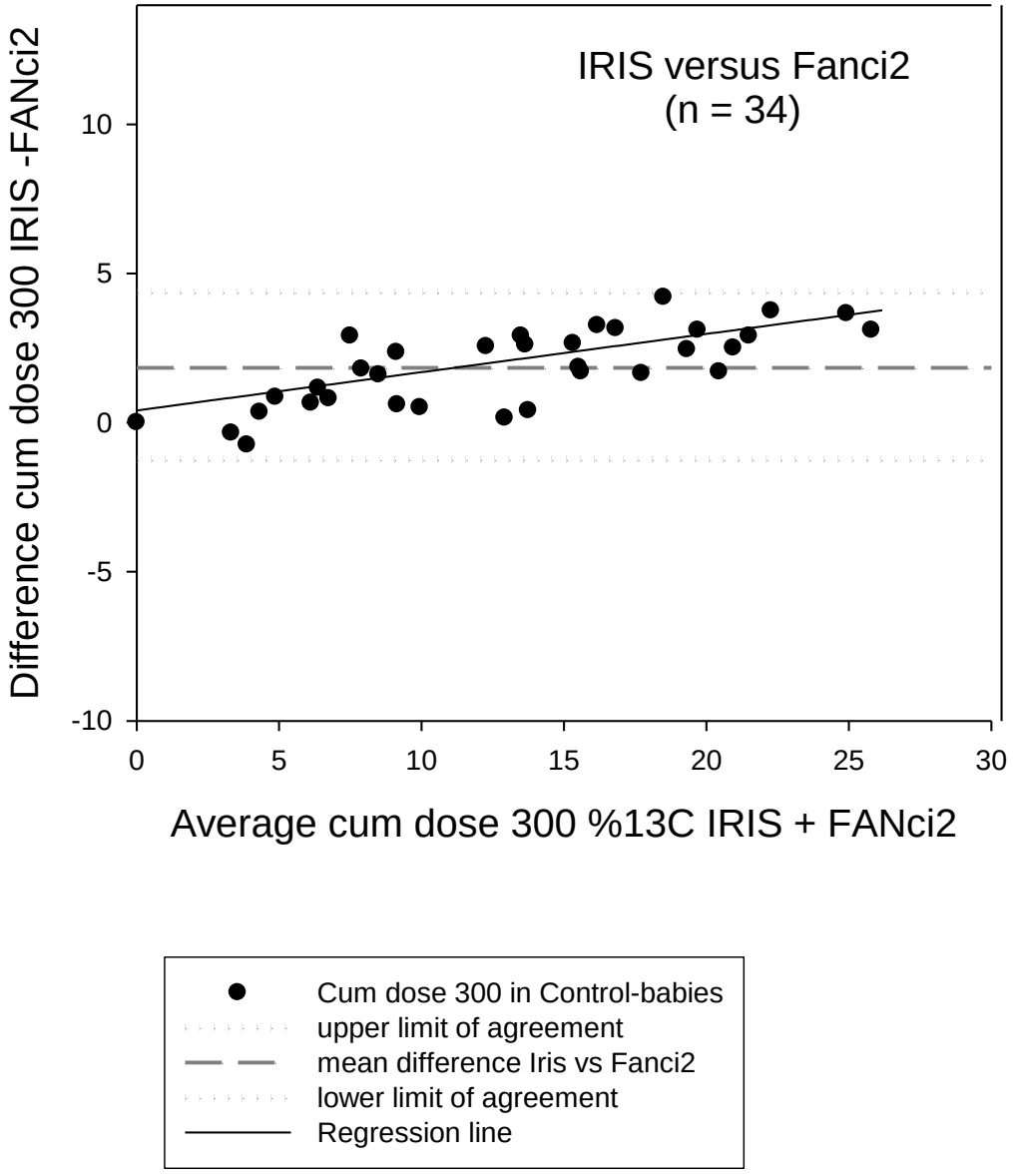


Black small dotted curve: minimum and maximum cPDR values of the reference range of the NDIRS system software IRIS®. Grey curve: mean cPDR results of the IRIS. Black curve: mean cPDR results for the FANci2. Positive and negative error bars represent the range of results: Grey error bars IRIS, Black error bars FANci2.

The lowest cPDR results over the 5-hour study period, on the IRIS device, were identical with the lowest cPDR results on the FANci2 device, with a cPDR result after 5 hours of 0%. Compared to the reference values of the system software IRIS®, mean cPDR results of 34 infants analysed on both NDIRS devices were inside the reference range from 0-60 minutes, and from 90-270 minutes continued to be below the minimum value of the reference range. The mean cPDR result after 300 minutes gave contradicting results: above the minimum of the reference range assessed with the NDIRS device IRIS and below with the NDIRS device

FANci2. Statistical analysis between both devices revealed significantly lower mean 5-hour cPDR results (12.2±6.7%) assessed with the FANci2 compared to the IRIS (14.0±7.2%) (p=0.00).

Figure 5 Bland and Altman analysis of 5-hour cPDR results of the IRIS vs FANci2



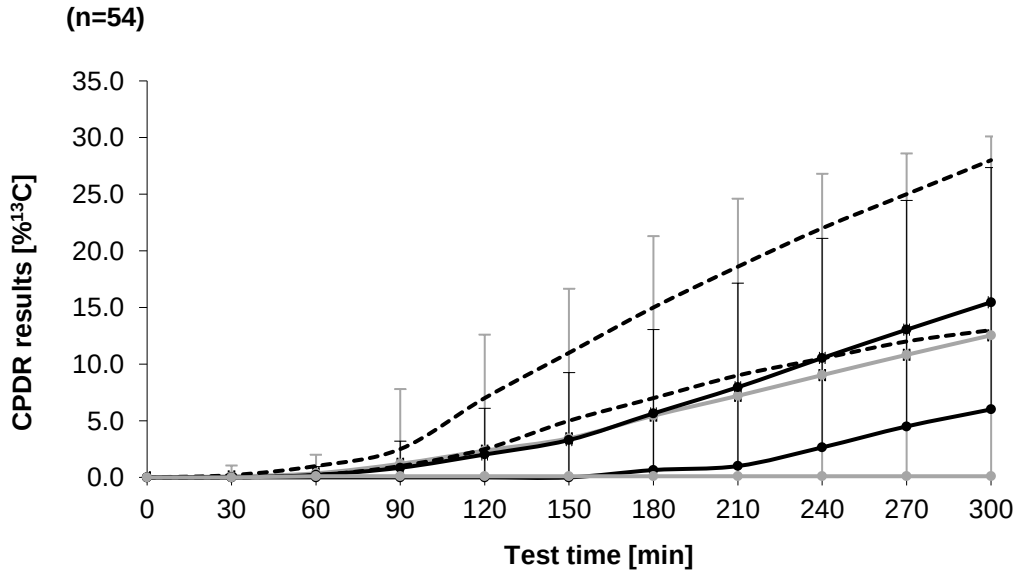
A correlation of 0.99 was found between both NDIRS devices, but Bland and Altman analysis to assess agreement disclosed a significant bias (p=0.00) between both NDIRS measurements and revealed that the measurements on the IRIS were on average 1.84% higher compared to the FANci2. Numerically, the difference was small and within the limits of agreement.

Of the total study group of 54 infants, the ^{13}C MTG breath test results of 39 infants were assessed with the IRIS device with a mean 5-hour cPDR of 13.3±7.3%. In comparison, 49 infants were assessed with the FANci2 device with a mean 5-hour cPDR result of 12.6±6.7%. Both devices presented results in the same range and no statistically significant difference was found comparing the total number of the infant breath test results assessed on both NDIRS devices (p=0.6).

3.7.3 Comparison between both groups of breast-fed and formula-fed healthy infants

To be able to compare results of the two different groups of breast-/and formula-fed infants, the 39 results assessed with the IRIS and the 15 results assessed with the FANci2 were combined and plotted against the reference range of the IRIS® system software below.

Figure 6 Mean %^{13}C cPDR results of both breast-/and formula-fed infants compared to the reference range set in the NDIRS system software IRIS®



Black dotted curve: minimum and maximum cPDR values of the reference range set in the NDIRS system software IRIS®. Black solid curves: mean and lower limit results in formula-fed infants (n=16). Grey solid curve: mean and lower limit results in breast-fed infants (n=38). The lower limit cPDR results in breast-fed infants are superimposed on the baseline. Error bars show range of cPDR results: Black error bars for formula-fed infants, Grey error bars for breast-fed infants.

Statistical analysis revealed no significant difference (p=0.2) between mean results in the two groups, breast-/and formula-fed (12.5±7.6% vs 15.5±6.3% cPDR after 5 hours, respectively). One breast-fed infant had a cPDR result of 0% after 5 hours and the second lowest cPDR result in this group was 2.5%. The lowest 5-hour cPDR result out of the formula-fed infant group was 6.0%.

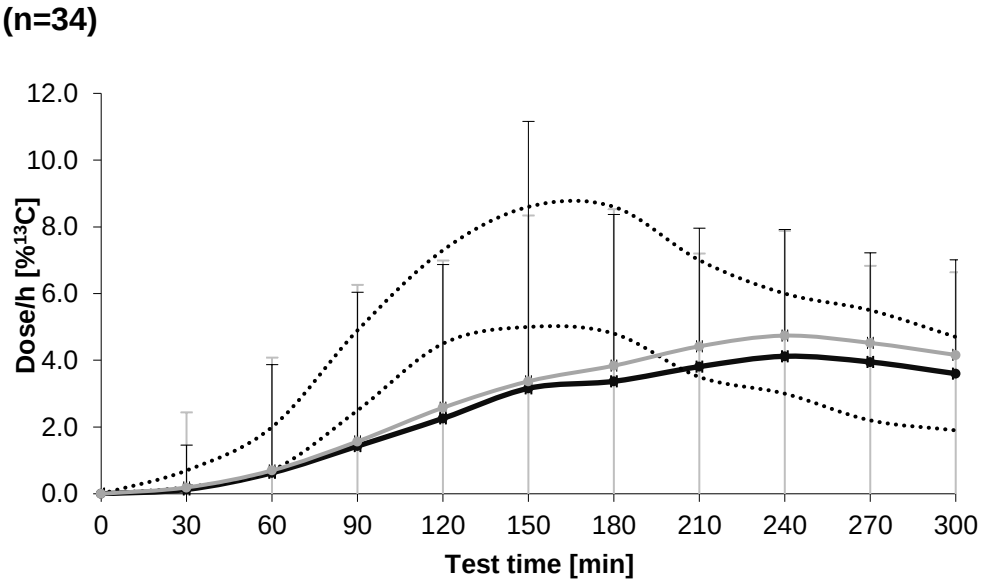
The minimum reference value in the IRIS® system software for a 5-hour cPDR is reported to be 13.0%. In the breast-fed baby group 21 out of 38 (55%) results were found to be lower than

the minimum value of the reference range and 5 out of 16 (31%) of formula-fed infants had a 5-hour cPDR test result below the 13% cut-off point.

3.7.4 Percentage of ^{13}C recovered dose/h of the initial amount of ^{13}C administered (%^{13}C dose/h)

In the third analysis results were expressed as the percentage of ^{13}C recovered dose of the initial amount of ^{13}C administered per hour. This analysis was performed on the 34 infants who had their $^{13}CO_2$ excretion measured on both IRIS and FANci2 devices. The average results over 300 minutes are shown in Figure 7.

Figure 7 Percentage ^{13}C of the original administered dose recovered/hour for IRIS and FANci2



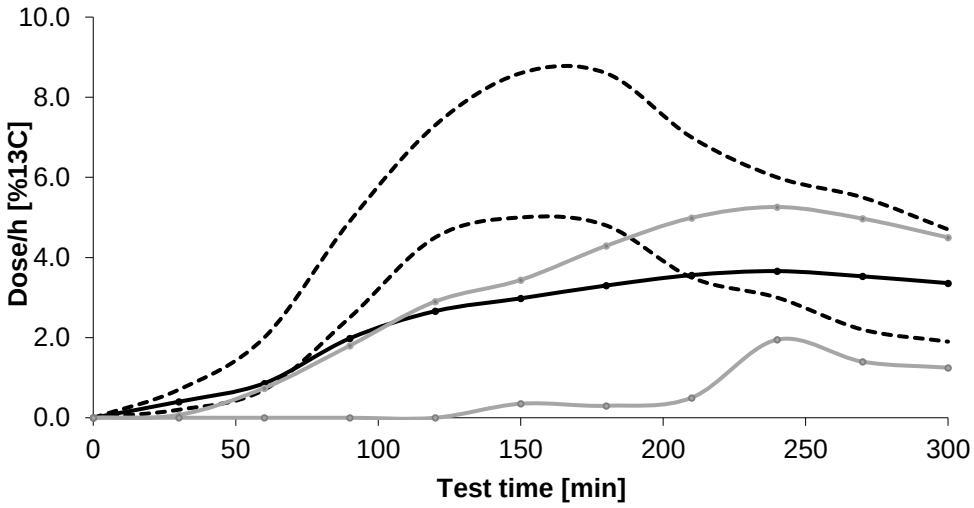
Black small dotted curve: minimum and maximum cPDR values of the reference range of the NDIRS system software IRIS®. Grey solid curve: mean % ^{13}C dose/h results of the IRIS. Black solid curve: mean ^{13}C dose/h for the FANci2. Positive and negative error bars represent the range of results: grey error bars IRIS, black error bars FANci2.

Between 180 and 300 minutes the results on FANci2 were significantly ($p < 0.05$) below IRIS. Bland and Altman analysis revealed a significant bias ($p = 0.000$) with an average 0.62% higher % ^{13}C dose recovered/h on IRIS compared with FANci2.

3.7.5 Percentage ^{13}C dose/hour results: comparison between breast-/ and formula fed infants

Results of the total 54 infants measured (39 on IRIS and 15 on FANci2) were combined to assess the mean % ^{13}C dose/h recovered of the original administered dose according to whether they were breast-/or formula-fed, as per Figure 8.

Figure 8 Mean %^{13}C dose/h in breast-/or formula-fed infants



Black dotted curve: minimum and maximum dose/hour (%^{13}C) of the reference range set in the NDIRS system software IRIS®, black curve: breast-fed baby results, grey curves: mean and lowest results in formula-fed babies

In both groups of breast-fed and formula-fed infants the results show a much slower increase of $^{13}CO_2$ excretion compared to the reference data of the IRIS®. A maximum momentary $^{13}CO_2$ breath excretion of 5.0-8.6% dose/hour is observed at 150 minutes in the reference range of the NDIRS system software IRIS®. Mean maximum momentary $^{13}CO_2$ peak excretion of breast-fed infants in the current study was 3.7±2.2% after 240 minutes compared to 5.3±2.3% for formula-fed infants, also after 240 minutes. Statistical analysis by Mann-Whitney U test revealed a significant difference (p=0.03) in peak ^{13}C excretion rates between the two groups of breast-fed and formula-fed infants after 4 hours. The lowest maximum momentary $^{13}CO_2$ peak breath excretion in breast-fed infants was 0.05% and in formula-fed infants 1.9% after 240 minutes. The minimum reference value in the IRIS® system software for a 4 hour % ^{13}C dose/h result is reported to be 3%^{13}C. In the breast-fed group 17 out of 38 (45%) results were found to be lower

than the minimum value of the reference range and in the formula-fed group 3 out of 16 (19%) had a 4 hour % ^{13}C dose/h result below the 3%^{13}C cut-off point.

3.8 Longitudinal ^{13}C MTG breath test assessment

Results for the longitudinal assessment of 53 control babies with at least three (39 babies had four) consecutive ^{13}C MTG breath test results are presented in **Table 3.**

Table 3: Longitudinal assessment of control babies - overview

	First breath test	Second breath test	Third breath test	Fourth breath test/yoghurt
Number of participants	48	52	52	47
Age (month)	3.4 ± 0.4 (2.5-4.8)	6.8 ± 0.7 (6.0-8.5)	9.7 ± 0.7 (9.0-11.5)	14.6 ± 3.3 (9.0-21.5)
Basal Delta 0 (‰)	-27.4 ± 2.6 (-33-21)	-27.6 ± 1.8 (-33-23)	-27.4 ± 2.2 (-32-23)	-25.9 ± 1.6 (-29-22)
5hour cPDR (%13C)	13.2 ± 7.1 (0-30.1)	13.6 ± 6.6 (0-28.9)	12.7 ± 5.9 (3.0-26.8)	14.9 ± 5.9 (5.6-29.6)

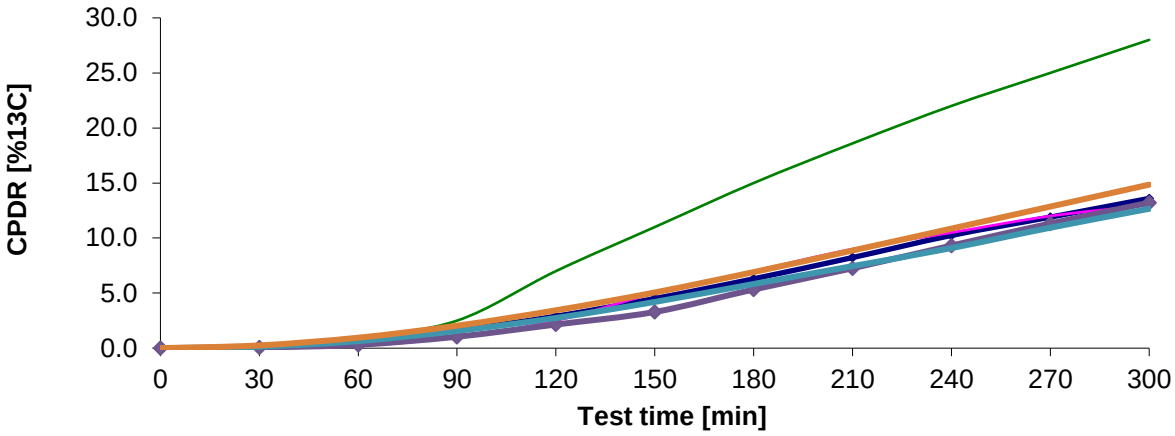
cPDR = cum percentage dose $^{13}CO_2$ recovered

Statistical analysis revealed no significant difference between mean results in babies < 5 months of age compared to the cPDR values in the older age groups; 6-9 months and 9-12 months and 9-24 months old, with the different test meal of yoghurt.

The database of the IRIS® system provides in its software the resultant data from a group of healthy individuals. The data includes the mean, maximal, and minimum values for ^{13}C cumulative dose recovered values and %^{13}C dose/hour values from a group of healthy subjects with normal fat absorption following the test procedure using ^{13}C MTG breath test with MS technique.

To be able to show mean cPDR values of control babies in the different age groups compared with the reference-values included in the IRIS® software, control baby results are plotted against the reference values in **Fig 9.**

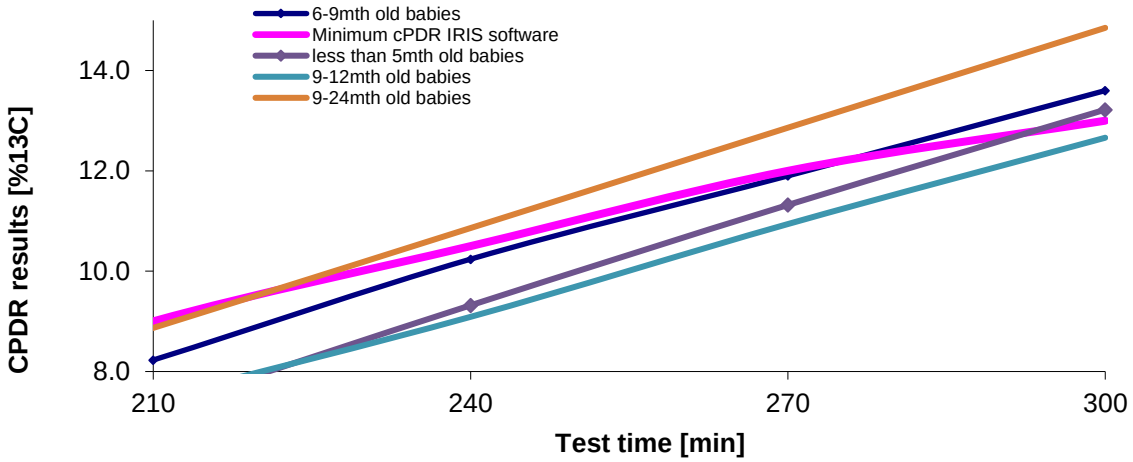
Figure 9A Mean cum dose of $^{13}CO_2$ recovered values of control babies in the different age groups compared to the reference-values in the IRIS software



Green line maximum cPDR and pink line minimum cPDR of reference values (IRIS®-software), purple line less than 5-month-old control babies, dark blue line between 6-9 month old control babies, light blue line between 9-12 month old control babies and orange line cPDR of 9-24 month old control babies.

Figure 9B Mean cum dose of $^{13}CO_2$ recovered values of control babies in the different age groups compared to the minimum cut-off reference-values in the IRIS® software

Figure 9B: Detailed overview



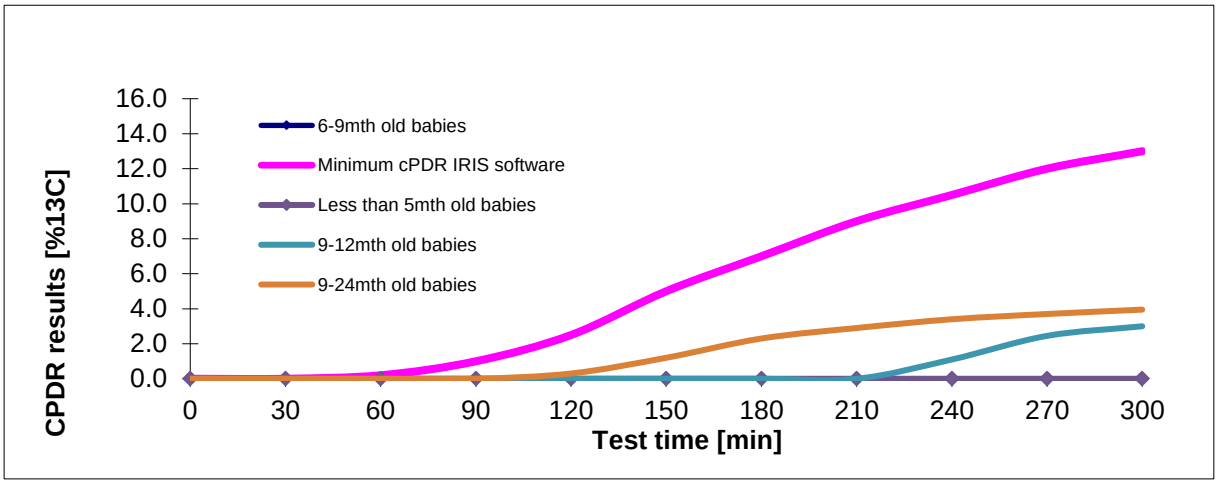
All cPDR values of all different age groups are around the minimum cPDR values of the reference range. In detail compared to the reference values of the IRIS® software mean cPDR values of control-babies at the age group of less than 5 months are within the reference values between 0-90min, below the minimum value of the reference range between 120-270min and the cPDR value of 300min is inside the reference range again. At the age group of 6-9 months, cPDR values between 0-120min are within the reference range, between 150-270min below the minimum reference range and the cPDR of 300min within the reference range again. At the age of 9-12 months cPDR between 0-120min are inside the reference range and cPDR values between 150-300min are below the minimum cPDR value of the reference range. And at the age of 9-24months cPDR between 0-150min are within the reference range, 180-210min below the minimum cPDR value of the reference range, and between 240-300min within the reference range again. The minimum reference value of the IRIS® software for a 5-hour cPDR is reported to be 13.0%. In all age groups, except for the age group of 9-12 months (cPDR of 12.7%), mean cPDR values are above the minimum reference cPDR values. Even though mean cPDR results are not significantly different from the minimum reference values of the IRIS software in the different age groups they are certainly lower than the mean cPDR reference values reported in the IRIS® software.

One should not compare mean results of the control babies with the reference values of the IRIS® software to decide if the reference range for older children and adults would be applicable

for babies. Individual 5-hour cPDR breath test results of control babies show, that a percentage of between 39% (9-24months old with the test meal of yoghurt) to 56% (9-12months old) are lower than the minimum reference range in all age groups.

Looking at the minimum cPDR results of babies in the different age groups compared to the minimum reference cPDR values in the IRIS software - as shown in **Fig 9C** - our results confirm that even up to an age of at least 24 months these reference values would be over diagnosing PI and therefore are not applicable in babies' first two years of life.

Figure 9C Minimum cum dose of $^{13}CO_2$ recovered values of control babies in the different age groups compared to the minimum cut-off reference-values in the IRIS® software



The min cPDR results of babies <5 months of age and babies of 6-9 months of age are superimposed on the baseline.

3.8.1 Repeatability of the MTG breath test in individual healthy babies

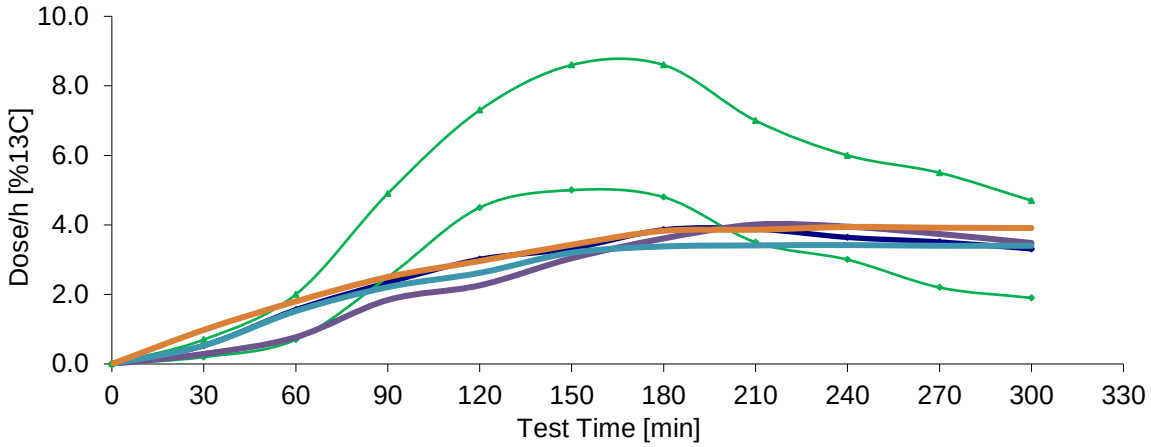
Out of the 53 babies with longitudinal ^{13}C breath test analysis, 14 babies had three consecutive breath tests in the different age groups and 39 babies had four consecutive breath test analysis. Of the 14 babies with three consecutive breath tests one baby had breath test results of <13% cPDR over the whole 3 breath tests, three babies had results >13% cPDR in one out of three analysis, eight babies had results >13% cPDR in two out of three breath test analysis and 2 babies had results >13% cPDR in all three breath test analysis.

Out of the 39 babies with four consecutive breath tests three babies had breath test results of <13% cPDR over the whole 4 breath tests, twelve babies had results >13% cPDR in one out of four analysis, eight babies had results >13% cPDR in two out of four breath test analysis, twelve babies had results >13% cPDR in three out of four breath tests, and 4 babies had results >13% cPDR in all four breath test analysis.

Only three out of 14 babies (21%) with three consecutive breath tests, and seven out of 39 babies (18%) with four consecutive breath test analysis, had consistent results, and only 6 babies out of the 53 assessed healthy babies had consistent results above the reference cut-off point of 13% over the whole series of three to four breath tests.

3.8.2 Percentage ^{13}C dose/hour results of control babies in the different age groups

Figure 10 Percentage ^{13}C dose/hour results of control babies of the different age groups compared to the reference values in the IRIS® software.



Green lines minimum and maximum dose/hour (%13C) of reference IRIS software data, purple line less than 5-month-old control babies, dark blue line between 6-9 month old control babies, light blue line between 9-12 month old control babies and orange line %^{13}C dose/hour results of 9-24 month old control babies.

In all age groups %^{13}C dose/hour results show values above the minimum dose/hour results compared to the reference range between 0-60 minutes, but then ^{13}C dose/ hour rates drop below the reference values between 90-180 minutes and are again above the reference values from the 210 to 240 minutes onwards until the end of the test at 300 minutes. %^{13}C dose/hour results in all age groups show a delayed increase after the 60-minute reference point, only reaches minimum ^{13}C dose/hour reference values by 210 minutes and in some age-groups increase even further until 300 minutes or drop slightly but not as much as the reference range would suggest.

A maximum momentary $^{13}CO_2$ breath excretion of 5.0-8.6 ‰dose/hour is observed at 150 minutes in the reference range of the IRIS® software. Maximum mean momentary $^{13}CO_2$ peak excretion of the control babies depends on the age group; less than 5 months 4.10‰ and between 6-9months 3.88‰ after 210 minutes; between 9-12 months 3.42‰ and between 9-12 months 3.94‰ after 240 minutes. This delayed peak excretion might be the reason of a slower gastric emptying rate of the babies compared to older children and adults.

3.9 Different test meals

The study also compared ^{13}C MTG breath test results between the test meal of apple gel and the test meal of yoghurt in babies of 9-12 months old: third breath test, and 9-24 months old: fourth breath test. CPDR values were slightly higher but not significantly higher when yoghurt was used as a test meal compared to apple gel. Comparison between maximum momentary $^{13}CO_2$ peak excretions showed that both test meals of apple gel and yoghurt lead to a maximum excretion at 240 minutes and the yoghurt showed a slightly 3.94‰dose/hour higher, but not significantly higher, excretion rate compared to the apple gel 3.42‰dose/hour.

4 DISCUSSION

This dissertation aimed firstly to evaluate the pancreatic function status by using both the ^{13}C MTG breath test with NDIRS technique and FE1 measurements with the monoclonal and polyclonal Elisa assays in breast-fed and formula-fed infants consecutively diagnosed with CF by neonatal screening and secondly the evaluation of the ^{13}C MTG breath test in healthy babies to assess whether the lower limit of normal pancreatic function as indicated by the manufacturer was applicable to healthy breast-fed and formula-fed babies in the first two years of babies life.

4.1 Evaluation of the ^{13}C MTG breath test to assess pancreatic function

Our study results indicate that ^{13}C MTG breath testing using NDIRS is not an adequate alternative assessment for the determination of pancreatic phenotype in CF infants compared to fecal fat balance studies. Specifically, the tests performed poorly in recognizing the PS phenotype as evident by ^{13}C MTG breath test's only 7 (option 1) and 4 (option 2) of the 13 PS infants on fat balance tests were correctly identified with a low sensitivity of 31-38%. Regarding the ^{13}C breath test these results are variably consistent with adult studies. For instance, the appearance rate of the ^{13}C tracer and pancreatic lipase secretion rate during a pancreatic stimulation test (PST) in adults with chronic pancreatitis or normal controls the sensitivity of $^{13}CO_2$ production for predicting lipase output were good at 89% and 81% respectively (42). Although another study confirmed that the ^{13}C MTG breath test sensitively reflected exocrine pancreatic insufficiency with fat malabsorption (92-100%), it demonstrated only limited sensitivity (46-62%) for the detection of mild cases with pancreatic impairment with normal fat absorption (94). The ^{13}C MTG breath test has also proved useful in older children for predicting fat malabsorption with over 90% sensitivity and specificity (145) but the study only included 6 PI children. Studies in neonates and young children are limited. One study used the ^{13}C MTG breath test with the technique of MS to measure development of fat digestion in infancy (97). They concluded that infants over one month of age have cPDRs within the normal range previously reported for adults and older children. However, neonates have cPDRs ranging from 0-32% in the first week of life to 7-26% in the last two weeks of the first month. In another study reference values for healthy children of different ages from premature infants (excluding breast-fed infants) to teenagers have aimed to be assessed and age-specific test meals and sampling methods defined (134). The cumulative dose recovered (cPDR) for healthy pediatric controls after 6 hours was reported to be 23.9 $\pm$ 5.2% in premature infants, 31.9 $\pm$ 7.7% in full-term infants, 32.5 $\pm$ 5.3% in children, and 28.0 $\pm$ 5.4% in teenagers. The mean value for adult controls was stated to be 35.6%, with a lower reference limit of 22.8% (134).

4.2 Evaluation of FE1 test to assess pancreatic function

Our findings also indicate that FE1 testing is not an adequate alternative assessment for the determination of pancreatic phenotype in CF infants as compared to fecal fat balance studies. Both methods of FE1 assays, the monoclonal and the polyclonal assay, demonstrated good sensitivity in CF PI infants of 100% - 92% respectively, however, in CF PS infants, poor sensitivity rates of 46/54% and 45% occurred. These results are consistent with other studies in older children and adults demonstrating high sensitivities (82-100%) for diagnosing PI but considerably more variable sensitivity (25-100%) in diagnosing PS (9, 91, 93, 126, 127, 137).

4.3 FE1 and longitudinal FE1 measurements in babies less than 2 years of age

Studies using FE1 to determine the pancreatic function status extensively in infants with CF using either the monoclonal ELISA or the polyclonal ELISA are limited. A pilot study of Wallis and colleagues (18) on three meconium samples of infants with CF homozygous for the ΔF508 mutation, showed zero activity of FE1 in all three newborns. Longitudinal testing over a subsequent period of 3 weeks proved FE1 concentration remained consistently zero. The authors stated that this pilot study highlights the potential role that this assay may play as a screening test for pancreatic function in neonates. On the contrary other studies disagreed. In a longitudinal analysis of pancreatic exocrine function in 27 CF infants carrying class 1 or 2 CFTR mutations by combining measurements of fecal fat excretion and FE1 were scheduled to be determined at diagnosis (3-4months of age), at 6 months and subsequently at 12 months of age. By 3-4 months of age, all infants had FE1 levels <200µg/g, yet steatorrhea was found in only 81.5% (fecal fat excretion >4g/d). At the age of 6 months, all screened CF subjects had FE1 <100µg/g with 96.3% having steatorrhea (fecal fat excretion >2g/d), and at 12 months of age all CF patients were pancreatic insufficient assessed with both measurements (140). Two further longitudinal studies on assessing pancreatic function status with FE1 in newborns have been published. A previous longitudinal study (11) in 236 CF infants under 2 years of age assessed with the monoclonal ELISA kit reported FE1 values >200µg/g stool in 122 patients (51% of CF infants), taken at day 195 (7-674), and <200µg/g in the remaining 114 patients, obtained at day 73 (5-606) in the first sample taken. Follow up of FE1 measurements obtained at day 207 (15-569) documented 18/122 (14.8%) CF infants had altered elastase concentrations leading to a PI diagnosis and in contrast 52/144 (45.6%) patients with a first measurement below the normal cut-off point, normalized their FE1 concentration. In another recent longitudinal study (112) pancreatic function was assessed in 61 CF infants during the first year of life with the polyclonal FE-1 ELISA kit. The study results showed 4 out of 48 infants with an initial FE1 level consistent

with PI ended up with a value in the PS range >200ug/g and 13 out of the 48 infants with an initial PI diagnosis had at least 1 value >200 µg/g during the year.

4.4 FE1 cut-off value between PI and PS

Others have suggested that the cut-off value or threshold between PI and PS for FE-1 of 200ug/g of stool is set too high (5, 93) and concluded even a value of 100ug/g or as low as 50ug/g would be more appropriate. In this regard in a study of non-CF adult chronic pancreatitis patients FE1 results were compared with fecal fat results and enzyme outputs during a PST (93). The patients were classified as having mild, moderate or severe exocrine pancreatic insufficiency (EPI) based on symptoms and PST results, noting those with mild or moderate disease had normal fat absorption i.e. were PS and severe EPI fat malabsorption or truly PI. However, in those with moderate disease (PS) over 50% had a FE-1 less than 50ug/g. In a similar but smaller study (137) in CF patients with PS on fecal fat collections had FE1 results varying from 54-162ug/g supporting the suggestion that 50 ug/g could be an appropriate cut-off for CF patients. Although the differences between the 2 studies is not readily apparent, they do support our findings that even with a cut-off of 100ug/g or greater for PI and PS patients there is still a large proportion (near 50%) of PS patients who would have been designated as PI over all age groups if using FE1 as the definitive test.

In summary, FE1 testing has to be used with caution. On the one hand FE1 reflects exocrine pancreatic status because it is well correlated with duodenal lipase, amylase, trypsin, and bicarbonates (27). It is a sensitive, specific, and relatively inexpensive, non-invasive test (38). Only a small, random sample is needed, making it more acceptable to patients and care providers than timed collections of stools. This protein is stable through a wide range of pH and temperature, up to one week at room temperature, making it ideal to collect at home, transport to central laboratory, and analyse electively (20). The human monoclonal elastase antibody does not cross-react with porcine elastase; thus, the test can be performed while patients are taking exogenous PERT (13, 18). However, on the other hand, CF is a heterogeneous disorder and the rare milder mutations associated with PS are not necessarily detected by FE1 testing (35, 138, 139). FE1 should only be measured on formed stool (20). The possibility of obtaining a false positive outcome is likely when the stool is diluted: in breast-fed infants an occurrence of loose or watery stools is not uncommon. A dilution effect can also happen as a result of factors such as infectious diarrhoea, severe enteropathies, short gut, or stool from an ileostomy, which applies to infants as well as older patients or in patients with severe intestinal enteropathy due to lack of stimulation to the pancreas (78). Significant variability in FE1 values has been described

in longitudinal studies assessing the pancreatic function status of CF infants during the first 2 years of life. Many infants were described with FE1 values assessed during the first and second year of life that were inconsistent with either initial or final phenotype evaluation. Clearly FE1 is not an ideal surrogate for a fat balance study in assessing pancreatic phenotype in CF patients. In spite of this evidence and cautions regarding its exclusive use (76) multiple units worldwide (11, 13, 18, 20, 27, 78, 112, 138, 139, 140, 142) have persisted with FE1 testing and it has become entrenched in protocols and guidelines (19, 121). Although satisfactory for the centres involved, with ease of performance of FE1 assays compared with the labour intensive and thus more expensive fat balance study which personnel are reluctant to perform, it is unsatisfactory for the majority of PS patients who are misdiagnosed as PI and placed unnecessarily on PERT. In this regard the largest and longest longitudinal study since the commencement of mutational screening programs (26) has demonstrated that 75% of PS patients have maintained their PS status to at least 10 years of age. Provision of the appropriate test to assess pancreatic function in these patients is mandatory and this is a role only filled currently by the fat balance study.

4.4 Control-baby data

4.4.1 Comparison between both NDIRS devices and reference values used in the system software IRIS®

The second main aim of this dissertation has been the evaluation of reference values, for the assessment of the adequacy of fat absorption set in the NDIRS system software IRIS® proposed for healthy children and adults using the ^{13}C-MTG breath test with MS technique and their applicability for healthy breast-fed and formula-fed as well as CF infants of less than 2 years of age with two different NDIRS devices. Firstly, our results demonstrate that there is a significant bias between the two NDIRS devices - IRIS and FANci2 - used for measuring $^{13}CO_2$, with slightly higher results measured with the IRIS compared to the FANci2. However, numerically the average differences between the devices were small (1.8% (for cPDR) and 0.62% for %13C dose recovered/h) and unlikely clinically relevant. Secondly, in a larger group of healthy infants the study shows that the ^{13}C cumulative dose recovered results after 5 hours in breast-fed and formula-fed infants are both below the normal range established in older children and adults with the technique of MS. In fact, as the range extends well below the normal minimum reference values for children and adults into a range seen in pancreatic insufficient patients, this NDIRS technology appears inappropriate for use in breast-fed and formula-fed infants with sufficient pancreatic function.

4.4.2 Use of the ^{13}C MTG breath test with NDIRS technique in breast-fed infants

To the authors' knowledge this study is the first using the ^{13}C MTG breath test in exclusively breast-fed infants. Other authors (67, 111) have been of the opinion that human milk might enhance fat digestion as human milk contains a bile salt stimulated lipase (BSSL), and have therefore excluded breast-fed infants (97, 134). The results of the current study reported a slightly, but not significantly, higher 5-hour cPDR result in formula-fed infants compared to breast-fed infants. However, when ^{13}C MTG breath test analyses between breast-fed and formula-fed infants were expressed as % ^{13}C peak recovery after 4 hours, a significantly lower result occurred in breast-fed compared to formula-fed infants. This discrepancy remains unexplained but needs to be confirmed using the gold standard measuring technique - mass spectrometry - prior to further investigation of the discrepancy.

4.4.3 Comparison of MS versus NDIRS technique

All published breath test studies using the ^{13}C MTG breath test and the MS technique (5, 68, 73, 75, 97, 129, 134, 136, 146) including the two studies on formula-fed infants, indicated mean cPDR results between 19-45% of ^{13}C recovered dose after 5 to 6 hours. Furthermore, others have demonstrated that it is possible to distinguish between pancreatic sufficiency and insufficiency status by using the ^{13}C MTG breath test and the technique of MS (136, 145, 146).

However, studies using the ^{13}C MTG breath test and the NDIRS are more like the current study results. One study in 10 healthy adults reported mean cPDR values of 30% after 5 hours, but the authors (79) stated that two out of ten healthy subjects had decreased ^{13}C exhalation, including one healthy subject having borderline results. Another study (3) assessed 20 healthy adult volunteers with the MTG breath test using NDIRS and indicated cPDR values after 4 hours of 14.6% (range 5.7-36.8%), and after 6 hours at 29.1% (range 10.3-59.3%). They also assessed a group of patients with chronic pancreatitis and concluded that the MTG breath test using the NDIRS technique cannot be recommended as a routine clinical method, because of marked data overlap between pathologic and normal values. These latter results are consistent with those of this dissertation in young babies.

Studies comparing the two techniques of NDIRS with the gold standard technique of MS using the MTG breath test are limited. One study in healthy adult volunteers and patients with chronic pancreatitis and exocrine pancreatic insufficiency concluded that the comparison of delta over base line and cPDR values by both techniques of NDIRS and MS showed a moderate to good linear correlation (14).

However, regarding the Bland and Altman analysis the limits of agreement were rather wide, leading to cPDR discrepancies of up to 18.9% between both methods. The authors of the study stated that high discrepancies could make MTG breath testing by NDIRS less suitable for exact evaluation of non-invasive indirect assessment of intraluminal duodenal pancreatic lipase activity. The current study results are in line with this contention that the NDIRS technique is not sufficiently sensitive, as shown by the normal values extending down to zero on the infant samples. The reason for this insensitivity of the NDIRS technique is not readily evident but could relate to the devices, the stability of the ^{13}C isotope MTG mixture, the amount of CO_2 expiry in a shallow breathing infant, slower gastric emptying in infants, or the ontogeny of the lipolytic pathways.

Results of this dissertation, measured on two different NDIRS devices, indicate that the reference range for the assessment of pancreatic function status set in the NDIRS system software IRIS® proposed for children and adults would fail as a good reference range for infants

either exclusively breast-fed or formula-fed, and babies in the first two years of life using NDIRS technique to determine pancreatic function status. All babies assessed in the current study were healthy, thriving well, and were without any symptoms of malabsorption. If the reference values of the IRIS® system software had been used for the assessment of fat digestion and subsequent absorption, 20 to 26 of the 54 breast-fed and formula-fed infants of less than 5 month of age would have been misclassified with pancreatic insufficiency. While the ^{13}C MTG breath test with the MS technique has the potential to be a suitable assessment of fat absorption in infants and babies, the technique of NDIRS appears too insensitive in this population.

4.4.4 Longitudinal assessment – repeatability of the MTG breath test

Studies assessing healthy volunteers or diseased patients longitudinally with a series of breath tests are limited. This dissertation found NDIRS technique to be too insensitive to assess pancreatic function with the ^{13}C MTG breath test in an infant population of less than 5 months of age. Therefore, the authors also wanted to investigate, whether with age increase of these previously assessed infants the ^{13}C MTG breath test would gain sensitivity. Our findings show that sensitivity of the ^{13}C MTG breath test with NDIRS technique could not be significantly increased with age during babies first two years of life, a percentage of between 39% (9-24 months old) to 56% (9-12 months old) of individual 5-hour cPDR results were found lower compared to the minimum reference point in all age groups. Our study group also examined intra-individual variation of the $^{13}CO_2$ response, in which no rate-limiting variation in pancreatic exocrine function was expected. However, out of the 53 longitudinally assessed babies with three to four consecutive MTG breath tests, only 6 babies (3%) had consistent results above the minimum ^{13}C cut-off point during the three to four breath test assessments with NDIRS.

Studies investigating repeatability or reproducibility of the MTG breath test in healthy or diseased volunteers are underlining the findings of this dissertation. The study of Murphy et al. (105) assessed the repeatability of breath tests based on the ingestion of ^{13}C-labeled lipid, involving [1-^{13}C] palmitic acid in healthy volunteers and reported poor repeatability. Also, in healthy adult controls and assessed with the technique of MS the study of Kalivianakis et al. (75) reported repeatability coefficient of the cumulative percentage ^{13}C excretion of 22.7%, meaning that with a 95% likelihood the second MTG breath test will be of 22.7% above or below the cumulative percentage ^{13}C excretion of the first test. They stated that these large repeatability coefficients are predominantly due to a considerable intra-individual variation and that it remains to be established whether the means and repeatability coefficients of diseased individuals allow a clear discrimination from unaffected individuals using MTG breath testing.

Swart et al (129) assessed the reproducibility of the MTG breath test on 17 controls mean age 31 (13-53y) and 8 adult CF pts on enzymes mean age 25 (18-31y). In controls statistical analysis revealed no significant difference between mean results in the first and second test: 35.0±5.5 vs 32.3±7.4%, mean difference in cPDR was 2.8±5.9. Calculating the standard deviation of difference according to Bland and Altman showed a value of 8 PDR for the combined group of controls and patients taken together. The authors stated that for a test that is intended to be used for clinical decision making this degree of reproducibility is reasonable but less than desirable. And the study of Herzog (68) assessed the discrimination capacity and repeatability on 9 CF patients on PERT and 10 healthy children who underwent the ^{13}C MTG breath test twice at a 2- to 4-week interval with MS technique. They reported that the test distinguished well between patients with severe exocrine pancreatic insufficiency on enzymes with mean (SD) cPDR (%) recoveries of 16.96 (7.1) in the first and 15.76 (6.8) in the second breath test in CF patients and 39.76 (5.1) and 43.75 (5.9) in healthy controls for test 1 and 2. However they also stated that repeatability of the ^{13}C MTG breath test proved to be poor. Herzog (68) concluded that technical improvement in vCO_2 quantification could contribute to the improvement of the test repeatability.

In summary, our study results are in line with other studies reporting a rather poor repeatability of healthy or diseased patients assessed with the MTG breath test with either technique of MS or NDIRS and therefore proven the MTG breath test to be a poor choice for the longitudinal assessment of CF patients. It needs to be born in mind that after application of ^{13}C-MTG, part of the ^{13}C-labeled tracer does not undergo β-oxidation, leading to formation of $^{13}CO_2$, that enters other metabolic pathways, for example, binding to ghrelin in gastric and pancreatic cells. In consequence, cumulative ^{13}C exhalation after application of ^{13}C-MTG does not amount to 100% of the dose. A good diagnostic test should have a low intra-individual variation and good repeatability, therefore, the fecal fat balance test remains the gold standard test in assessing CF patient's pancreatic function longitudinally to determine their change over from PS to PI.

4.5 Conclusion

Neither the ^{13}C MTG breath test nor FE1 tests accurately diagnose CF PS patients and cannot be relied upon to fulfil a surrogate role for fecal fat determinations. However, the relative ease of performance of both tests is of merit and modification and further research using modifications of both tests would be of value. To take advantage of the full potential of breath tests, further standardization and improvement are warranted with respect to choice and dose of tracer, nutrient dose, composition of test meal, conditions of performance and identification of the optimal parameters for analysis that may differ for evaluation of pancreatic exocrine function and fat absorption. Also, for instance the use of mass spectrometry instead of NDIRS for the ^{13}C MTG breath test and assessing FE1 post feeds or on a recurrent basis e.g. at 6 and 12 months of age may improve the current diagnostic uncertainty instead of a spot neonatal sample as currently recommended. All modifications would still require validation by fat balance studies and until this type of research is performed fat balance studies will remain the gold standard measurement for the assessment of pancreatic function.

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