UNIVERSITY OF LIVERPOOL

FACTORS RESPONSIBLE FOR THE DEVELOPMENT OF DIABETES IN ADULTS WITH CYSTIC FIBROSIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree M.D.

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Declaration

No portion of the work referred to in this dissertation has been submitted in support of an application for a degree qualification of this or any university or institute of learning.

The design of the work presented in this thesis is mine and my supervisor Professor Martin Walshaw. The original research idea and development of the protocol were the contributions of Professor Walshaw, Dr Kamlesh Mohan and myself. I recruited subjects, performed experimental work including the ultrasound scans, collected blood samples, analysed and presented the data in this thesis. The laboratory analyses of blood samples were carried out at accredited laboratories in Liverpool and London. Statistical support was provided by Mr James Mcshane at Liverpool Heart and Chest Hospital.

Glossary

ADA. American Diabetes Association

CFRD. Cystic Fibrosis Related Diabetes

CFTR. Cystic Fibrosis Trans-membrane Regulator

CGM. Continuous Glucose Monitoring

DI. Disposition Index

DPP4. Dipeptidyl Peptidase-IV

FPG. Fasting plasma glucose

GIP. Gastric Inhibitory Peptide

GLP_1. Glucagon like peptide-1

HbA1c. Glycated Haemoglobin

IDDM. Insulin Dependent Diabetes Mellitus

IFCC. International Federation of Clinical Chemistry

IFG. Impaired Fasting Glucose

IGT. Impaired Glucose Tolerance

MMT. Mixed Meal Tolerance Test

NGT. Normal Glucose Tolerance

NIDDM. Non-Insulin Dependent Diabetes Mellitus

OGTT. Oral Glucose Tolerance Test

PYY. Pancreatic Polypeptide

T1DM. Type 1 Diabetes Mellitus

T2DM. Type 2 Diabetes Mellitus

WHO. World Health Organisation

ABSTRACT

As survival in Cystic Fibrosis (CF) has improved dramatically, Cystic Fibrosis Related Diabetes (CFRD) has now come to the forefront, with an increasing prevalence. CFRD is associated with worse pulmonary function, under nutrition and an increase in early mortality. The pathophysiology of CFRD is complex and not fully understood, with CFRD unlike other types of diabetes.

In CF, gastric emptying and motility are likely to be altered, changing the way in which nutrients are presented to the gut compared to normal individuals. Then, the relative progressive insulinopaenia that occurs in CF may result in diurnal changes in glucose and other nutrient handling, and abnormalities in the small intestine may alter incretin secretion and associated pancreatic enzymes.

The aim of this thesis was to enhance understanding of the physiology and regulation of gastric emptying, glucose handling and pancreatic hormones in CF individuals.

A series of experiments involving 10 healthy volunteers and 20 CF patients are presented in this thesis. Firstly, gastric emptying was measured using a novel easy technique and the responses compared to healthy control subjects, throughout the day. Secondly, glucose handling and secretory patterns of insulin, c-peptide, glucagon, incretin hormones (GLP_1 and GIP) and pancreatic polypeptide (PYY) throughout the day were compared.

The main conclusions that can be drawn from this thesis are: (1) There is delayed gastric emptying throughout the day in CF subjects (2) The novel and inexpensive bedside technique used in this study, provides a simple method of assessing gastric emptying (3) The mixed meal is more physiological in CF (4) In CF subjects without frank diabetes, there is deficient glucose handling with differences in the afternoon and testing in the evening deserving more attention (5) The quantity of insulin secreted is similar in CF and healthy subjects with an insulin lag in CF subjects and glucagon does not appear to contribute to elevated blood sugars (6) Insulin sensitivity is highest in the afternoon and appears to play a significant part in

improving glucose handling in the afternoon (7) β cells take longer in in the evening to produce insulin, a concept I refer to as 'pancreatic fatigue' (8) There is no difference in GLP_1 or GIP secretion following a mixed-meal, but GIP hypersecretion exists early in the response to the OGTT in the CF group (9) CF subjects have lower PYY levels likely to be secondary to existing pancreatic insufficiency.

Areas of potential future research based on this thesis are also outlined.

Acknowledgments

I am greatly indebted to Professor Martin Walshaw for his constant encouragement, guidance and inspiration without which this work would not have been possible. His enthusiasm and dedication to high quality research and clinical care and improving the care of patients with CF has been inspirational. He has been a wonderful mentor to me in more ways than just in medicine and I will always be grateful for the most important thing he taught me - humility with patients.

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1.1 Diabetes

1.1.1 History of Diabetes

The earliest recorded descriptions of 'Diabetes' date back to around 1550 BC. The term Diabetes is derived from the Greek word for 'Siphon' and 'Mellitus' or sweet was a subsequent addition to the term to distinguish it from another polyuric condition 'Diabetes Insipidus', in which urine was tasteless.

Diabetes was linked to hyperglycaemia and defects in glucose metabolism for many centuries and the first linking of diabetes to the internal secretion of the pancreas was by Minkowski and Von Mering in 1890.

A major landmark was the discovery of islet cells by Paul Langerhans in 1869 and subsequently the most significant development with relation to Diabetes Mellitus was in 1921: the discovery of insulin by Banting and Best [1]. Eventually Frederick Sanger in 1955 identified the insulin sequence and the treatment of diabetes mellitus was revolutionised [2]. Following on from this discovery, over the last six decades there have been major developments in the understanding of the aetiology, types, classification and management of diabetes with the development of more refined forms of insulin and oral agents.

However, the management of diabetes is still a challenge for most physicians.

1.1.2 Diagnosis of diabetes

Since 1965 the World Health Organisation (WHO) has published guidance and criteria for the diagnosis and classification of diabetes. In 1965, a fasting blood sugar (glucose) value ≥7.2 mmol/L was used for diagnosis [3]. Subsequent revisions were made over the next three decades. In 1979, the National Diabetes Data Group, proposed new criteria and introduced the terms 'Non-Insulin Dependent Diabetes Mellitus' (NIDDM) and 'Insulin Dependent Diabetes Mellitus' (IDDM) to distinguish the two main types of diabetes and also included the term 'Impaired Glucose Tolerance' (IGT) [4].

The American Diabetes Association (ADA), in 1997, revised the criteria for diagnosis to a new lower threshold for fasting blood glucose of 7 mmol/L and introduced a new category 'Impaired Fasting Glucose' (IFG) for those with fasting glucose values between 6 and 6.9 mmol/L [5].

Although similar, there are small differences between the WHO and ADA criteria. The ADA consider a cut-off value of 5.6 mmol/L for IFG based on studies on Pima Indians and other ethnic groups [6] and the WHO cut-off value remains at ≥6.1 mmol/L. The WHO recommendations are based upon diagnostic criteria being able to distinguish a group with significantly increased premature mortality and increased risk of microvascular and cardiovascular complications.

1.1.3 Diagnostic tests to define glycaemic status

The WHO recommends that venous plasma glucose be the standard for measuring and reporting.

Oral Glucose Tolerance Test (OGTT): There is considerable debate about the role of the OGTT in the diagnosis and classification of diabetes. A number of studies have reported that fasting plasma glucose and 2-hour post-glucose plasma glucose do not identify the same people as having diabetes. Using only fasting plasma glucose criteria will fail to diagnose approximately 30% of people with diabetes [7]. There are documented increased rates of mortality and worse outcomes in relation to diabetes diagnosed on the basis of the 2-hour plasma glucose result. The Hoorn study [8] showed that all cause cardiovascular mortality over a 8 year follow-up was significantly elevated in those with a 2 hour plasma glucose ≥11.1 mmol/L but not in those with a fasting plasma glucose ≥7 mmol/L. The 2-hour plasma glucose is also important for microvascular complications with an increased incidence of retinopathy [9] in newly diagnosed diabetics with 2-hour values ≥11.1 mmol/L and even in those with a fasting value ≥7 mmol/L. Hence the recommendation by the WHO that the OGTT is an important test and is the only means of identifying people with IGT and is an important exclusion test in asymptomatic people. The WHO recommends that the OGTT be

used, with fasting glucose values between 6.1 and 6.9 mmol/L to determine glucose tolerance status. Figure 1-1 on page 4 shows the diagnostic values for diabetes mellitus.

The test is recommended by the WHO [10] and although the ADA acknowledges the OGTT as a valid diagnostic test, in clinical practice it is inconvenient, costly and poorly reproducible [11].

Glycated Haemoglobin (HbA1c): HbA1c was identified in 1969 as an unusual haemoglobin in patients with diabetes [12] and subsequently numerous studies correlating HbA1c to glucose measurements suggested it could be used as an objective measure of glycaemic control.

Since the 1980s HbA1c has been widely used in clinical practice and reflects average plasma glucose over the previous 8 – 12 weeks [13]. It soon became the preferred test for assessing glycaemic control in diabetics as it could be performed any time in the day and did not require any special preparation or fasting. In addition, the HbA1c measure bypassed the problem of variability of daily glucose levels. The WHO, after initially rejecting the test, revised its guidance to include this as a diagnostic test. An HbA1c value of 6.5% (48 IFCC) is recommended as the cut off point for diagnosing diabetes.

Diabetes	
Fasting plasma glucose 2–h plasma glucose*	≥7.0mmol/l (126mg/dl) **or** ≥11.1mmol/l (200mg/dl)
Impaired Glucose Tolerance (IGT)	
Fasting plasma glucose 2–h plasma glucose*	<7.0mmol/l (126mg/dl) **and** ≥7.8 and <11.1mmol/l (140mg/dl and 200mg/dl)
Impaired Fasting Glucose (IFG)	
Fasting plasma glucose 2–h plasma glucose*	6.1 to 6.9mmol/l (110mg/dl to 125mg/dl) **and (if measured)** <7.8mmol/l (140mg/dl)

* Venous plasma glucose 2–h after ingestion of 75g oral glucose load

* If 2–h plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded

Figure 1-1: Diagnostic criteria for diabetes

1.1.4 Classification of diabetes

The first classification of diabetes was by the WHO in 1980. This has then been modified over the last three decades. Since 1998, a new classification based on collaboration between the WHO and ADA groups have been in use [6, 11]. Diabetes is classified into four major groups – Type 1, Type 2, gestational and other types of diabetes (Table 1-1) and their main differences are shown in Figure 1-2.

Type 1 and Type 2 diabetes are briefly discussed below.

Table 1-1: The classification of diabetes

Type 1 (beta cell destruction, usually leading to absolute insulin deficiency) • Autoimmune • Idiopathic
Type 2 (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance)
Other specific types • Genetic defects of beta cell function • Genetic defects in insulin action • Diseases of the exocrine pancreas • Endocrinopathies • Drug or chemical induced • Infections • Immune-mediated diseases • Other genetic syndromes sometimes associated with diabetes
Gestational diabetes

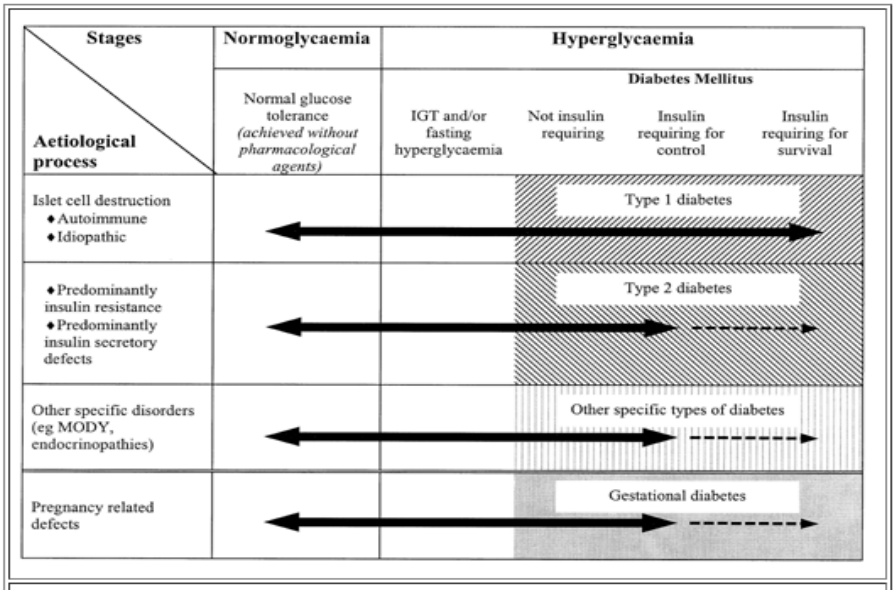


Figure 1-2: Differences in types of diabetes

From Diabetic Med. 15: 539-553 (1998)

Type 1 Diabetes (TIDM)

T1DM usually diagnosed in children and young adults is a chronic autoimmune disease in which destruction or damage of β cells in the islets of Langerhans results in insulin deficiency and hyperglycaemia. Autoimmunity is the predominant mechanism of T1DM, but may not be its primary cause. T1DM precipitates in genetically susceptible individuals, very likely because of an environmental trigger.

The many types of insulin replacement available have dramatically increased the quality of life of T1DM patients and insulin remains the major treatment in the short and long term.

Type 2 Diabetes (T2DM)

T2DM is a chronic metabolic disorder with steadily increasing worldwide prevalence. As a result, it has become an epidemic in some countries with the number of people affected

expected to double in the next decade due to increases in an ageing population. A number of lifestyle factors are known to be important to its development - physical inactivity, sedentary lifestyles, cigarette smoking and excess alcohol consumption [14]. There is a strong genetic association, where having first-degree relatives with T2DM significantly increases the risk of developing the condition. Concordance among monozygotic twins is close to 100%, and about 25% of those affected have a diabetic family history [15].

T2DM is characterized by insulin insensitivity as a result of insulin resistance, declining insulin production, and eventual pancreatic β cell failure [16, 17]. This leads to decreases in glucose transport into liver, muscle, and fat cells, and increases in breakdown of fat with hyperglycaemia. Moreover, the involvement of impaired α cell function has also been recognized in the pathophysiology of T2DM [18].

1.1.5 Complications of diabetes

The complications of diabetes involve different organ systems and may be found at the time of diagnosis of diabetes, or many years after the onset of diabetes. Many complications are secondary to persistently elevated levels of blood glucose. These complications can broadly be divided into acute and chronic (See Table 1-2 on page 8) and are discussed below.

Acute complications

Acute complications occur as a result of an absolute or relative lack of insulin that lead to changes in glucose metabolism [19]. These can broadly be divided into those associated with hypoglycaemia and hyperglycaemia. Acute complications of hyperglycaemia include diabetic keto-acidosis seen in Type 1 diabetics and non-ketotic hyper-osmolar coma (HONK) seen mainly in Type 2 diabetics. HONK is characterised by very high blood sugars, dehydration and shock. Both conditions need managing rapidly and aggressively with fluid and insulin therapy.

Hypoglycaemia, most often an acute complication secondary to glucose lowering drug therapy (insulin or oral hypoglycaemic agents) is also common.

Chronic complications

Long-term exposure to hyperglycaemia results in significant end-organ damage, classified in two categories – microvascular and macrovascular damage. In the general population, diabetes is the leading cause of blindness (retinopathy), end-stage renal disease (nephropathy) and small nerve damage (neuropathy). The cause of this end-organ damage involves several mechanisms leading to the accumulation of advanced glycation end-products [20]. These complications are often progressive and irreversible. However, increased awareness and advances in our understanding of the pathogenesis of diabetes has allowed earlier detection and interventions to slow the progression of diabetic complications.

Table 1-2: Complications of diabetes

<table>
<tr><td>Acute</td><td colspan="2">Diabetic ketoacidosis
Hyperosmolar hyperglycaemic non-ketotic coma
Hypoglycaemia</td></tr>
<tr><td rowspan="2">Chronic</td><td>Macrovascular
Coronary disease
Cerebrovascular disease
Peripheral vascular disease</td><td>Microvascular
Retinopathy
Nephropathy</td></tr>
<tr><td>Neuropathy
Autonomic
Somatic</td><td>Pulmonopathy (described later)</td></tr>
</table>

1.1.6 Factors affecting glucose handling

In the fasted and postprandial states, factors which affect rates of glucose entry into the circulation are more important for maintaining normal glucose homeostasis than those that affect the rate of removal of glucose from the circulation. The liver is responsible for glycogenolysis and some gluconeogenesis. Postprandially, almost all endogenous glucose release is via gluconeogenesis [21] - the liver and kidney contributing approximately 8 and 2 μmol/kg/min, respectively; Of the total glucose released into the circulation (10 μmol/kg/min) the brain, splanchnic tissue, muscle, adipose tissue, and blood cells account for approximately 5, 2, 1.5, 0.5, and 0.5 μmol/kg/min of glucose uptake (See Figure 1-3).

The regulation of glucose entry into the circulation is complex, being influenced by hormones, the sympathetic nervous system, and meal composition and substrates (i.e., free fatty acid concentrations and availability of gluconeogenic precursors). Of these, insulin and glucagon are most important both in fasting and postprandial states. Incretins that form the entero-insular axis contribute by altering gastric emptying, insulin and glucagon secretion. These hormones are discussed individually in subsequent sections of this review.

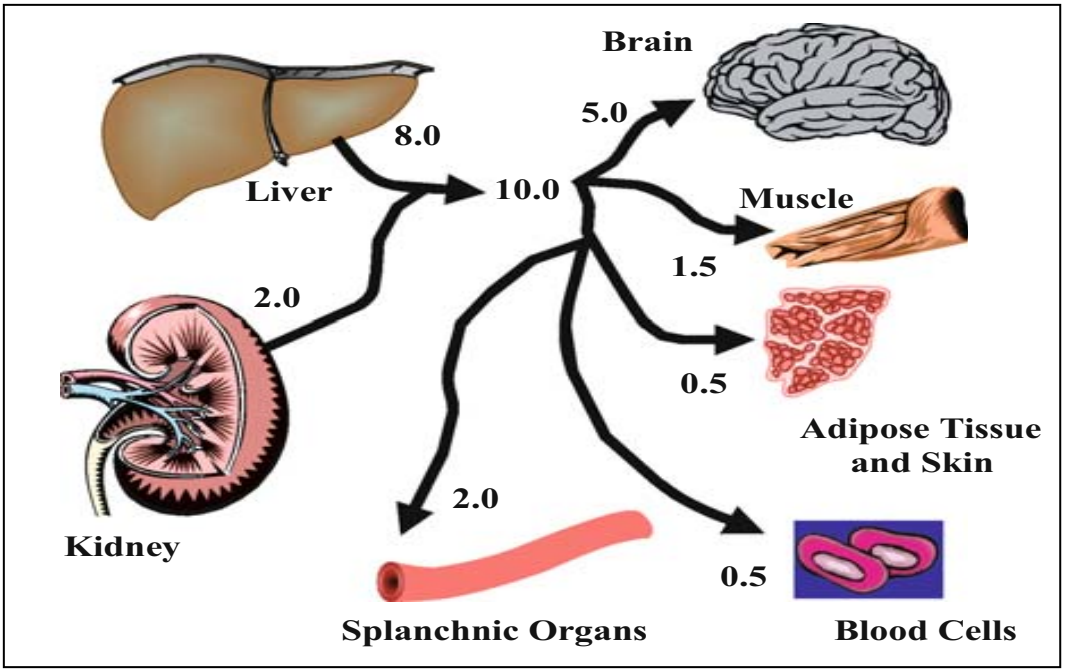


Figure 1-3: Glucose utilization and production in the post-absorptive state

From: Gerich J.E. Normal Glucose Homeostasis. In: Poretsky L, editor. Principles of Diabetes Mellitus: Springer; 2010 [21]

1.1.7 Management of diabetes

The importance of tight glycaemic control in the management of diabetes has been firmly established and classed into the following categories [*Adapted from Management of diabetes, Quick Reference Guide 2010, (SIGN) Scottish Intercollegiate Guidelines Network*].

- Lifestyle management
  - *access to structured education programmes*
  - *smoking cessation*
  - *weight loss*
  - *self-monitoring of blood glucose*
- Systematic screening for diabetic retinal disease
- Assess risk of developing a foot ulcer
- Psychosocial factors
  - regular assessment of a broad range of psychological and behavioural problems (e.g. anxiety, depression and eating disorders)
- Management of Type 1 diabetes
  - either regular human or rapid-acting insulin analogues
  - basal insulin analogues are recommended in adults with type 1 diabetes who experience severe or nocturnal hypoglycaemia
  - tailored insulin regime
  - optimisation of glycaemic control towards a normal level
- Management of Type 2 Diabetes (Pharmacological)
  - HbA1c target of 7.0% (53 mmol/mol)
  - Consideration of oral hypoglycaemic agents
  - DPP-4 inhibitors may be used to improve blood glucose control
  - GLP_1 agonists (such as exenatide or liraglutide) may be used to improve glycaemic control in obese adults
  - When commencing insulin therapy, bedtime basal insulin is recommended with dosage titration against morning (fasting) glucose

1.2 Cystic fibrosis related diabetes (CFRD)

The first recognition of cystic fibrosis related diabetes (CFRD) as a unique entity was in 1955 [22] and since then its association with a number of comorbidities and increased mortality has been well described. As survival in CF has improved dramatically over the last 50 years, CFRD has now come to the forefront. The median age of onset of CFRD is 20 years [23-25] with females more susceptible, tending to develop CFRD at a younger age [24, 26]. With newer diagnostic methods, CFRD can be present in about 20% of CF adolescents and up to 50% of adults [27] and is associated with increasing age, worse pulmonary function, under nutrition, liver dysfunction and steroid use [28, 29].

CFRD shares features of both Type 1 (occurs in young people and associated with insulin insufficiency) and Type 2 (mild insulin resistance present and ketosis is rare) diabetes. Unlike those with T1DM, those with CFRD do not develop a complete absence of insulin secretion and there is some retention of basal insulin secretion [30]. However, few CF patients have truly normal glucose metabolism. The differences between CFRD and the most common types of diabetes – Type 1 and 2 are shown in Table 1-3 (page 13). The presence of CFRD is associated with an increase in early mortality of up to six fold [31] and importantly weight and lung function decline have been shown to precede diagnosis of CFRD by standard criteria [32]. Insulin treatment confers a short-term benefit and in the long-term enhances nutritional status and pulmonary function, on average delaying decline in FEV_1 by 34 months [33].

Table 1-3: **Differences between Type 1, Type 2 and CF related diabetes**

	Type 1 DM	**Type 2 DM**	**CFRD**
Onset	Acute	Insidious	Insidious
Peak age of onset	Children and adolescents	Adults	18-24 years
Antibody presence	Yes	No	Probably No
Insulin secretion	Eventually absent	Decreased	Severely decreased but not absent
Insulin sensitivity	Somewhat decreased	Severely decreased	Somewhat decreased
Treatment	Insulin	Diet, oral medications, insulin	Insulin
Microvascular complications	Yes	Yes	Increasing incidence
Macrovascular complications	Yes	Yes	No
Cause of death	Cardiovascular disease and nephropathy	Cardiovascular disease	Pulmonary disease

1.2.1 Pathophysiology of diabetes in CF

The pathophysiology of CFRD is complex and not fully understood.

Autopsy findings have demonstrated pancreatic fibrosis and atrophy with a 50% reduction in pancreatic islet mass [34] and progressive β cell loss is thought to cause abnormal glucose handling [35, 36]. There is delayed insulin secretion following a glucose load in people with CF when compared to normal matched controls [37] and an impairment in glucagon release (**discussed in the subsequent chapter**). The role of insulin sensitivity is unclear, as is that of insulin resistance that is not thought be the primary pathology. However, not all those with pancreatic insufficiency develop CFRD and a genetic predisposition might exist [38].

The role of CFTR in the pancreas is still unclear. Discordant observations have been made about the presence of CFTR in the pancreas. Strong et al [39] and later Rolon et al [40] found no apparent expression of CFTR protein in the islets of Langerhans of CF patients, whereas Polychronakos [41] found the highest level of CFTR expressed in these islets. However, this could be due to a reflection in the sensitivity of the methods used to localise CFTR in the human pancreas.

Genetics: While abnormal chloride channel function induced by CFTR mutations is necessary for CFRD to develop, the twin and sibling study by Blackman [42] involving 1366 individuals at 109 centres, from which were derived 68 monozygous twin pairs, 23 dizygous twin pairs, and 588 sibling pairs, all with CF showed that genetic modifiers are the primary cause of diabetes in CF subjects. Data from the UK CF registry of 8,029 individuals, demonstrated CFTR class I and II mutations increase the risk of diabetes independent of other risk factors [43].

Histopathology: It is believed that CFRD results from a combination of chronic pancreatic inflammation and loss of the islet cells, pancreatic duct obstruction leading to interstitial oedema and ischaemic changes of the endocrine pancreas [29, 44, 45]. The islet cells are initially preserved but destroyed by fibrous fatty tissue over time and this could explain why CFRD is more common later in life [46].

Comparisons can be made with non-CF obese T2DM subjects where a 63% decrease in β cell mass was observed, compared with an obese non-diabetic group [47]. In the same study, a 40% decrease in β cell mass was observed in those with impaired fasting glucose. It is established [48] that a 50% pancreatectomy in humans causes loss of normal glucose regulation and the degree of β cell loss observed in post-mortem studies in T2DM is sufficient to account for at least some part of hyperglycaemia. The degree of cell death and islet cell dysfunction are only marginally associated [49] adding to the difficulty in identifying a single causative process and this is even more pertinent in the CF condition.

1.2.2 Complications associated with CFRD

The most serious acute complication of CFRD is hypoglycaemia. Hypoglycaemia not requiring assistance from another individual is common even in CF patients without CFRD. It can occur in the fasting state, where it may reflect malnutrition and/or increased energy needs due to inflammation and infection, and postprandially, where it is related to delayed and discordant insulin secretion [50]. In those with CFRD, hypoglycaemia can occur as in any patient on insulin therapy, although severe hypoglycaemia may be less common in CF [51] probably secondary to an inadequate glucagon response to hypoglycaemia, a brisk catecholamine response and normal hypoglycemia awareness [52].

Chronic complications of CFRD include microvascular disease that typically does not appear to become clinically apparent until individuals have had the disease for at least 5 years and have developed fasting hyperglycaemia [53-55]. Renal failure due solely to diabetes is uncommon in CF, but the reported incidence of microalbuminuria is 4 – 21% in individuals with CFRD [53-55]. Tight glycaemic control and treatment of microalbuminuria with ACE inhibitors or angiotensin receptor blockers combined with optimal control of hypertension are thought to delay progression of microvascular damage in CFRD as in T1DM and T2DM.

Diabetic retinopathy occurs in 10 – 23% of patients with CFRD but diabetic neuropathy does not seem to occur commonly in CFRD [53, 54]. Current data suggest the severity of this microvascular complication may be less in CFRD [53], but gastroparesis is common in CF patients both with and without CFRD. It is thought CFRD aggravates gastroparesis.

However, this has been difficult to determine [53] due to complexities in assessment. Also, gastroparesis may make good glycaemic control difficult to achieve in those with CFRD.

Hypertension is not uncommon in adult CF patients, particularly after transplantation [54] and although atherosclerotic vascular disease has not been described in CF, hypertension is a known risk factor for diabetic kidney disease. Hyperlipidaemia is rare in CF but may occur, especially after transplantation or in pancreatic-sufficient individuals.

1.2.3 Glucose handling and its impact on the lungs

Significant morbidity and mortality associated with CFRD usually occurs before diabetic retinopathy develops (unlike in T1DM and T2DM). The most important organ in CF is the lung and diabetes can harm the lung, at levels far below the current diabetic biochemical thresholds: a process described as 'pulmonopathy' [56]. It has been argued that hyperglycaemic thresholds based on specific features of pulmonary function decline would be of greater relevance to those with CF than any based on the statistics for developing microvascular disease as in T1DM. Diagnostic criteria based on lung function therefore need to be developed in order to decide on the level of plasma glucose that should be the cut-off in a screening programme [56].

Diabetes can affect the lung in a number of ways – an increase in infections, reduced gas diffusion and increased lung stiffness resulting in an increased effort of breathing.

Non-CF subjects with impaired glucose handling at diabetic thresholds have been shown to have higher morbidity and mortality from pulmonary infection than those with normal blood glucose levels [57-59]. There is loss of lung elasticity and recoil in diabetics and a greater rate of decline in lung function with age compared with non-diabetic subjects. As a result, the lungs become stiffer and harder to inflate and deflate resulting in a decrease in FEV_1 and FVC. The diffusion capacity (DLCO; diffusion of carbon monoxide from the alveoli, across the epithelium and into the blood) is slightly reduced, probably owing to thickening in the alveolar epithelium and the pulmonary capillary basal lamina. Changes have been seen in

arterioles and capillaries of the lung, which are similar to those in the diabetic kidney, although less marked [60].

There are a number of mechanisms by which increases in glucose levels within airway secretions contribute to increased frequency and severity of pulmonary infection [61]. The air spaces are lined with a thin layer of fluid which normally contains little or no glucose [62], but the level can be increased by both hyperglycaemia and inflammation, both of which occur in CF. This presence of glucose increases proliferation of colonising and infective microorganisms with increased virulence. It may also foster virulence [61] leading to altered immune proteins and impairment of epithelial cell function [62]. Hence, the optimisation of glycaemic control and maintenance of normal glucose concentrations in the airways is a significant factor in protecting patients with CF from acute and chronic microbial infection.

In CF, we are now well aware of the deterioration in pulmonary function in those with CFRD [28, 63, 64]. Reductions in FEV_1 and FVC in those with CFRD and IGT have been noted [65]. It is also well established that this decline is seen from at least two to six years prior to diabetes being diagnosed using the standard OGTT [25, 32].

In following up 343 CF patients, Schaedel et al [66] demonstrated declines in pulmonary function, even before the classical definition of diabetes mellitus was achieved and an associated faster decline in those with diabetes, but this was seen only in those over 15 years of age. In their studied population, all those with diabetes had pancreatic insufficiency raising the possibility that the mechanism of glucose intolerance is via under nutrition, leading to poor lung function.

Brennan et al [67] studied breath condensates in healthy volunteers (n = 23), CF patients with (n = 10) and without (n = 10) CFRD, and non-CF diabetic subjects (n = 17). In their study, glucose levels in breath condensates were low in healthy volunteers, but raised in other groups. However, the levels were higher in those with CF (both with and without CFRD) than in the non-CF diabetic group. The highest levels were seen in those with CFRD suggesting airway glucose is raised by both hyperglycaemia and inflammation.

More recently, measures of sputum glucose in 88 CF patients with and without CFRD were studied [68]. Interestingly, the sputum glucose measurement was highest in subjects with normal glucose tolerance, suggesting the dynamics of glycaemic control, sputum glucose and pulmonary infections are more complex than previously thought.

In a review of CF related diabetes studies, Milla et al [63] noted the cause-and-effect relationship between insulin deficiency and decline in health, but most studies were retrospective, making it difficult to determine whether glucose intolerance accelerated decline or whether sicker patients were more likely to get diabetes.

Subsequently, they carried out a prospective study [63] over 4 years of 152 CF patients and divided them into three groups based on the OGTT: NGT (45%), IGT (39%) and CFRD without fasting hyperglycaemia (16%). Over the follow-up period lung function declined in those with IGT and CFRD without fasting hyperglycaemia, but not in those with baseline NGT. They also demonstrated an association between baseline insulin production and lung function decline, with the highest decline in those with lower baseline insulin. However, insulin levels did not correlate with glucose groups, suggesting in part a direct relationship between insulin and lung function perhaps due to the catabolic effect of insulin deficiency [63] and hence is probably why insulin supplementation slows declines in lung function [33].

The lung is the organ most at risk with CF and hyperglycaemia has several adverse effects on the lung occurring early and one of the earliest manifestations of hyperglycaemia might be weight loss. With damage occurring early and glucose levels well below the threshold for the usual definition of diabetes by current criteria, there is a need to screen and intervene at an earlier stage, making the choice of a screening test important.

1.2.4 Screening and diagnosing CFRD

The diagnosis of CFRD can be difficult to detect biochemically and clinically. The classical symptoms of 'polyuria, polydipsia and poor weight gain' have been reported to occur in only 33% of patients with CFRD [23]. Therefore, the diagnosis of CFRD purely on the basis of

symptoms would fail to recognize the vast majority of diabetics. Hence the inclusion of a diabetic test in the CF annual screen has been advocated.

Several screening tests have been used to screen for CFRD: 50-g glucose challenge test, 75-g OGTT, OGTT 60-minute glucose level, continuous glucose monitoring (CGM), fasting plasma glucose, HbA1C, serial capillary blood glucose monitoring and combinations of these tests.

Fasting blood glucose (FPG) is insensitive, as fasting hyperglycaemia occurs late in CFRD [23, 69, 70] thereby making this a poor test to diagnose CFRD. A raised FPG may detect only 16% of cases [23], however it is still one of the screening tests recommended CFRD by the ADA [71] .

Random blood glucose elevations tend to occur across the spectrum of CFRD but a single elevated recording is of no value in CF.

Glycosylated haemoglobin (HbA_1C) reflects blood glucose levels over the preceding 2-3 months and a level ≥ 6.5 % is the globally accepted standard [72] to diagnose T2DM due to the association with the prevalence of diabetic retinopathy above this level. An elevated HbA_1C is suggestive of CFRD but again is a late occurrence and levels are often spuriously low in those with CF [23]: hence, reliance on this as a screening test will miss a significant number of diabetics [73] and cannot be used as a reliable test to diagnose CFRD.

There are two possible reasons why the HbA1C is a late occurrence in individuals with CF. Firstly, the initial step of glycation of Hb is a non-enzymatic reversible reaction, and therefore requires prolonged hyperglycaemia for Hb to get irreversibly glycated (the HbA1C) and therefore not high in CF until late in CFRD. Moreover, rebound hypoglycaemia common in CF will reverse any glycation initially. Secondly, the increased red cell turnover in CF might be responsible for a lesser time for Hb to become glycated.

Fructosamine, a glycated serum protein measured in conditions where hormonal changes cause greater short-term fluctuation in glucose concentrations (e.g. pregnancy) reflects blood glucose levels over a short period of two to three weeks. It has been demonstrated that

neither HbA1c nor fructosamine correlate with mean blood glucose levels in CF [74], thereby providing limited value.

Postprandial glucose monitoring is a more accurate reflection of changes on a day-to-day basis. It is easy to carry out and if performed serially and correctly is a valuable screening test [75].

OGTT: Current, UK CF Trust [76] and ADA [77] guidelines on the management of diabetes recommend an OGTT to diagnose CFRD. The values of an OGTT test indicating normal and abnormal glucose tolerance (NGT <7.8, IGT ≥7.8 - <11.1 and diabetes ≥11.1mmol/L at 120-minutes) are drawn from a non-CF diabetic population who are geno-typically different. The existing diagnostic criteria for diabetes were based on the WHO criteria and the risk of developing micro-vascular complications in T2DM [78] rather than CF specific outcomes. In T2DM an OGTT involves only baseline and 120-minute values but in CF the 120-minute value fails to discriminate between healthy controls and those with CF [75]. The earliest glycaemic abnormality associated with clinical decline in CF is not known and it is common practice to carry out sampling at 30-minute intervals between baseline and 120-minutes, but the value of each time point is not yet established.

In impaired glucose-tolerant and T2DM patients the OGTT glucose values at 60-minutes is thought to be a good indicator for decreased insulin sensitivity and secretion for the prediction of future T2DM than the 120-minute value [79]. The importance of the 60-minute value has also been suggested in CF - in studies of 89 paediatric CF patients [80] and 240 adult CF patients [81], where the higher 60-minute value was associated with worse pulmonary function. This higher 60-minute value has also been associated with a higher future risk of CFRD occurrence and patients increased their BMI following early insulin introduction [82, 83].

OGTTs are labour intensive, time-consuming and cause discomfort due to repeated sampling. In the CF population, glucose tolerance is often variable and a single OGTT based on guidelines may be misleading. Decline in weight and lung function tend to precede the diagnosis of CRFD by current criteria by months to years [84-86]. The OGTT has poor

sensitivity and specificity in the diagnosis of CFRD [87] questioning the applicability of this as a screening test in CF.

Continuous Glucose Monitoring (CGM) has recently emerged as a useful validated tool to screen for CFRD [75, 88-90]. CGM was developed for monitoring glucose control in those with T1DM and uses a tiny subcutaneous filament probe that records interstitial fluid glucose levels every 5-minutes generating a detailed profile over a course of three to five days of usual diet and activity. It provides profiles of glucose handling at any time of the day or night and is particularly useful in CF, as the CF diet frequently exceeds the carbohydrate load of a standard OGTT. A study of 17 children with CF and 14 controls demonstrated that CGM is a useful tool in detecting early glucose derangements in those with CF. Importantly, CGM was the stronger predictor of the development of CFRD in a 2.5 year follow up period, when compared to an OGTT [91]. CGM time ≥ 4.5% of glucose values ≥ 7.8 mmol/L is associated with declining weight and lung function in the preceding 12 months [69] and is perhaps the most useful test of glucose handling in CF.

There is a need for a new accurate screening tool appropriate for individuals with CF that can alert a CF physician to commence insulin to prevent clinical deterioration.

1.2.5 Treatment of CFRD

Nutrition: A high calorie, high fat diet is recommended in CF patients [92]. There is no evidence to support reductions in carbohydrate load in patients with CFRD or to avoid high glycaemic index foods as this might reduce the total energy intake and impair nutritional status in those individuals with CF.

Oral hypoglycaemic agents: Sulphonylurea derivatives act by increasing insulin secretion, but progressive destruction of β cells make this treatment of limited value and there is an associated high incidence of hypoglycaemia [93], hence not recommended for use in CF. Insulin resistance is not a major factor in CF and agents that primarily work by reducing insulin resistance are not adequately effective. Drugs such as metformin tend to cause

significant side effects such as nausea, diarrhoea and abdominal discomfort and most patients with CF do not tolerate these side effects [64].

Insulin treatment: Currently, insulin is the only treatment recommended in the management of CFRD. The choice and regime of insulin depends on the individual needs of the patient. A basal bolus regime provides the required background insulin and a continuous anabolic effect. Short acting insulin helps control post-prandial hyperglycaemia and allows for variable eating patterns [94]. Patients on overnight feeds might require more insulin at night.

1.2.6 Benefits of insulin in CF

Patients with CFRD are insulin insufficient, and insulin is the only recommended treatment. Those with CF on insulin therapy who achieve glycaemic control demonstrate improvements in weight, fat free mass, pulmonary function and survival [27, 83].

Insulin improves lung function after decline resulting from the pre-diabetic condition in patients with CF [95]. Previous data from 42 patients from this CF unit [33] has shown that prior to treatment, over a period of 5 years, the annual rate of change in FEV_1 was -3.2%, FVC -2.5%, and BMI -0.07%. Three months following commencement of insulin treatment, there were significant improvements in all parameters, that were maintained at 1 year for FEV_1 (55.1%; $p < 0.002$), 2 years for FVC (72.1%; $p<0.01$) and 3 years for BMI (20.4%; $p < 0.002$). After 3 months, FEV_1 declined at a rate similar to that before treatment (-3.2 vs. -3.1% per year; $p=0.77$), such that the mean FEV_1 after treatment returned to pre-treatment baseline values at 34 months, suggesting, insulin enhances the nutritional state and temporarily improves pulmonary function in CFRD patients, on average delaying the decline in FEV_1 by 34 months.

The subsequent chapter discusses pancreatic endocrine cell function and its assessment in CF.

1.3 Pancreatic endocrine cell function

The pancreas is responsible for the release of several hormones contributing to carbohydrate metabolism and glucose handling. The pancreatic islets of Langerhans secrete glucagon (α cells), insulin (β cells), somatostatin (D cells) and pancreatic polypeptide (F or D cells) that modulate insulin and glucagon secretion, and are all polypeptides. Insulin is only secreted by β cells whereas the other hormones are also secreted by gastrointestinal mucosa and somatostatin is also found in the brain. Insulin (and c-peptide), glucagon and pancreatic polypeptide (PYY) are discussed in greater detail below.

1.3.1 Insulin and C-peptide

Insulin is synthesized in β cells in the form of pre-proinsulin the precursor, and the gene for the same is located on chromosome 11. Within a-minute after synthesis, pre-proinsulin is discharged into the space of the rough endoplasmic reticulum where it is cleaved into proinsulin by proteolytic enzymes. Proinsulin with a C (connecting) chain linking A and B chains is then transported by microvesicles to the Golgi apparatus. Proinsulin is released in vesicles and the conversion of proinsulin to insulin continues in maturing granules through the action of pro-hormone-convertase 2 and 3 and carboxy-peptidase-H. In the conversion process, a C-peptide chain is removed from the proinsulin molecule producing the disulfide-connected A and B chains that are insulin [96, 97]. Maturing granules are translocated with the help of microtubules and microfilaments. A normal healthy β cell, maintains in excess of 10,000 granules, but not all granules are functionally equivalent. Roughly 1,000 granules are attached to the plasma membrane, and some of these are in a readily releasable pool. Only a small proportion of total insulin is released even under maximal stimulatory conditions [98]. These granules are dynamic structures and around 10% granules turn over every hour during active secretion. Granule formation and turnover are precisely controlled processes and involve regulation and expression of multiple genes.

C-peptide reflects insulin synthesis and low levels of C-peptide are seen when insufficient insulin is produced by β cells or when production is suppressed.

Insulin secretion is pulsatile and regulated by a variety of stimulatory and inhibitory factors, most related to glucose metabolism and effects of cAMP. Insulin secretion is stimulated by high blood glucose levels and reduced when blood glucose is low. Other stimulatory factors include several amino acids, intestinal hormones and acetylcholine (reflecting parasympathetic stimulation). Once in circulation, insulin is degraded within-minutes in the liver and kidneys. Insulin binds with specific membrane receptors found in almost all cells, forming an insulin-receptor complex taken into the cell by endocytosis. The insulin-receptor, a tetramer, is made up of two alpha and two beta glycoprotein subunits. The β subunit is a protein kinase that catalyzes phosphorylation of proteins, an activity resulting in change in the number of "transporters". The insulin-receptor complex enters lysosomes where it is cleaved, the hormone internalized and the receptor recycled. Increased circulating levels of insulin reduce the number of receptors and decreased insulin levels up-regulate the number of receptors. The number of receptors per cell is increased in starvation and decreased in conditions such as obesity and acromegaly; receptor affinity is decreased by excess glucocorticoid.

There are several actions of insulin:

- Facilitation of glucose transport through membranes (adipose and muscle cells)
- Stimulation of the enzyme system for conversion of glucose to glycogen (liver and muscle cells)
- Decreases the rate of gluconeogenesis (liver and muscle cells)
- Regulation of lipogenesis (liver and adipose cells)
- Promotion of protein synthesis and growth (general effect)

Diabetes results from the failure of β cells to produce or increase insulin secretion in response to an increasing demand for insulin from peripheral tissue.

The normal physiological release of insulin following ingestion of a meal is the rapid release of preformed insulin from storage granules within β cells. This occurs in 2 phases, the first within 2 - 5-minutes of a glucose load being ingested and continues for 10 to 15-minutes. This "first phase" of insulin secretion promotes peripheral utilization of nutrient load, suppresses

hepatic glucose production and limits postprandial elevation of glucose. The remaining secretion is post-prandial. The second phase of prandial secretion follows and is sustained until normoglycaemia is restored. In non-diabetic individuals, approximately 50% of total daily insulin is secreted during basal periods. It is well recognized in T2DM that decreased first-phase insulin secretion is an early marker of β cell dysfunction, appearing long before significant changes in absolute glucose concentrations are apparent [99, 100]. This is also true in patients with CF and there is impairment in first phase of insulin release in response to glucose and this has been demonstrated even in non-diabetic CF patients [37, 101, 102] in part contraindicating the hypothesis that reduced β cell mass is solely responsible for the development of CFRD. These points to other possible mechanisms that might contribute to the development of CFRD such as the role of peripheral insulin sensitivity or altered β cell function.

1.3.2 Insulin sensitivity and resistance

Studies using gold standard hyper-insulinaemic clamp techniques to directly assess insulin sensitivity have demonstrated conflicting results. Cucinotta et al [102] could not demonstrate reduced sensitivity in diabetic CF patients, whereas, Ahmad et al [103] demonstrated increased sensitivity in CF subjects compared to controls. However, in this study subjects were not stratified according to their glucose tolerance. In contrast, Bergman [104], Lanng [105] and Holl [37] reported significant reductions in insulin sensitivity in those with IGT, while CF subjects with NGT did not differ from healthy controls. Data from my CF unit [101] demonstrated no differences in insulin sensitivity or resistance among three CF groups (NGT, IGT and CFRD) confirming the co-existence of altered secretory kinetics and delayed peak insulin concentration. A number of studies have shown no significant changes in insulin resistance in those with CF [45, 102, 103, 105-108] suggesting glucose intolerance is characterized by qualitative and quantitative defects in insulin secretion and not insulin resistance.

Therefore, in the long term, early identification of β cell defects need to be identified to prevent clinical decline in CF.

1.3.3 Measuring β cell function

The hyperinsulinaemic / euglycaemic glucose clamp method is the widely-accepted gold standard to evaluate insulin sensitivity. However, its use of an unphysiological dose of insulin, variations in test methodology, cost and complexity limit its role in clinical practice [109, 110]. To circumvent this, a variety of methods of deriving surrogate measures of insulin sensitivity and secretion from non-invasive methods such as the OGTT have been developed to calculate both insulin sensitivity and pancreatic β cell function in basal and dynamic states. These methods correlate well with clamp techniques and are accepted as providing reliable estimates of insulin sensitivity and β cell function and perform reasonably well for discrimination between groups and individuals with differing levels of insulin secretion, sensitivity and resistance [110-113].

Methods such as the updated homeostatic model assessment (HOMA2) estimates insulin sensitivity and β cell function using fasting glucose and insulin levels [114, 115]. This method, developed in 1985 and subsequently computerised [116] and updated in 1996, is used to measure pancreatic β cell function and insulin resistance [114] from paired glucose and insulin concentrations in basal states. This model is used in numerous studies, has been well validated and is available as a computer spread sheet application (*http://www.dtu.ox.ac.uk/homa/)* known as the HOMA *Calculator*© (The University of Oxford 2004). The model is based on non-linear equations and mathematical assessments of interaction between the above two variables. HOMA2 has been widely used in normal and diabetic patients, including those with CF.

Stumvoll and colleagues developed indices that allow simultaneous estimation of insulin sensitivity and β cell function in dynamic states using glucose and insulin values during an OGTT [117, 118]. First and second phase insulin secretion is calculated using the Stumvoll equation (1283 + 1.829 x Ins_{30}- 138:7 x $Gluc_{30}$ + 3:772 _x Ins_0) validated using the euglycaemic hyperinsulinaemic clamp [117].

Another index, the Matsuda Index [111] had been designed to indicate values which are comparable to rates of disappearance of plasma glucose measured by insulin clamp methods.

Retnakaran et al [119] demonstrated a hyperbolic relationship between the Matsuda index of insulin sensitivity and the ratio of the total areas under the curve (AUC) for insulin and glucose (AUC ins/glu) over 120-minutes derived from OGTT data in subjects with normal and impaired glucose tolerance referred to as the Disposition Index (DI) [ΔI0–30/ΔG0–30 and 1/fasting insulin]. In prospective studies, the DI declines well before glucose levels rise into the diabetic range: a low DI is an early marker of inadequate β cell compensation and predicts development of future diabetes above and beyond fasting and 2-hour glucose levels over 10 years [120]. Within CF, the only study [121] evaluating β cell function measured by DI showed DI to be reduced compared to non-CF controls. The DI was reduced even in those CF subjects with NGT and decreased further in those with CFRD. It remains to be seen whether DI proves to be a predictor of the development of CFRD in larger studies enabling identification of CF patients who are at particularly high risk, allowing early interventions aimed to delay or prevent CFRD.

1.3.4 Oral mixed meal tolerance test (MMT):

The patho-physiology of diabetes has usually been studied using the OGTT, but this is un-physiological and may not necessarily give information that is relevant to responses to meals. Dietary constituents other than glucose affect insulin secretion. The addition of protein, amino acids or fat to carbohydrate is known to enhance insulin secretion; hence the administration of a standard mixed meal is considered a more physiological test for evaluating subjects both at the time of diagnosis and follow-up and has been proposed for clinical practice [122].

A near normal incremental plasma glucose pattern and more rapid insulin response to a standard breakfast have been reported in mild diabetics [123].

The MMT is commonly used to assess residual insulin secretion. Randomized studies [124] comparing the MMT with a Glucagon Stimulation Test (GST) showed the MMT was more sensitive of residual β cell function, the peak C-peptide response being significantly higher compared to the GST, an effect likely to be due to the inherently greater response to

combined stimuli of a mixed-meal. Repeat testing also demonstrated the MMT was more reproducible. Therefore, the standardized MMT is the preferred test to assess β cell function.

Various types and formulations of mixed meals have been used. One study [125] used a mixed meal consisting of 15 g Weetabix, 100 g skimmed milk, 250 mL pineapple juice, 50 g white meat chicken, 60 g whole meal bread and 10 g polyunsaturated margarine (75 g carbohydrates; total, 500 Cal; calorie contribution: 55% carbohydrate, 30% fat, and 15% protein) to study pancreatic ß cell responsiveness. Subjects were required to consume the meal within 10-minutes. Another study [122], comparing an OGTT and mixed meal, used a standard mixed meal whose composition was similar to a continental breakfast. The meal consisted of 125 g fruit juice, 75 g ham and 80g white bread (590 kcal, 44% carbohydrates, 41% lipids and 15% proteins) with sampling times comparable to an OGTT.

1.3.5 Glucagon

Glucagon is synthesized and secreted by the pancreatic islet α cells. Variation in blood glucose levels cause changes in glucagon secretion. This is achieved directly at the level of α cells via adequate glucose sensing, and the rate of exocytosis of glucagon-containing granules. Glucagon release is also regulated by systemic, extrinsic factors to the islet and mediated by the autonomic nervous system [126]. Glucagon levels are highest in venous drainage of the pancreas and hepatic portal vein with the liver being the principle target. The glucagon receptor (GlcaR) is highly specific and abundantly expressed by hepatocytes. Hepatic clearance of glucagon is 20-30% of portal content, with the kidney contributing to a major portion of glucagon removal.

Abnormalities of glucagon secretion are encountered in diabetic patients. Insufficient secretion in response to hypoglycaemia underlies disorders of counter-regulation in long-term T1DM subjects. The almost complete loss of β cells profoundly alters islet architecture but is not accompanied by changes in α cell mass [127]. It is widely recognized that T2DM patients show elevated plasma glucagon levels relative to their high glucose levels [128, 129]. Excessive glucagon secretion, with background insulin insufficiency and resistance, causes fasting hyperglycaemia by promoting glucose production in the liver. Insufficient and

delayed inhibition of glucagon secretion after meals contributes to postprandial hyperglycaemia. Owing to decreases in β cell mass, the α to β cell ratio is increased in many, though not all T2DM subjects, but the α cell mass is similar to that of non-diabetic subjects [130].

In both T1DM and T2DM there is impaired glucagon suppression after glucose ingestion [131, 132]. This is an important factor contributing to failed suppression of hepatic glucose release after meal ingestion [133, 134].

There are a number of studies demonstrating elevated fasting glucagon levels in those with IGT [134-136] suggesting that there is early α and β cell dysfunction in pre-diabetic states. This is pertinent in CF where early detection of α and β cell dysfunction is important.

Within CF, the evidence has been limited and conflicting. Fasting levels of glucagon have been found to be normal in patients with CF. In one of the first studies in CF looking at glucagon and insulin responses [137] in 17 children with CF and in 9 control children, glucagon responses did not parallel those of insulin. Glucagon output varied in CF children and there was diminished insulin secretion in the CF group, but diminished secretion was only noted when some insulin secretion was preserved. Subsequently, Lanng at al [36] demonstrated glucagon suppression decreases along with decreasing glucose tolerance. Lippe [138] and Moran [52] demonstrated normal fasting glucagon levels in CF patients, but these subjects were not able to appropriately increase glucagon secretion in response to arginine, consistent with reduced α cell mass leading to a blunted glucagon response.

1.3.6 Pancreatic polypeptide (PYY)

PYY is a 36–amino acid peptide produced by pancreatic F cells located in the Islets of Langerhans. It can be measured in plasma by radio-immuno assay (RIA), has a basal level of 10-30 pM and exhibits a rapid increase after food ingestion peaking at 15-30 min followed by a lower sustained phase that lasts 4-5 hours in humans [139-141], with the vagus nerve as the main stimulator of secretion [142]. Intravenous infusions of amino-acids, glucose, or lipids do

not significantly alter circulating PYY levels, indicating the possible contribution of an entero-PYY axis.

PYY has effects on GI motility, metabolism and food intake. In the gastrointestinal tract, PYY inhibits gastric emptying rate, pancreatic exocrine secretion, and gallbladder motility [143], and in contrast to its central effects, intraperitoneal administration of PYY decreases food intake and increases energy expenditure [144]. Decreased postprandial secretion of PYY has been observed in individuals with morbid obesity [145], whereas subjects with anorexia nervosa are characterized by an exaggerated postprandial release of PYY [146, 147]. In humans, PYY inhibits gastric emptying of solid food and delays postprandial rises in plasma glucose and insulin [148]. PYY is suggested to have a physiological role in the pancreatic postprandial counter regulation of gastric emptying and insulin secretion.

Raised plasma PYY levels have been reported in both maturity onset and juvenile diabetes [141] where hyperplasia of F cells is seen [149].

Within CF, early studies demonstrated absent PYY secretory responses to protein-stimulated meals in those with exocrine pancreatic insufficiency, suggesting defects in the PYY secretory mechanism [150]. PYY measurements in response to secretin may be a convenient and useful means of following the course of pancreatic disease in a chronic illness such as CF and abnormal PYY secretion might be considered an indirect index of pancreatic damage in CF [151]. Subsequently, Moran et al [52] showed peak PYY responses to hypoglycaemia distinguished CF patients with and without exocrine insufficiency. Those with exocrine disease had reduced F cell function suggesting exocrine disease causes some endocrine dysfunction in CF or that a common pathogenic process simultaneously and independently impairs exocrine and endocrine function.

Within CF, no studies exist looking at early derangements of PYY secretion, its role in glucose handling and its repose to stimuli in the CF population.

1.3.7 Incretins

La Barre in 1932 was the first to refer to the term 'incretin': an extract from upper gut mucosa that produced hypoglycaemia but did not alter exocrine secretion. Since then significant progress on the incretin concept has been made. John C Brown in 1971 isolated a peptide from intestinal mucosa and exogenous administration of this peptide inhibited gastric secretion in dogs and was called Gastric Inhibitory Peptide (GIP) [152]. This was subsequently found to have insulinotropic properties and was called glucose-dependent insulinotropic peptide [153]. The two main incretins are discussed below in more detail.

Incretins are hormones released from the gut into the blood stream in response to ingestion of food and modulate the insulin secretory response to products within nutrients in food. Insulin secretory responses to incretins, called the incretin effect, accounts for two-thirds of the insulin response to an oral glucose load and is due to potentiating effects of gut-derived hormones [154]. The incretins GLP_1 and GIP released post-prandially have been shown to be modulators of glucose homeostasis and play a major role in insulin secretion [155]. Moreover, responses vary according to glucose tolerance status and may differ depending on the nature of the stimulus.

GIP and GLP_1, the two incretin hormones have a number of similarities and differences [156] and these are outlined in Figure 1-4 below.

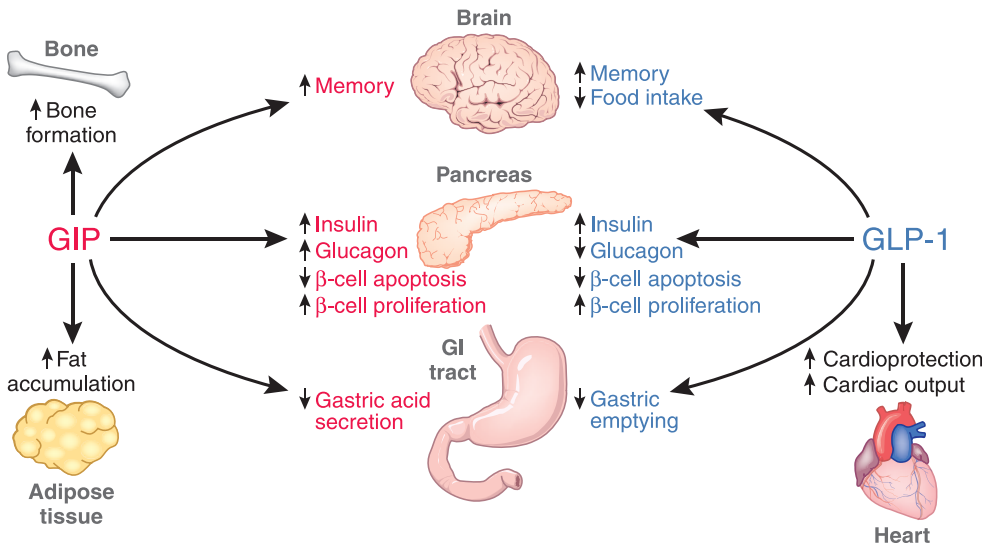


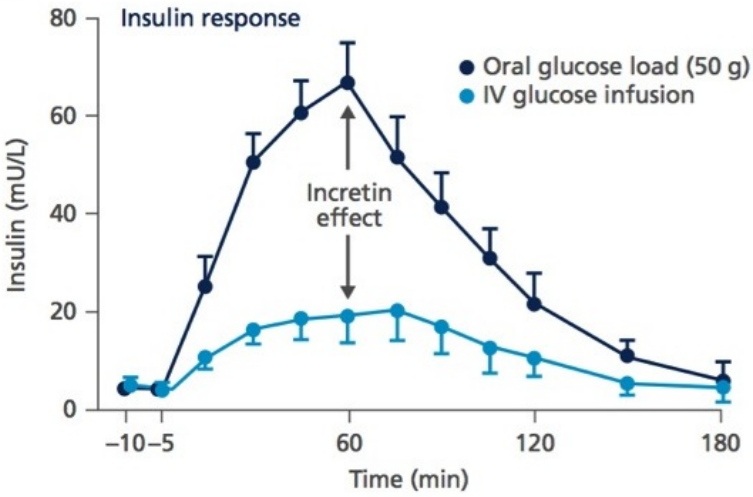
Figure 1-4: Actions of incretin hormones

Reproduced from: Seino Y, Fukushima M, Yabe D. GIP and GLP-1, the two incretin hormones: Similarities and differences. Journal of diabetes investigation. 2010 Apr 22;1(1-2):8-23.

1.3.7.1 The incretin effect

The incretin effect is the β cell secretory response to factors other than glucose. This is represented by differences in response of plasma insulin, C-peptide or insulin secretion rate, measured during oral glucose ingestion versus isoglycaemic intravenous glucose infusions (See Figure 1-5 below).

In healthy subjects, the incretin effect accounts for up to 70% of the total amount of insulin released in response to an oral glucose load. This amplification of glucose induced insulin secretion is the result of actions of incretins which potentiate glucose induced insulin secretion and, therefore, play an essential role in the regulation of glucose homeostasis - in particular, postprandial glucose levels. It is known that the incretin effect is severely impaired or absent in patients with T2DM and this has fuelled interest in the development of therapies that target the incretin system.



Plasma glucose after an oral glucose load (50 g/400 ml) and during "isoglycaemic" intravenous glucose infusion

Figure 1-5: The incretin effect

From: Nauck M, Stockmann F, Ebert R, Creutzfeldt W. Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. Diabetologia. 1986 Jan;29(1):46-52.

1.3.7.2 Glucose-dependent insulotropic peptide (GIP)

GIP is a single 42-amino acid peptide structurally like secretin, glucagon and vasoactive intestinal peptide. It is synthesized and released in response to nutrients from entero-endocrine (K) cells mainly in the duodenum and jejunum. GIP is glucose dependent and exerts its effects following a glucose load - ensuring prompt insulin mediated glucose uptake into tissues.

In the fasted state, GIP levels are low compared to levels after a meal and this release is stimulated by food ingestion containing glucose or fat [153, 157]. GIP exerts its effect by binding to specific receptors (GIPR) on β cells and facilitates secretion of insulin via activation of proximal signal transduction pathways. GIPR activation leads to membrane depolarization and increases in intracellular Ca^{2+} concentration leading to a direct effect on insulin exocytosis. In addition to potentiating the release of insulin from β cells, GIP replenishes insulin in β cells by increasing insulin gene transcription and biosynthesis, and enhances the glucose-sensing system by increasing expression of components of β cell glucose sensors. GIP also affects fat metabolism in adipocytes by enhancing insulin-stimulated incorporation of fatty acids into triglycerides and modulates fatty acid synthesis [158].

GIP synergizes with glucose, causing more insulin to be released from β cells both in vivo and in vitro, and the amount of insulin released from β cells is proportional to rates of glucose metabolism [159], determined by plasma membrane glucose transporters, enzymes responsible for glucose metabolism and their activity [160]. There is some evidence indicating GIP acts synergistically with glucose as a growth- and anti-apoptotic factor by protecting β cells from cell death induced by various stimuli and serum or glucose deprivation [161, 162], providing another mechanism by which GIP promotes long-term accommodation of β cell function to changing nutritional status.

Ingestion of oral fat alone without any carbohydrate induces GIP secretion, but is insufficient to stimulate insulin at fasting glucose concentrations, indicating the effects of GIP on insulin release do not occur if plasma levels of glucose are also not concurrently increasing [163].

The GIP response to a mixed meal was found to be 186% larger than that elicited by an oral glucose load [164] thereby suggesting the role of these hormones in the breakdown of a meal.

GIP secretion in response to oral glucose ingestion or different test meals has been quantified in many studies. In humans, fasting plasma total GIP, assayed from peripheral venous blood collection, is approximately 9 - 11pM and peak plasma concentrations are 50 - 120pM, achieved after eating, dependent upon the health status of the individual, and the quantity and quality of food consumed [165]. Once released, GIP is degraded very quickly by the enzyme di-peptidyl-peptidase-4 (DPP4), which is bound to lymphocytes and endothelial cells in blood vessels of gut and liver. The elimination rates of GIP are similar in subjects with type 2 diabetes and those without [166]; hence, more rapid degradation and elimination of GIP is unlikely to be a factor in defective insulinotropic effects seen in T2DM.

In T2DM, concentrations of GIP have been found to be normal or increased [165], however the insulinotropic effect is diminished. T2DM is characterised by a severely impaired or absent GIP insulinotropic effect [167] that most likely results in worsening insulin secretion. Recent studies suggest that hyperglycaemia alters physiological responses by down-regulating GIPR expression / activity [168, 169].

However, it seems unlikely that T2DM results from deficient GIP secretion. One explanation for using DPP4 inhibitors such as Gliptins in T2DM is that they lead to "normalisation" of incretin levels that are supposedly reduced compared with non-diabetic subjects [170]. However, it is far from certain that incretin secretion is reduced in T2DM. Based on OGTT and MMT data, GIP secretion and fasting levels seem to be increased, in both impaired and diabetic states [164, 171] whereas the insulinotropic effect is almost totally lost. Although different radio-immunoassays have been used over the years, most studies seem to agree that secretion of GIP is normal or even higher in patients with T2DM compared with healthy control subjects [166, 172-175].

1.3.7.3 Glucagon-like peptide 1 (GLP_1)

In 1985, another peptide - GLP_1 was found to be insulinotropic and was the second incretin to be identified. GLP_1 is produced in entero-endocrine L cells that are scattered among enterocytes throughout the small bowel and ascending colon, most in distal parts of the intestine, where they are secreted into the bloodstream in response to nutrient ingestion [176, 177]. Potential mechanisms of stimulation include upper gut signals such as stimulation of the autonomic nervous system [178-180], neurotransmitters such as Acetylcholine (Ach) [181] and gastrin-releasing-peptide [180], suggesting intramural enteric nervous system involvement in mediating release of GLP_1 when appropriate nutrients enter the duodenum or upper jejunum.

In addition, GLP_1 inhibits gastric emptying, decreases food intake [182], inhibits glucagon secretion [183] and slows the rate of glucose production [184]. Again, GLP_1 has been shown to protect β cells from apoptosis [185] and continuous GLP_1 treatment in T2DM has been shown to normalise blood sugar, improve β cell function, and restore first-phase insulin secretion and glucose competence to β cells [186, 187]. Hence, GLP_1 receptors have become therapeutic targets for the treatment of T2DM.

GLP_1 levels reach maximum secretion 17 to 20-minutes after oral glucose administration and 60 to 90-minutes after a mixed meal, followed by a slow decline toward fasting levels. Typical basal (fasting) levels of bioactive GLP_1, measured from peripheral veins, are in the range of 5 to 10 pM and increase by 2- to 3-fold after meal ingestion, depending on size and composition of meal [188].

GLP_1 is one of the most potent substances known to stimulate glucose-dependent insulin secretion and, like GIP, its stimulatory activity is exerted via binding to its receptor on β cells. This binding result in activation of adenylyl cyclase with consequent production of cAMP, subsequent activation of Protein Kinase A (PKA) and cAMP-binding proteins (also known as *Epac*), which lead to inhibition of K^+ ATP channels, elevation of intracellular Ca^{2+} levels, increases in mitochondrial ATP synthesis, and enhanced exocytosis of insulin from insulin-secretory vesicles.

Data from the large Baltimore Longitudinal Study of Aging showed GLP_1 secretion is not deficient in either the fasting state or after oral glucose ingestion in glucose-impaired or diabetic subjects not taking any drugs affecting glucose homeostasis. Vollmer et al. [164] found a trend toward higher plasma GLP_1 levels in 17 well-controlled subjects with T2DM after a mixed meal. In their study, the mean HbA1c level was 6.8%, and as HbA1c is a measure used in clinical practice to monitor long-term blood glucose control in diabetes management (levels <7% indicate good control), it is evident that T2DM develops in the setting of normal incretin secretion. Hence, reduced secretion cannot be justified as causing the disease. Older studies, using patients with worse metabolic control, on multiple drugs and suffering from diabetes for longer times, have found impairments in GLP_1 secretion [165, 189] and so chronic hyperglycaemia and its metabolic consequences may be the cause of slightly impaired GLP_1 secretion seen in previous studies. The study by Toft-Neilsen et al [189] reported an increase in total GLP_1 in 33 healthy participants and 54 T2DM subjects after 60-minutes and in the second hour, peak GLP_1 levels were maintained in healthy participants, but returned to lower levels in the T2DM group. Overall, this reflected a 53% reduction in integrated GLP_1 concentrations in T2DM patients, relative to healthy controls. Based on this, it has been implied that slightly reduced GLP_1 concentrations after a meal in those with impaired oral glucose tolerance and more severely impaired GLP_1 secretion in T2DM may translate into a progressive loss of the ability to secrete GLP_1 with advancing T2DM as part of disease progression [190].

In addition, in T2DM, insulin secretion is typically reduced just after ingestion of glucose or a meal (referred to as defective early-phase insulin secretion), but studies showing deficient GLP_1 secretion found reductions in secretion at 60 to 150 min, which is well after early-phase insulin secretion has occurred and at a time where there is actually exaggerated insulin secretion; therefore, defective insulin secretion did not coincide in time with defective GLP_1 secretion, so one cannot postulate cause and effect [165]. With relation to GLP_1 and insulin, it seems possible to conclude that abnormalities of incretin secretion are unlikely to be a primary pathogenic factor in the development of T2DM and are instead a consequence of the diabetic state [191-193]. It is also clear that the incretin effect of GLP_1 in T2DM is better preserved, in contrast to that of GIP [194], because an infusion of GLP_1 in T2DM so as to

reach pharmacologic concentrations in plasma can normalize fasting [195-197] and postprandial [196-198] glucose concentrations, resulting from increases of glucose-stimulated insulin secretion, decrease of glucagon secretion and slowing of gastric emptying [195, 199, 200]. Continuous intravenous infusion of GLP_1 also lowers postprandial plasma glucose levels in subjects with type 1 diabetes by delaying gastric emptying [199]. These effects of GLP_1 have been consistently shown in a number of human studies [194, 201, 202]. In particular, continuous subcutaneous infusions of GLP_1 for 6 weeks in T2DM subjects was associated with significant reductions in both fasting and post-prandial blood glucose as well as HbA1c with a slight decrease of body weight [187]. Prolonging the GLP_1 infusion for 3 months in patients with T2DM resulted in restoration of first-phase insulin secretion as well as an improvement of late-phase secretion during a glucose clamp, but no significant change in body weight and plasma glucagon levels were noted [203]. In another study, repeated i.v. infusions of GLP_1 normalized fasting blood glucose in patients with T2DM [204]. Thus, there has been considerable interest in an incretin-based therapeutic approach for treating T2DM. However, continuous GLP_1 infusion or repeated GLP_1 injections are impractical and expensive ways to lower blood glucose and so the above strategies have been developed.

1.3.7.4 Incretins in Cystic Fibrosis

The role of incretins in CF has received limited attention. Lanng et al [36], observed normal basal GLP_1 and GIP levels across the spectrum of glucose tolerance in CF but GIP hyper-secretion during an OGTT was found in adults without CFRD [205].

More recently Kuo et al [206] described impaired GLP_1 and GIP secretions in five adults with CF without CFRD: however, their study subjects' glucose tolerance and peak insulin secretion were not well characterised.

In another study in CF patients, Anzeneder et al reported no differences in GLP_1 or insulin responses to OGTT in adults with CF and NGT, IGT, and in newly diagnosed CFRD compared to healthy controls [207]. The authors evaluated responses to an oral glucose load and not a mixed meal, so possible impairments in fat and protein stimulated incretin secretion would

not have been detected. In addition, they combined pancreatic exocrine sufficient and insufficient subjects in the analyses.

We now know that pancreatic enzyme replacement improves glucose excursion and GLP_1 secretion in response to a mixed meal [206] and this was studied in greater detail recently [208] where, although pancreatic enzyme supplementation restored GLP_1 and GIP secretion, the latter was still not normalized in the adult group. This perhaps suggests suboptimal mixing of enzymes and nutrients in the most proximal small intestine, from which most of GIP is derived.

In summary, the evidence relating to GLP_1 and GIP secretion is limited and conflicting in CF, probably reflecting methodological inconsistencies in the studies described above. Moreover, the variation in incretin secretion through the day and its effect on blood glucose has not been studied in CF.

Thus, in CF more work needs to be done to assess those with normal glucose tolerance.

1.4 Diurnal Variation in glucose handling

In humans, the phenomenon of diurnal variation in glucose tolerance is well known with glucose intolerance increasing throughout the day, and also as the pancreas ages [209-215]. It has been suggested that impaired glucose tolerance in the afternoon or "afternoon diabetes" is an early sign or a forerunner of frank diabetes mellitus [216] and in the general population, a reduced glucose tolerance in the evening appears to reflect both decreases in insulin sensitivity and insulin secretion [217].

Early morning rises in fasting glucose levels and insulin requirements known as the 'dawn phenomenon' have been seen in patients with diabetes [218] and in some patients with non-diabetes [219, 220]. Nocturnal elevations in growth hormone and early morning increases in cortisol secretion have been explored as contributors to this phenomenon [221-223]. However, a number of studies have shown that blood glucose levels in response to a mixed meal are markedly higher in the late afternoon or evening compared to morning and both the size of responses and diurnal variation are more pronounced when the meal has a high carbohydrate content [224, 225]. This could be partially mediated by circadian variations in circulating concentrations of cortisol, a counter-regulatory hormone [226]. Furthermore, it has been postulated that if testing was carried out in the afternoon using current oral glucose tolerance test (OGTT) criteria for the diagnosis, half of all cases of undiagnosed diabetes would be captured [227] and this may be important in CF.

It is possible to attribute the loss of glucose tolerance to a diminished pancreatic response to a glycaemic stimulus. Freinkel et al [228] observed a diurnal variation in plasma insulin levels during periods of total fasting, with mean levels in the morning exceeding those in the afternoon. Lambert and Hoet [229] also noted a diurnal pattern in insulin levels. They found high levels during the night, even though daytime meals were presumably stimulating insulin release from the pancreas. It is possible that insulin liberated post-prandially in the morning conserves a certain activity at the moment of the next meal, and still intervenes in maintaining glucose homeostasis. Later in the day, however, glucose homeostasis would necessitate a new synthesis of insulin.

1.4.1 Diurnal variation in CF

Within the CF population, screening later in the day might be able to identify CFRD earlier. Moreover, the caloric intake in those with CF is observed to be greater later in the day compared to the general population [230].

Using classical testing methods, 40% of adults will have severe enough glucose intolerance **in the morning** to be labelled with CFRD. However, it is not known what happens to glucose tolerance and pancreatic β cell function in CF patients through the day.

The current diagnostic criteria for diabetes are based on plasma glucose levels recorded in the morning after an overnight fast of at least 8 hours. However, in the CF population, many patients are seen late in the morning, are in an uncertain duration of fasting, are encouraged to eat regularly, tend to be anorexic in the morning and usually have their largest meal later in the day - thereby making the early morning assessment of blood glucose un-physiological.

It is unclear whether the current diagnostic criteria for diabetes in CF (a single point test in the morning) can be applied uniformly to all patients and especially those who are tested for diabetes at other times of the day.

1.5 Gastric Motility and Glucose Handling

Gastric emptying is central to post-prandial glycaemic control [231, 232]. In healthy individuals, gastric emptying accounts for around a third of the variation in the peak glucose response after an oral glucose load [233]. A number of factors might influence the rate of gastric emptying, including meal composition and volume, posture, illness, glycaemia and medications.

Factors that promote gastric emptying

- Gastric volume: Increased food volume in the stomach promotes increased emptying and antral distension stimulates vasovagal excitatory reflexes leading to increased antral pump activity.
- Liquid vs solid food: Clear fluids are emptied rapidly, usually half within 30-minutes. Solids stay in the stomach longer.
- Types of food: Protein empties fastest, followed by carbohydrates. Fats take longest to empty.
- Hormonal factors: Gastrin has mild to moderate stimulatory effects on motor functions in the body of the stomach. This enhances activity of the pyloric pump. In addition, motilin released by epithelium of the small intestine enhances the strength of the migrating motor complex which is a peristaltic wave that begins within the oesophagus and travels thru the whole gut every 60-90 min during the inter-digestive period.
- Neural: Parasympathetic innervation via the vagus nerve stimulates motility as does the local myenteric reflex.
- Drugs: Prokinetics such as domperidone, cisapride, erythromycin and metoclopramide promote gastric motility.

Factors that inhibit gastric emptying

- Duodenal distension results in inhibitory enterogastic reflexes that slow or even stop stomach emptying if the volume of chyme in the duodenum becomes too much.

- Osmolarity of chime: Iso-osmotic gastric contents empty faster than hyper or hypo-osmotic contents due to feedback inhibition produced by duodenal chemoreceptors.
- Types of food: Fat and protein breakdown products in the small intestine inhibit gastric emptying.
- Acid: pH of chyme in the small intestine of < 3.5-4 activates reflexes to inhibit stomach emptying until duodenal chyme can be neutralized by pancreatic and other secretions.
- Temperature: Cold liquids (4^{o}C) empty more slowly.
- Hormones: Cholecystokinin released from the duodenum in response to breakdown products of fat and protein digestion, blocks the stimulatory effects of gastrin on antral smooth muscle. Secretin released from the duodenum in response to acid has a direct inhibitory effect on gastric smooth muscle. Other hormones that decrease emptying are somatostatin, vasoactive intestinal peptide (VIP) and gastric inhibitory peptide (GIP).
- Neural: Sympathetic nerves via the celiac plexus inhibits motility.
- Physiological factors: Pregnancy, anxiety and pain.
- Disease states such as diabetes mellitus (autonomic neuropathy), post-operative bowel surgery with resultant ileus and high intra-abdominal pressure.
- Drugs such as opioids

Disordered gastrointestinal motor and sensory function occur frequently in diabetes mellitus and has substantial implications for the morbidity and effective management of patients with diabetes.

Gastric emptying is abnormally delayed in 30–50% of patients with T1DM or T2DM leading to potential consequences of gastrointestinal symptoms, impaired nutrition, poor glycaemic control, and delayed absorption [234]. This abnormal motility in humans has traditionally been attributed to irreversible autonomic neuropathy, but the association between delayed gastric emptying and the presence of cardiovascular autonomic neuropathy is relatively poor [235]. However, acute changes in blood glucose concentration — both hyper- and hypoglycaemia — have a marked, reversible, effect on gut motility [234].

1.5.1 Gastric Motility in CF

Gastric motility within CF has received little attention.

Variables of relevance to CF are pancreatic enzyme supplementation, glycaemia and the high fat/high energy diet prescribed for these individuals. Gastric emptying is itself influenced by acute changes in blood glucose concentrations, with hyperglycaemia delaying gastric emptying, which in turn slows the absorption of ingested carbohydrate and reduces the propensity for further hyperglycaemia [236].

Only a limited number of studies have evaluated gastric emptying in CF, with inconsistent results, attributable to differences in subject characteristics, meal composition, use of pancreatic enzymes and the methodology used to measure gastric emptying. The results have been conflicting with Collins [237] and Kuo [206] reporting rapid emptying, Pauwels [238] reporting delayed emptying, or gastric emptying being no different from healthy control subjects [239-241]. A delayed or rapid gastric emptying rate (GER) in patients with CF can have important clinical consequences.

In addition, circadian changes in glucose handling are well described in the normal population [209, 212, 213, 217] and gastric emptying, which is in turn slowed by hyperglycaemia [242] may influence these changes. However there have been no studies of circadian changes in gastric emptying in CF subjects, a group with poor glucose handling.

Hence, there is a need to explore gastric motility in CF and the effects this might have on glucose handling and CFRD.

1.5.2 Assessment of Gastric motility

Several different methods have been used to evaluate gastric emptying in adults. Oesophageal manometry, intubation and epigastric impedance are invasive tests and need specialised equipment. They also need sedation or anaesthesia and hence are poor tests to study dynamic changes. Barium contrast studies are not practical due to the large amounts of radiation and the effect of gravity. More recently paracetamol absorption studies, looking at

plasma paracetamol levels after ingestion of a fixed amount of the drug have been used. Although good in assessing gastric motility in type 1 and 2 diabetics, it is not a good test in CF due to co-existing liver disease. The gold standard test is scintigraphy, a nuclear medicine scan wherein, the subjects consume radio-tracer labelled food and images are taken every few-minutes for upto 4 hours. Subjects need to remain in a fixed position for the duration of the study. Hence, none of these tests are practicable for daily use and there is need for a safe non-invasive method that is easy to use and has practical applications.

Assessment of gastric emptying using this ultrasound method is safe, easy to use and requires a standard ultrasound transducer and gives real time results. This method has been standardized by determining gastric antral cross-sectional area in a single section of the stomach [243]. The gastric antrum is considered to be the most reliable and reproducible portion of the stomach demonstrating a 'distinct' sonographic appearance when empty and after a meal, when visualised with a curvilinear probe.

The advantage of trans-abdominal ultrasonography is the ability to measure several parameters of gastric motor function. Serial changes in antral cross-sectional area provide an index of gastric emptying. Gastric emptying is considered complete when the antral area returns to the fasting baseline level.

This method of assessment has been validated against barium studies [244] and the gold standard test – scintigraphy [245, 246]. In a non-CF population, this method has been used to demonstrate delayed gastric emptying following the ingestion of fat before a carbohydrate meal and the increased postprandial rise in glucose, insulin and GIP, in T2DM. Within CF, gastric ultrasound to evaluate emptying has been used in the paediatric population [247] to demonstrate gastric dysmotility and compare the benefits of H_2 blockers to those of pro-kinetic agents.

1.6 Indications for current research

This literature review has demonstrated that CFRD is unlike other types of diabetes for several pertinent reasons:

1. Gastric emptying and motility are likely to be altered in the condition, changing the way in which nutrients presented to the gut compared to normal individuals.

2. The relative progressive insulinopaenia that occurs in CF over time may result in diurnal changes in glucose and other nutrient handling that has not previously been explained.

3. Abnormalities in the small intestine due to the condition may alter the secretion of incretins and the associated pancreatic enzymes compared to non-CF individuals.

1.7 Aims and objectives

The aims and objectives of this study are:

1. Measure gastric emptying using a novel easy technique in CF and compare responses to healthy control subjects, throughout the day.

2. Determine whether 'pancreatic fatigue' contributes to a delayed insulin release and peak that is seen in patients with cystic fibrosis.

3. Explore glucose handling in CF and characterise the secretory pattern of insulin, c-peptide, glucagon, GIP and GLP_1 and PYY throughout the day.

1.8 Hypothesis

The hypotheses this study aims to test are as follows:

1. Insulin sensitivity and gastric motility are altered in CF and this is influenced by the type of meal.

2. Relative gradual insulinopenia in CF, results in pancreatic fatigue throughout the day.

3. The entero-insular axis is intact and altered glucose handling is due to a beta cell defect.

2 Subjects, Materials and Methods

2.1 Ethical approval

R&D approval was obtained from the sponsor of the study (Liverpool Heart and Chest Hospital, UK.).

Ethical approval was obtained from the National Research Ethics Service (NRES Committee North West - Greater Manchester East) on 18 June 2012 [(REC reference 11/NW/0552); [Appendix 3].

2.2 Subjects

Ten (5 males, 5 females) healthy volunteers and 20 CF (17 males, 3 females) patients from the large (n=281) [See Figure 2-1 on page 51] regional adult unit in Liverpool, UK (281 patients) were recruited. CF subjects were recruited in the outpatient department during their usual CF clinic review. Control subjects were recruited through a poster advertisement in the hospital. No subjects needed to be withdrawn from the study or requested withdrawal before completion of the study protocol.

2.2.1 Inclusion criteria

CF subjects

- Male or female with a confirmed diagnosis of CF defined by
    - Clinical features consistent with a diagnosis of CF AND
    - Sweat chloride ≥60mmol/L by pilocarpine ionotophoresis; OR
    - Genotypic confirmation of CFTR mutation
- Aged 18 – 50 years
- Outpatients from the regional adult unit in Liverpool
- Clinically stable over the preceding 4 weeks i.e. no indication for iv antibiotics, steroids or hospital admissions
- Haemoglobin value >10g/dl at their last CF annual screen
- Subjects known to be pancreatic insufficient as documented in their clinical record

Control subjects

- Male or female without a known diagnosis of CF
- Aged 18 – 50 years
- Clinically stable over the preceding 4 weeks
- Haemoglobin value ≥10g/dl (done on the morning of visit 1)

2.2.2 Exclusion criteria

- Subjects with known diabetes or on glucose lowering medications (insulin, oral hypoglycaemic agents)
- On-going acute illness
- Long-term oral steroids or on steroids at the time of the test
- Pregnant or intention to become pregnant
- Those on immunosuppressive treatment
- History of, or planned organ transplant
- Known clinically significant abnormal findings on haematology or clinical chemistry
- Subjects known to have significant CF related liver disease as documented in their clinical record
- Subjects with documented or suspected, clinically significant, alcohol or drug abuse. The determination of clinical significance was determined by the investigator
- History of malignant disease
- Any serious or active medical or psychiatric illness, which in the opinion of the investigator, would interfere with subject treatment, assessment, or compliance with the protocol

2.2.3 Subject selection

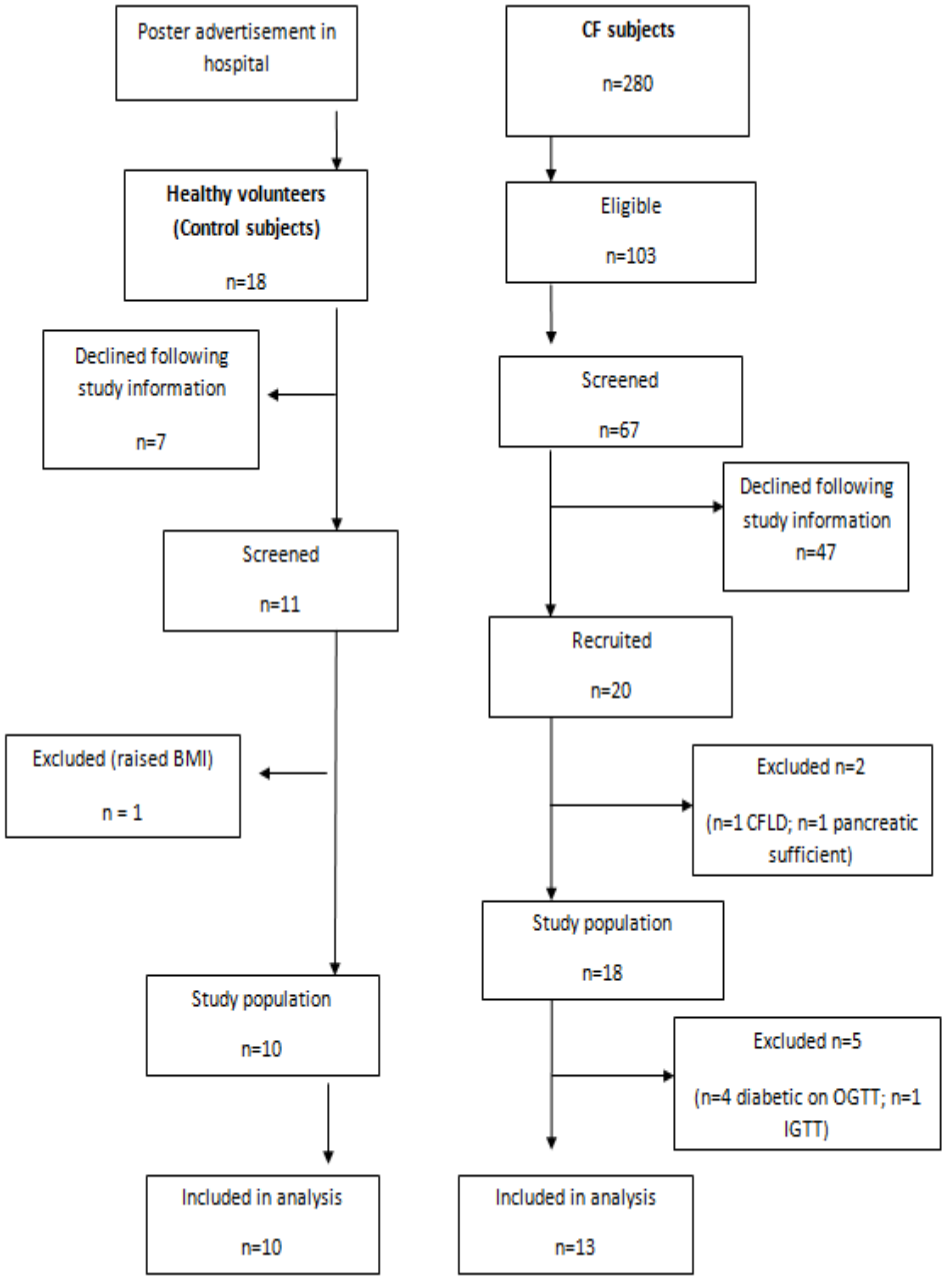


Figure 2-1: Subject selection

2.2.4 Demographic data

I recruited 10 control subjects (5 males and 5 females) and 20 CF subjects [See Table 2-1 below and Appendix 1]. Of the CF subjects, four were diabetic and one had IGT based on the OGTT, one subject with NGT was subsequently found to be pancreatic sufficient and one subject was found to have significant liver disease (See CONSORT Flow diagram - Figure 2-1 on page 51). These subjects were excluded from the analysis.

The analysis includes 10 control subjects (age range 23 – 41 years) and 13 CF subjects (age range 18 – 38 years). Their characteristics are shown in Table 2-2 below.

The 13 CF subjects had a FEV_1 of 80 ± 16 (% predicted) and a FVC of 95 ± 15 (% predicted).

Table 2-1: Subject distribution

	n	M	F
Controls	10	5	5
CF-NGT	14	12	2
CF-IGT	1	1	0
CFRD	5	4	1

Table 2-2: Subject characteristics

	Age (yrs)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (IFCC)
Controls	29.7 ± 4.8	70.9 ± 10.8	1.7 ± 0.1	24.3 ± 3.4	34.2 ± 3.1
CF	25.9 ± 5.5	71.8 ± 7.3	1.7 ± 0.1	23.9 ± 2.1	37.6 ± 3.8

Control subjects (n=10); CF subjects (n=13). [Mean ± SD]

2.3 Consent

All subjects provided written consent in triplicate in accordance with Good Clinical Practice (GCP). [See appendix for approved REC documents].

2.4 Power calculation

This work was preliminary and exploratory and hence no statistical power analysis was carried out. Based on previous studies in other populations it was recognized that large cohort sizes would be necessary to detect weak associations.

I initially set out to undertake a study of 12 CF patients as a realistic and pragmatic target for recruitment from the existing population at the CF centre. Moreover, this patient number would make the study feasible considering funding, time and effort.

Following the University Advisory Panel meeting in May 2012, it was suggested that the CF subjects be increased to 20 to make the study more powerful and this amendment to the protocol was approved by the NHS REC committee.

2.5 Statistical analysis

Data were collated on a spreadsheet (MS Excel 2010) and analysed using StatsDirect® version 3.1.14®. Data were analysed for distribution using the Shapiro-Wilk test. For normally distributed data, comparisons between groups were carried out with an independent t-test and differences within groups with a paired t-test. For non-normally distributed data, comparisons between groups were carried out with a Mann-Whitney test and differences within groups with Wilcoxon's Signed Ranks Test. Differences over time were examined using repeated analysis of variance (ANOVA). Significant F-tests were followed by Bonferroni adjusted pairwise comparisons. Statistical significance was accepted at the 5% level and results presented as means ± standard deviation (SD) in all data tables. All figures in the results chapters with charts represent the mean ± confidence interval (CI) and one half of each error bar has been omitted for clarity (unless otherwise stated).

2.6 Study protocol

See Figure 2-2 on page 56.

Subjects consenting and fulfilling the inclusion criteria were recruited and entered into a database with full details of demographics, anthropometry (height and weight) and baseline spirometry (FEV_1, FVC).

On 2 occasions, following an overnight fast of at least 12 hours, subjects underwent a standard OGTT at 0800 hrs and then within a week, a MMT at 0800, 1300 and 1800 hours.

Part 1 (OGTT – Liquid meal) – Visit 1

Subjects were asked to take any routine medication at least 1 hour prior to the start of the test (0800 hrs).

Subjects then consumed 113 mL *Polycal*® (Nutricia Clinical, UK) made up with extra water to a total volume of 200mL. This was the routine OGTT (liquid meal) providing subjects 75g of CHO.

Part 2: (MMTT - Mixed meal) – Visit 2

This was carried out within a week of the initial test and after a 12 hour overnight fast. As in visit 1, the subject took any routine medication at least 1 hour prior to the start of (each) test.

The mixed meal (see Table 2-4 on page 58) was consumed by the fasted subject within 10-minutes. All the tests as in part 1 were carried out.

Following completion of the first MMT at 1000 hrs, a second MMT was carried out after three hours (1300 hrs), followed by a third MMT after another 3 hours (1800 hrs). Subjects remained fasted between meals.

On each occasion, blood was drawn and ultrasound measurements performed as in Part 1. These timings were chosen to physiologically match the time most individuals have their meals. The study was considered complete at the collection of the last blood sample and recording of the gastric antral diameters at 120-minutes after the completion of the third meal.

Prior to both visit 1 and 2 the following were obtained prior to the commencement of the study

- Written consent (visit 1)
- Inclusion and exclusion criteria reviewed
- Medical history reviewed
- Medications and concomitant medications reviewed
- BMI
- Routine physical examination
- Vital signs: blood pressure, heart rate, respiratory rate, oxygen saturation (SpO_2)
- Blood samples for FBC (visit 1)
- Asked overnight fasting period (minimum 12 hours required)

For each part of the experiment, blood samples were collected at 0 (baseline), 30, 60, 90 and 120-minutes. Gastric motility, using ultrasound, was measured as outlined above at 15, 30, 60, 90 and 120-minutes. This part of the study was considered complete at the collection of the last blood sample and recording gastric antral diameters at 120-minutes (See Experiment 1). At visit 1, the above was carried out once and at visit 2, was carried out thrice.

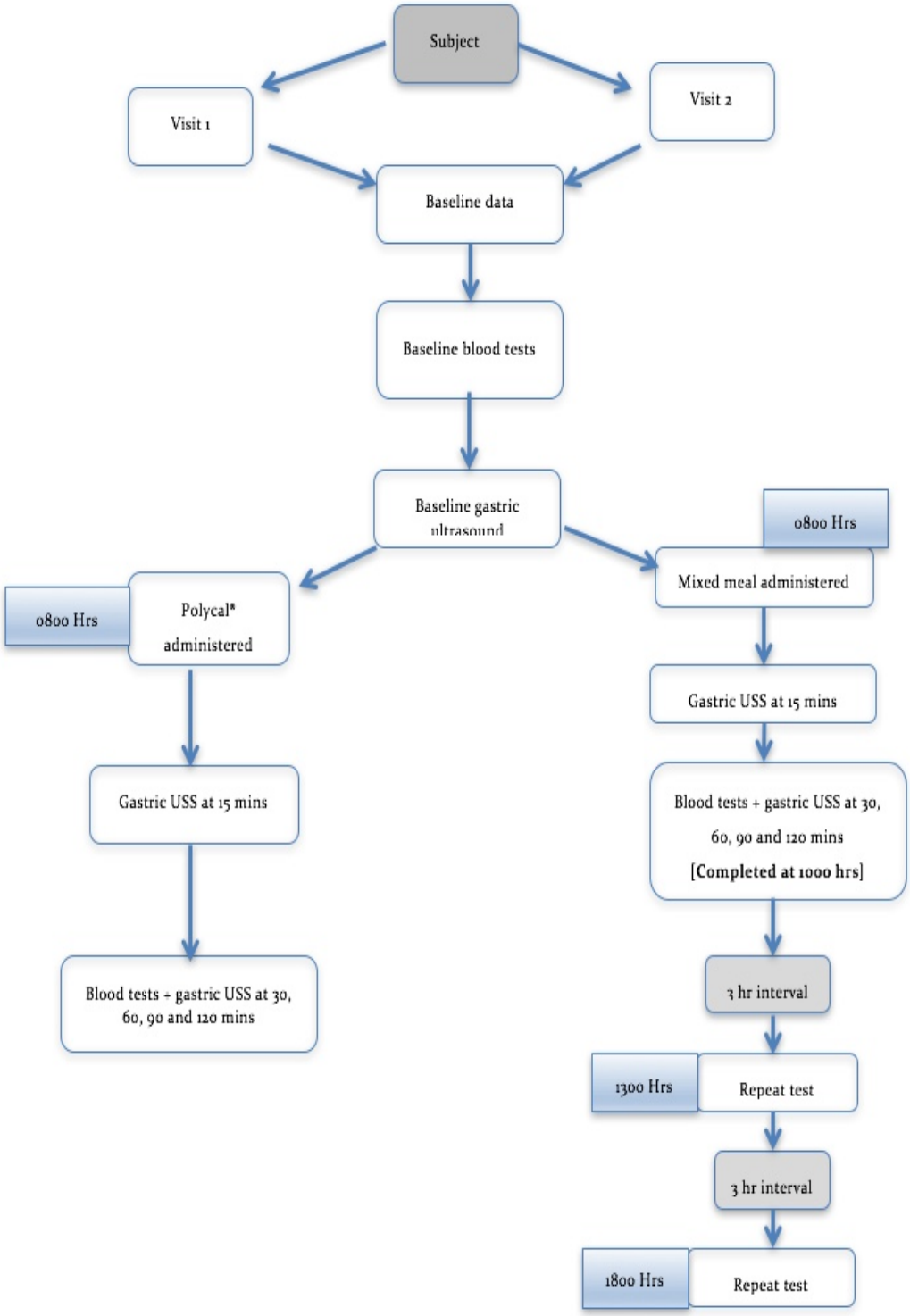


Figure 2-2: Study protocol

Table 2-3: Schedule of tests

		Time Points (mins)	0	15	30	60	90	120
Visit 1	**0800 – 1000 hrs**	**Blood collection**	x		x	x	x	x
		Ultrasound study	x	x	x	x	x	x
Visit 2	**0800 – 1000 hrs**	**Blood collection**	x		x	x	x	x
		Ultrasound study	x	x	x	x	x	x
	1300 – 1500 hrs	**Blood collection**	x		x	x	x	x
		Ultrasound study	x	x	x	x	x	x
	1800 – 2000 hrs	**Blood collection**	x		x	x	x	x
		Ultrasound study	x	x	x	x	x	x

2.7 Mixed meal composition

A standardized mixed meal (see Table 2-4) consisting of carbohydrate, lipids and protein was formulated. Each meal resembled a standard continental breakfast and provided the subject 741.07 kcal. The components of the mixed meal were determined using the nutrient analysis software system Microdiet® (Downlee Systems Ltd, UK).

The meal provided nutrients that are shown in Table 2-5.

Table 2-4: Composition of meal administered

Food name	Quantity
White bread, sliced	2 (72 g)
Unsmoked standard ham (Tesco®)	75 g
Margarine (soft, polyunsaturated)	2 (14 g)
Orange juice (sweetened)	1 (150 g)
Cheddar cheese slice (Tesco®)	1 (45 g)
Digestive biscuits, plain	30 g
Total	386 g

Table 2-5: Nutrients in meal

Nutrient	Amount (g)	Nutrient	Amount (g)
Water	231.03	Total Monounsaturates	12.27
Total Nitrogen	5.54	Total polyunsaturates	6.8
Protein	34.13	Non-starch polysaccharides	2.03
Carbohydrate	73.75	Total sugars	26.51
Sodium	1.79	Total saturates	15.12

2.8 Blood sample collection

A cannula (20 or 22 G) was inserted in the ante-cubital fossa or the forearm. Blood was collected using BD vacutainer® tubes (See Table 2-6 below) from the cannula after discarding the first 3 ml. Following collection of each sample, the cannula was flushed with 5 mls of 0.9% Sodium Chloride.

Blood samples were separated immediately by centrifugation at 3000 rpm for 10-minutes at 4 °C. Aliquots of serum and plasma were stored at -80°C until analysis in batches at the end of the study.

In summary, the various tests carried out through the study are outlined in Table 2-3 on page 57 and the summary of the protocol outlined in Figure 2-2 on page 56.

Table 2-6: Blood tubes used for sample collection

Test	Tube
Plasma glucose	Potassium oxalate/ sodium fluoride tube
Serum biochemistry	Clot activator and gel for serum separation
Full blood count	Liquid K_3EDTA
Gut and pancreatic cell hormones	Spray-coated K_2EDTA

2.9 Laboratory methods

Analyses of all samples were carried out at the listed below (Table 2-7) and the methods employed detailed in the relevant chapters.

Table 2-7: Sample analysis location

Test	Laboratory
• Glucose	Clinical Biochemistry, Royal Liverpool University Hospital, Liverpool L7 8XP, UK.
• Insulin • C-peptide	Clinical laboratory, Liverpool Heart and Chest Hospital, Thomas Drive, Liverpool L14 3PE, UK.
• Glucagon • GIP • GLP_1 • Pancreatic polypeptide (PYY)	Clinical biochemistry laboratory, Department of Genomics of Common Disease, Hammersmith Hospital, Imperial College London, Burlington-Danes Building, Du Cane Road, London, W12 0NN, UK. [Dr Paul Bech]

3 Experiment 1: The evaluation of gastric emptying in Cystic Fibrosis using bedside ultrasonography

3.1 Assessment of gastric motility

Ultrasound measurements were performed in real time using a 3.5-MHz abdominal transducer probe [243] connected to a M-TURBO Ultrasound machine® (Sonosite, Washington , United States).

I was trained and competency assessed by an expert ultra-sonographer (Dr Hilary Fewins MBBS, FRCR, Consultant Radiologist, Liverpool Heart and Chest Hospital), prior to the start of this study.

Measuring GER using this method involved marking of the gastric antrum (See Figure 3-1 on page 62) by identifying the liver and the aorta: once these landmarks are identified, the gastric antrum comes into view and when located, 2 perpendicular diameters (D_1 and D_2) were measured and the mean of 3 consecutive recordings taken at each time point.

The antral cross-sectional area determined using the formula

$$A_{(Antrum)} = \pi \times (D_1 \text{ mean} \times D_2 \text{ mean}) / 4$$

was obtained 15-minutes after ingestion of the meal (liquid / mixed), as the stomach is largest at 15-minutes, allowing for gastric accommodation.

From this, the emptying rate was obtained by determining the % change from 15-minutes to any time point

$$GER = [1-(A_{\text{area x mins}} / A_{\text{area 15 min}})] \times 100$$

A GER of > 45% at 60-minutes (GER_{60}) for a liquid meal and > 63% at 90-minutes (GER_{90}) for the mixed meal [243] is considered normal.

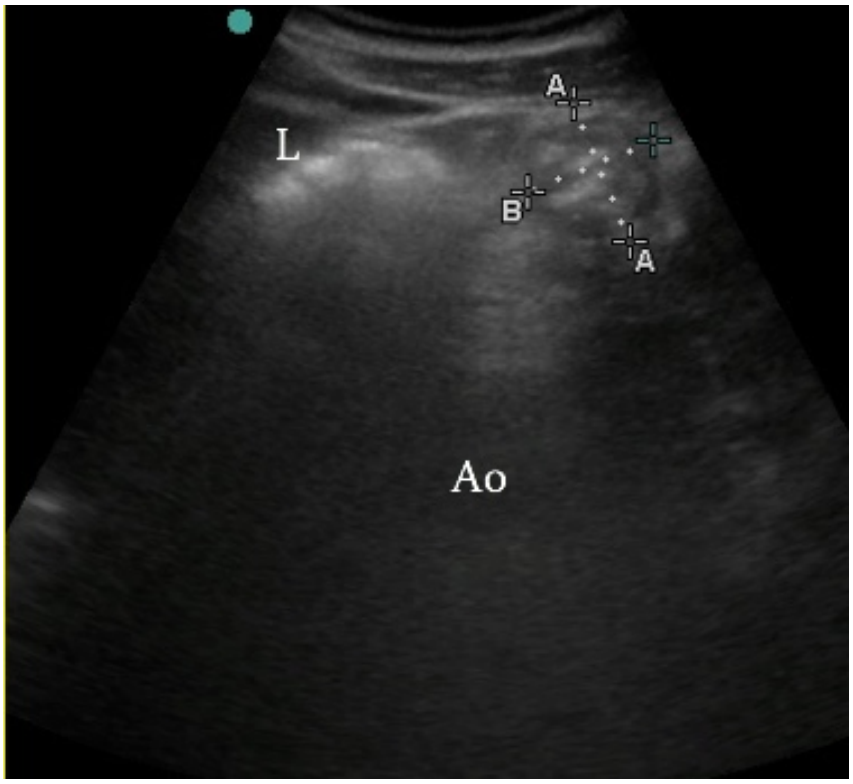


Figure 3-1: Identification of gastric antrum

The figure illustrates the calculation of gastric volume by identifying the landmarks of the (L) Liver and (Ao) Aorta and measuring the perpendicular diameters (A, B) of the gastric antrum. This image is that of control subject 4 after the liquid meal (OGTT) at 15 minutes.

3.2 Results

Ten control and 13 CF subjects underwent ultrasonographic assessment of GER. It was not possible to reliably identify the gastric antrum in 1 CF subject, so data are reported for the remaining 12.

3.2.1 Oral Glucose Tolerance Test (liquid meal)

See Table 3-1 on page 64 and Figure 3-2 on page 65

A. Controls

These had a mean gastric emptying at 60-minutes (GER_{60}) of 51 %, which is within the normal range. None had delayed gastric emptying. Individual data are given in the appendix (Appendix 2 on page IV).

B. Cystic Fibrosis

These had a mean GER_{60} of 46%, which is also within the normal range. However, 5 (42%) subjects had delayed gastric emptying. Individual data are given in the appendix (Appendix 2 on page IV).

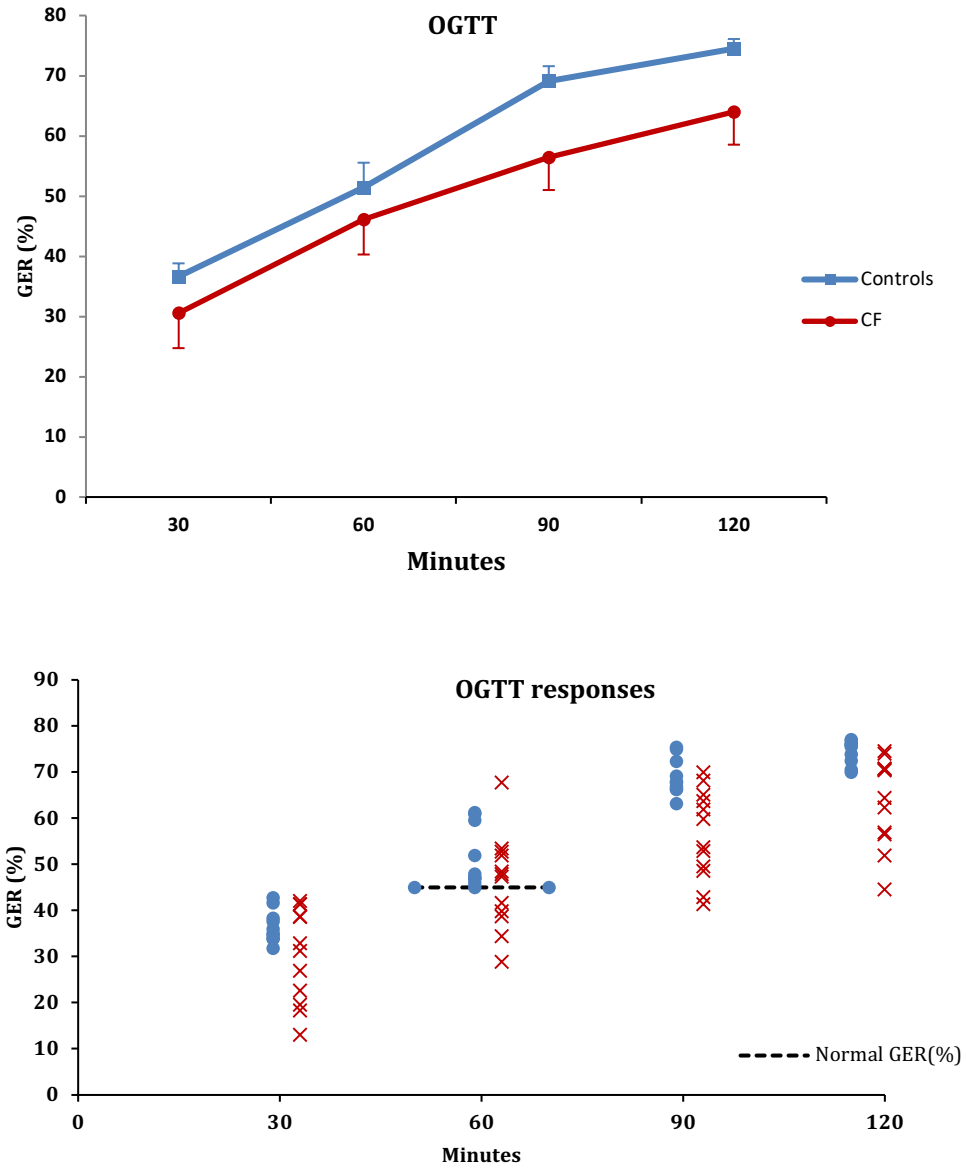
C. Controls v Cystic Fibrosis

CF subjects had a lower baseline GER (GER_{30}), but this did not achieve statistical significance compared to controls (p=0.07). However, GER worsened over time, becoming significant at GER_{90} (p=0.001) and GER_{120} (p=0.003). Individual data are given in the appendix (Appendix 2 on page IV).

Table 3-1: GER following the OGTT

	GER (%)			
	30 mins	**60 mins**	**90 mins**	**120 mins**
Controls	37 ± 4	51 ± 8	69 ± 4	74 ± 3
CF	31 ± 10	46 ± 10	56 ± 10	64 ± 10
p	0.07	0.22	0.001	0.003

Comparison of gastric emptying rate (GER%) of a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=12) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Mann-Whitney U test.



(Top panel): Comparison of gastric emptying rate (GER%) of a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=12) subjects (mean ± CI). (Bottom panel): Comparison of the individual GER% over 120 minutes, following the liquid meal. [• Controls x CF]

Figure 3-2: GER: Response to the OGTT

3.2.2 Mixed Meal Test 1 (solid meal)

See Table 3-2 on page 66 and Figure 3-3 on page 67

A. **Controls**

These had a mean gastric emptying at 90-minutes (GER_{90}) of 72%, which is within the normal range. None had delayed gastric emptying. Individual data are given in the appendix (Appendix 2 on page IV).

B. Cystic Fibrosis

These had a mean GER_{90} of 56%, which is below the normal range; only 2 had normal emptying. Individual data are given in the appendix (Appendix 2 on page IV).

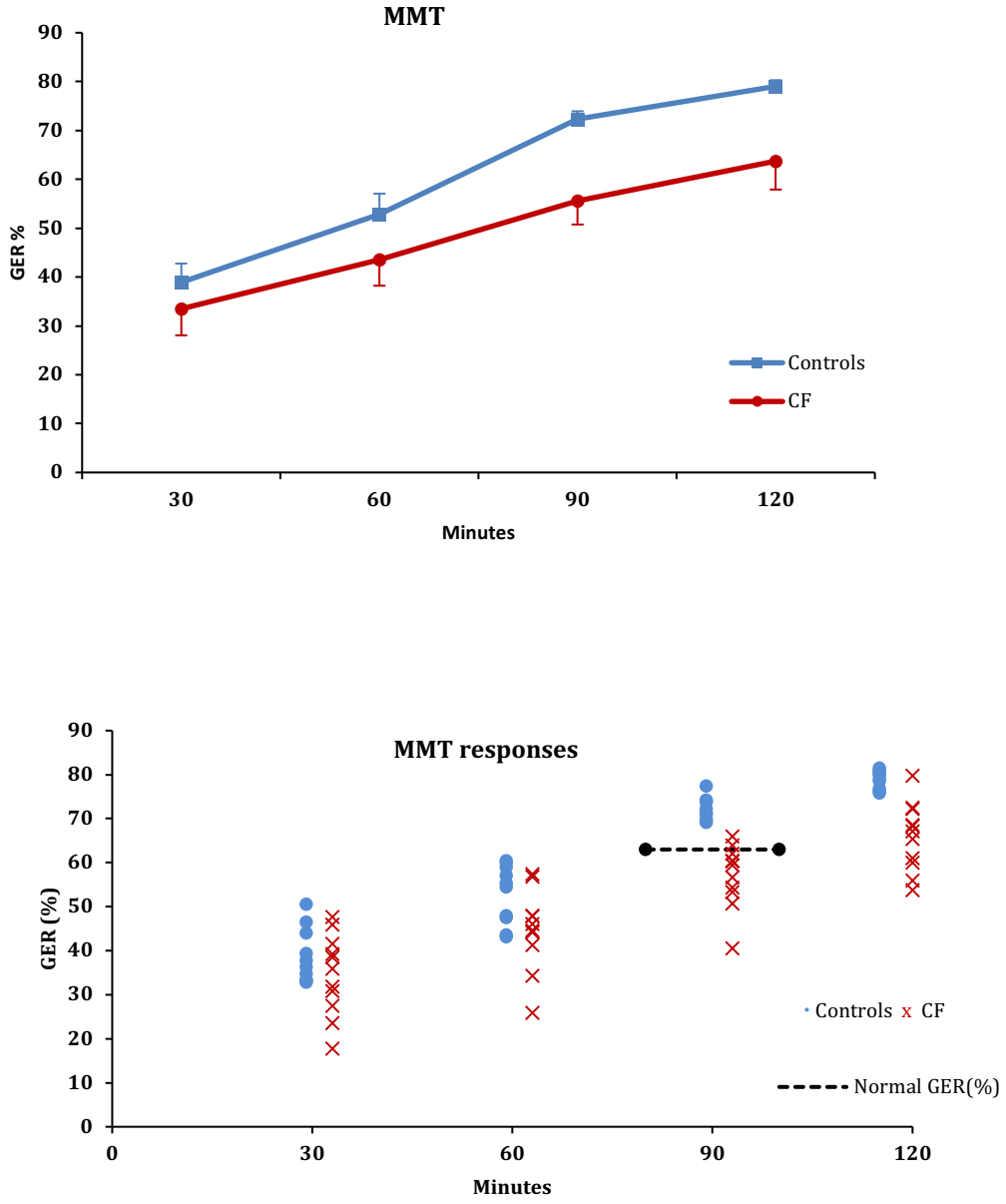
C. Controls v Cystic Fibrosis

There was no difference at GER_{30} (CF 33% vs controls 39%, p=0.12), but GER in CF worsened over time, becoming significant at GER_{60} (CF 44% vs controls 53%, p=0.01) until GER_{120} (64% vs 79%, respectively, p=0.0003). Individual data are given in the appendix (Appendix 2 on page IV).

Table 3-2: GER following the MMT

	GER (%)			
	30 mins	**60 mins**	**90 mins**	**120 mins**
Controls	39 ± 6	53 ± 7	72 ± 3	79 ± 2
CF	33 ± 10	44 ± 9	56 ± 9	64 ± 10
p	0.12	0.01	0.002	0.0003

Comparison of gastric emptying rate (GER%) of a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=12) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Mann-Whitney U test.



(Top panel): Comparison of gastric emptying rate (GER%) of a mixed meal (mean ± CI) over 120 minutes between control (n=10) and CF (n=12) subjects. (Bottom panel): Comparison of the individual GER% over 120 minutes, following the mixed meal (MMT). [• Controls x CF].

Figure 3-3: GER - Response to the MMT

3.2.3 Comparison between the OGTT and MMT

See Table 3-3 on page 68 and Figure 3-4 on page 69

I then compared the differences in gastric motility in both groups in response to the OGTT and the MMT1.

A. Controls

Control subjects had normal emptying for both the liquid (GER_{60}) and the mixed meal (GER_{90}) until 90-minutes. The GER_{30}, GER_{60} and GER_{90} were no different between the liquid and mixed meals. However, the GER_{120} was higher after the MMT compared to the OGTT ($p<0.0001$).

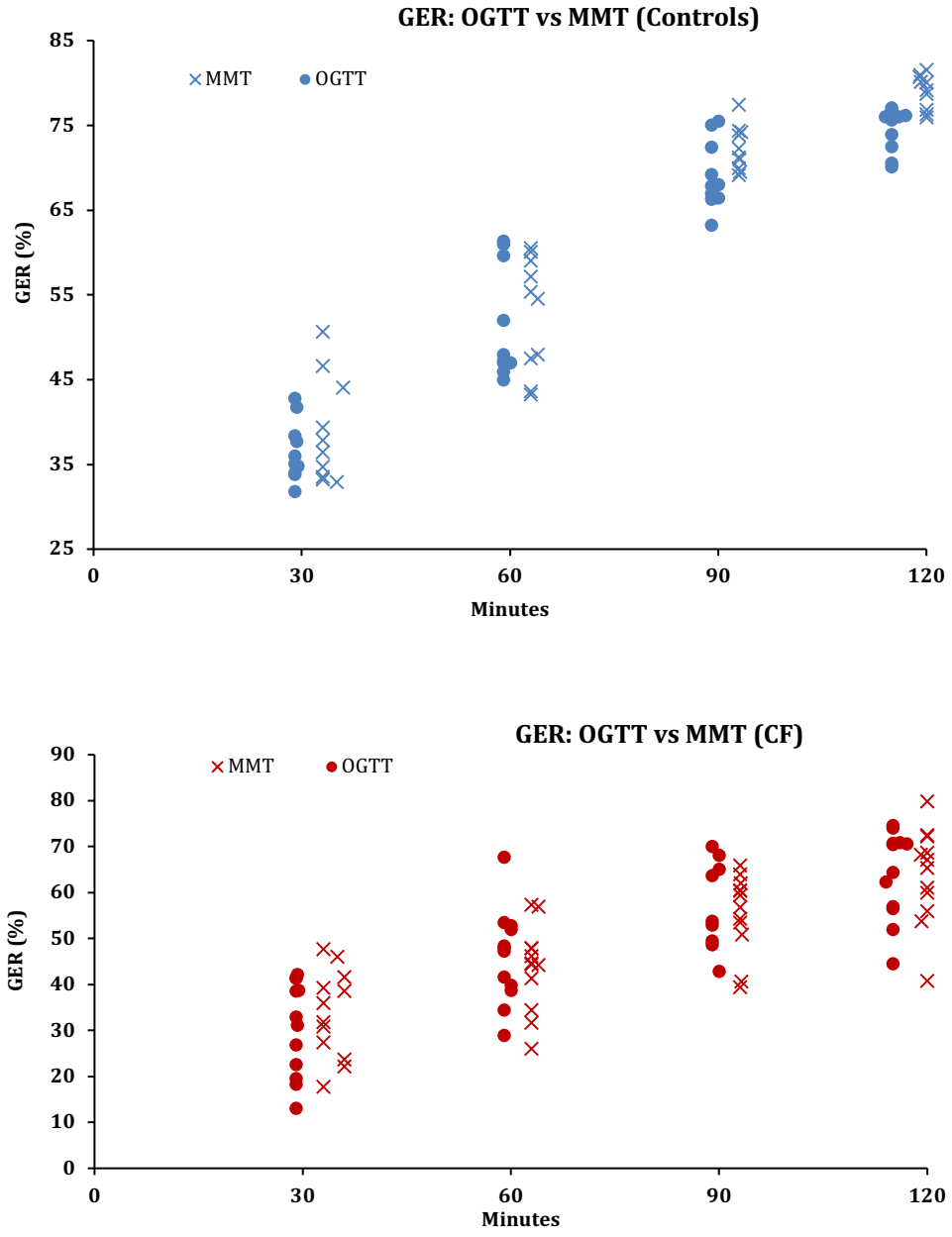
B. Cystic Fibrosis

There were no differences between individual time points in the group between the 2 studies. However, CF subjects had decreased gastric emptying following the MMT compared to the OGTT (Fisher's exact test, $p = 0.049$).

Table 3-3: GER - Comparison between the OGTT and MMT

		GER (%)			
		30 mins	**60 mins**	**90 mins**	**120 mins**
Controls	**OGTT**	37 ± 4	51 ± 8	69 ± 4	74 ± 3
	MMT	39 ± 6	53 ± 7	72 ± 3	79 ± 2
	p	0.38	0.47	0.08	<0.0001
CF	**OGTT**	31 ± 10	46 ± 10	56 ± 10	64 ±1 0
	MMT	33 ± 10	44 ± 9	56 ± 9	64 ± 10
	p	0.36	0.47	0.68	0.93

Comparison of gastric emptying rate (GER%) of a liquid (OGTT) and mixed (MMT) meal over 120 minutes in control (n=10) and CF (n=12) subjects (mean ± SD). Statistical comparisons between tests were carried out using the Wilcoxon signed-rank test.



Comparison of the individual gastric emptying rate (GER%) over 120 minutes, following the liquid meal (OGTT) and mixed meal (MMT) in control (n=10, upper panel) and CF subjects (n=12, bottom panel [• OGTT x MMT].

Figure 3-4: GER - Comparison between OGTT and MMT

3.2.4 Diurnal Studies (MMT 2 and MMT 3)

See Table 3-4 and Figure 3-5 on page 72

A. Controls

In the afternoon (MMT2), control subjects had gastric emptying [GER_{90}] of 70%, which is within the normal range.

In the evening (MMT3), control subjects had the same emptying (GER_{90}) of 70%. Again, none had delayed gastric emptying. Individual data are given in the appendix (Appendix 2 on page IV).

B. Cystic Fibrosis

In CF subjects, the GER_{90} in the afternoon (MMT2) was 58%, again indicating reduced emptying. Only 1 subject had normal emptying at this time. In the evening (MMT3) GER_{90} was 59%, indicating reduced emptying in the evening too. Two subjects had normal emptying at this time.

C. Controls v Cystic fibrosis

When the 2 groups were compared in the afternoon (MMT2), CF subjects showed no difference in the GER_{30} (CF 31% vs controls 36%, p=0.20), but lower GER_{60} (CF 43% vs controls 54%, p=0.003) and lower GER_{120} (CF 67% vs controls 77%, p=0.0003) compared to control subjects.

When the 2 groups were compared in the evening (MMT3) CF subjects now had lower GER_{30} (CF 26% vs controls 35 %, p=0.003), lower GER_{60} (CF 43% vs controls 50%, p=0.02) and lower GER_{120} (CF 71% vs controls 76%, p=0.001) compared to control subjects.

Diurnal changes

I then compared the incremental changes in GER through the day and evaluated the changes in GER at 30 (GER_{30}), 60 (GER_{60}), 90 (GER_{90}) and 120 (GER_{120}) minutes in both groups (See Figure 3-5 on page 72 and Table 3-4 on page 72).

A. Controls

When assessed for changes during the day, there was no difference in GER_{90} throughout the day (p=0.07) in control subjects. There was also no difference throughout the day for the GER_{30} (p=0.37) and GER_{60} (p=0.28). GER_{120} appeared to worsen throughout the day (p=0.04), but the post-hoc statistical tests were not significant for specific times of the day.

B. Cystic Fibrosis

In CF subjects, although there was no difference in GER_{90} throughout the day (p=0.33), GER_{30} worsened (p=0.02) with GER_{30} lower in the evening (MMT3) (26%) compared to the morning (MMT1) (33 %) [p=0.01]. There was no difference in GER_{60} (p=0.97), but conversely GER_{120} appeared to improve throughout the day (p=0.03). The evening GER_{120} (MMT3) (71%) although higher than the corresponding time in the morning (MMT1) (64%), was statistically not significant (p=0.036) on the post-hoc test.

3.2.5 Repeatability of tests (GER %)

Since control subjects had gastric motility assessed on several occasions, I used this group to evaluate repeatability of the measures. An ANOVA comparing GER_{30} in the morning, afternoon and evening studies (MMT1, MMT2, MMT3: Appendix 2) showed good similarity [F (variance ratio) = 0.84, p=0.44] indicating that the method was repeatable.

Table 3-4: GER through the day

	Morning			Afternoon			Evening		
	Control	**CF**	**p**	**Control**	**CF**	**p**	**Control**	**CF**	**p**
30 mins	39 ± 6	33 ± 10	0.12	36 ± 9	31 ± 6	0.2	35 ± 6	26 ± 7*	0.003
60 mins	53 ± 7	44 ± 9	0.01	54 ± 9	43 ± 7	0.003	50 ± 4	43 ± 8	0.02
90 mins	72 ± 3	56 ± 9	<0.0001	70 ± 2	58 ± 5	<0.0001	70 ± 2	59 ± 4	<0.0001
120 mins	79 ± 2	64 ± 10	0.0003	77 ± 3	67 ± 7	0.0003	76 ± 4#	71 ± 3£	0.001

*Comparison between gastric emptying rates (GER%) over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=12) subjects at each time point were carried out using the Mann-Whitney U test and a repeated measures ANOVA was performed to study differences within the group through the day. [Comparison to morning: *p=0.02; £ p=0.03; #p=0.02]*

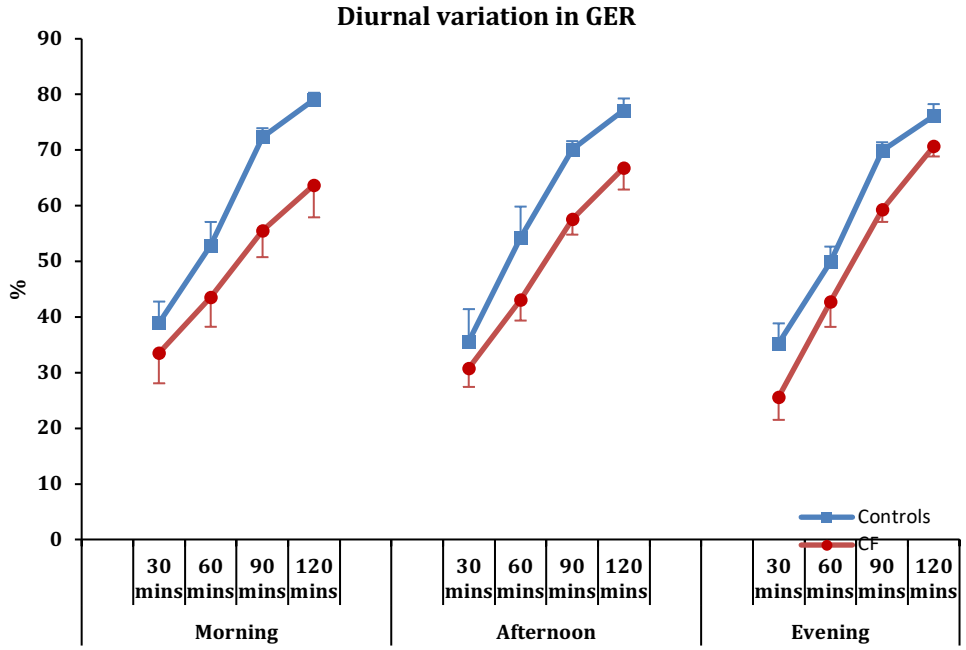


Figure 3-5: GER through the day

Comparison of GER of a mixed meal (mean ± SEM) over 120 minutes between control (n=10) and CF (n=12) subjects in the morning, afternoon and evening.

3.3 Discussion

This gastric emptying ultrasound technique is inexpensive, the equipment is widely available and moreover it can be carried out at the bedside. The use of a 3.5-MHz abdominal transducer probe to measure the antral cross-sectional area and volume allows gastric emptying to be assessed in real time and this can be applied to both liquid and mixed meals.

The technique has been shown to be accurate with good intra- and inter-observer agreement [243]: I was trained and had a competency assessment carried out by a validated experienced ultrasound radiologist. In this experiment, I was able to reliably measure GER in 22 of 23 subjects (96%).

Although my experiment did not seek to evaluate reproducibility and repeatability, four complete studies for each subject were carried out on two different days, three of which were performed on the second day. ANOVA of these duplicated tests gave a variance ratio (F) of 0.84 ($p=0.44$), indicating an acceptable level of repeatability.

With regards to reproducibly, I had initially set out to compare the GER_{30} in the morning on the 2 separate days. However, since the volumes of substance used to make up the OGTT and MMT were different, stomach distension was dissimilar such that comparison was not possible. Furthermore, it was not possible to blind the study given the nature of the experimental subjects.

This is the first study to use ultrasound to assess gastric emptying in the adult CF population. Using this method, I have shown that healthy control subjects have normal gastric emptying for both liquid and mixed meals after an overnight fast, in keeping with previous literature using ultrasound to evaluate GER [248].

However, for the first time I have shown that although adult CF subjects without known CFRD have a normal antral size, following an overnight fast they have delayed gastric emptying for both liquid and mixed meals.

Only one previous study has evaluated GER throughout the day in normal subjects. Goo et al [249] measured GER in 16 healthy males at 8am and 8pm on separate occasions (2 – 21 days apart), and showed that GER in the evening was greater for solids but not liquids when compared to the morning. My results partly concur with this, since my normal subjects had normal GER for both solids and liquids throughout the day, but at 120-minutes the emptying in the evening appeared reduced compared to the morning, although this was not statistically significant on the post-hoc analysis, possibly due to the small sample size of my study. However, these two studies are not comparable – unlike the work of Goo, my subjects were studied on the same day with fasting between each meal, and the constituents of the meals used were different.

In my study cohort, although the mean gastric emptying rate in response to a liquid meal was normal, 5 CF subjects (42%) had decreased gastric emptying at 60-minutes. These 5 subjects also had reduced emptying of a mixed meal. I explored possible reasons as to why these individual subjects responded differently. I reviewed the possible factors that might have reduced gastric emptying in these subjects. On review of their medications, 3 subjects were on a PPI (1 Omeprazole and 2 Lansoprazole). The other factors that might affect gastric emptying were controlled by the nature of the experiment – all subjects had the same volume and type of meal at room temperature, none reported any significant pain or anxiety and all subjects had their usual dose of pancreatic supplements.

There have been no previous studies of gastric emptying in adult CF patients throughout the day. I have shown that not only do CF individuals without known CFRD have delayed gastric emptying in the morning, but this delay continues throughout the day. Additionally, I have shown that more CF subjects have decreased gastric emptying in response to a mixed meal compared to a liquid meal, demonstrating that the mixed meal test is the more physiological test in CF individuals.

Gastric emptying is a complex process influenced by gastric contents and volume, hormonal actions, and vagal activity. A number of studies have also demonstrated that pre-existing hyperglycaemia slows gastric emptying in healthy humans [232], Type 1 [250-252] and Type 2 diabetics [231]: I chose CF individuals without known CFRD to minimise this and also

prevent the possibility of occult gastroparesis due to diabetic-related autonomic neuropathy. Similarly, by using the same carefully measured constituents in each experiment I sought to control for differences in food composition and volume. Nevertheless, these CF subjects still had a delay in gastric emptying: the experiments in subsequent chapters aim to explore dynamic glucose handling and the influence of incretins and other hormones following both physiological (MMT) and non-physiological (OGTT) nutrient loads that might influence gastric emptying.

Furthermore, gastric ultrasound may also be useful in the clinical setting, by providing a simple cheap non-invasive way of assessing gastric emptying, for example to aid the prescription of pro-kinetic agents used as an adjunct to improving nutrition in gastroparesis or preventing reflux and potential micro-aspiration.

In summary, this is the first study to use ultrasound to assess gastric motility in the adult CF population and demonstrates delayed gastric emptying throughout the day in CF subjects, compared to matched controls, for both liquid and mixed meals. Moreover, this study shows that this novel inexpensive bedside technique provides a simple method of assessing GER in CF and will have a role in the clinical setting.

4 Experiment 2: Glucose handling in CF and its diurnal variation

4.1 Insulin and C-peptide assay

Insulin and c-peptide assays employed sandwich based techniques and c-peptide assays were based on the principle of competitive binding, using commercial kits [DRG® Insulin ELISA (EIA-2935); DRG® C-Peptide ELISA (EIA-1293)].

The dynamic measuring ranges of the assays were 1.76 - 100 µIU/ml for insulin and 0.06 – 16ng/ml for c-peptide. Actual mean within and between batch precision levels (% CV) were 5.77 and 14.44 % for Insulin and 6.41 and 7.07 % for c-peptide.

All ELISA assays employed absorbance based detection systems at 450nm, measured on a Wallac Victor 2 plate reader.

4.2 Glucagon assay

Glucagon was measured using established in-house (Hammersmith Hospital) RIAs [253, 254]. All samples were assayed in duplicate. Glucagon was purchased from Bachem Ltd (Switzerland). All other reagents and materials were supplied by Sigma (Poole, Dorset, UK). The glucagon labels were prepared by Professor M. Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) who iodinated the peptide using the iodogen method [255] and this was purified by reverse-phase HPLC.

Assays were performed in veronal buffer (1 litre distilled water containing 10.3g sodium barbitone, 0.3g sodium azide), at pH 8.0 with 0.3% BSA (VWR International, UK). Standard curves were prepared in assay buffer of 0.5 pmol/ml, added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 µl.

The glucagon antibody (RCS5) was raised in rabbits against the C-terminal of glucagon and is therefore specific for pancreatic glucagon. In this assay, the antibody was used at a dilution of 1:50000.

Experimental samples of 50 µl, 100 µl glucagon antibody solution and 100 µl of glucagon label solution were used and all tubes were buffered to a total volume of 700 µl with assay buffer. The assays were incubated for 96 hours at 4°C. Free peptide was separated from bound using charcoal adsorption. To each tube, 4 mg of charcoal, suspended in 0.06 M phosphate buffer with gelatine was added immediately prior to centrifugation. The samples were then centrifuged at 1500 rpm at 4° C, for 20-minutes. Bound and free label were separated and both the pellet and supernatant counted for 180 seconds in a γ-counter (Model NE1600, Thermo Electron Corporation). Plasma glucagon concentration in the samples were calculated using a non-linear plot (RIA Software, Thermo Electron Corporation) and results calculated in terms of the standard.

4.3 Results

As outlined in the subject demographics and selection, subjects with a 120-minute value >11.1 mmol/L were excluded from analysis. Data are reported on 10 controls and 13 CF subjects.

4.3.1 Oral Glucose Tolerance Test (liquid meal)

4.3.1.1 Glucose:

See Table 4-1 below and Figure 4-1 on page 79

A. Controls

In response to the OGTT, control subjects had normal baseline (4.52 ± 0.44 mmol/L) and 120-minute (4.59 ± 1.48 mmol/L) glucose levels.

B. Cystic Fibrosis

CF subjects had normal baseline (4.61 ± 0.46 mmol/L) and 120-minute (5.06 ± 1.13 mmol/L) glucose levels.

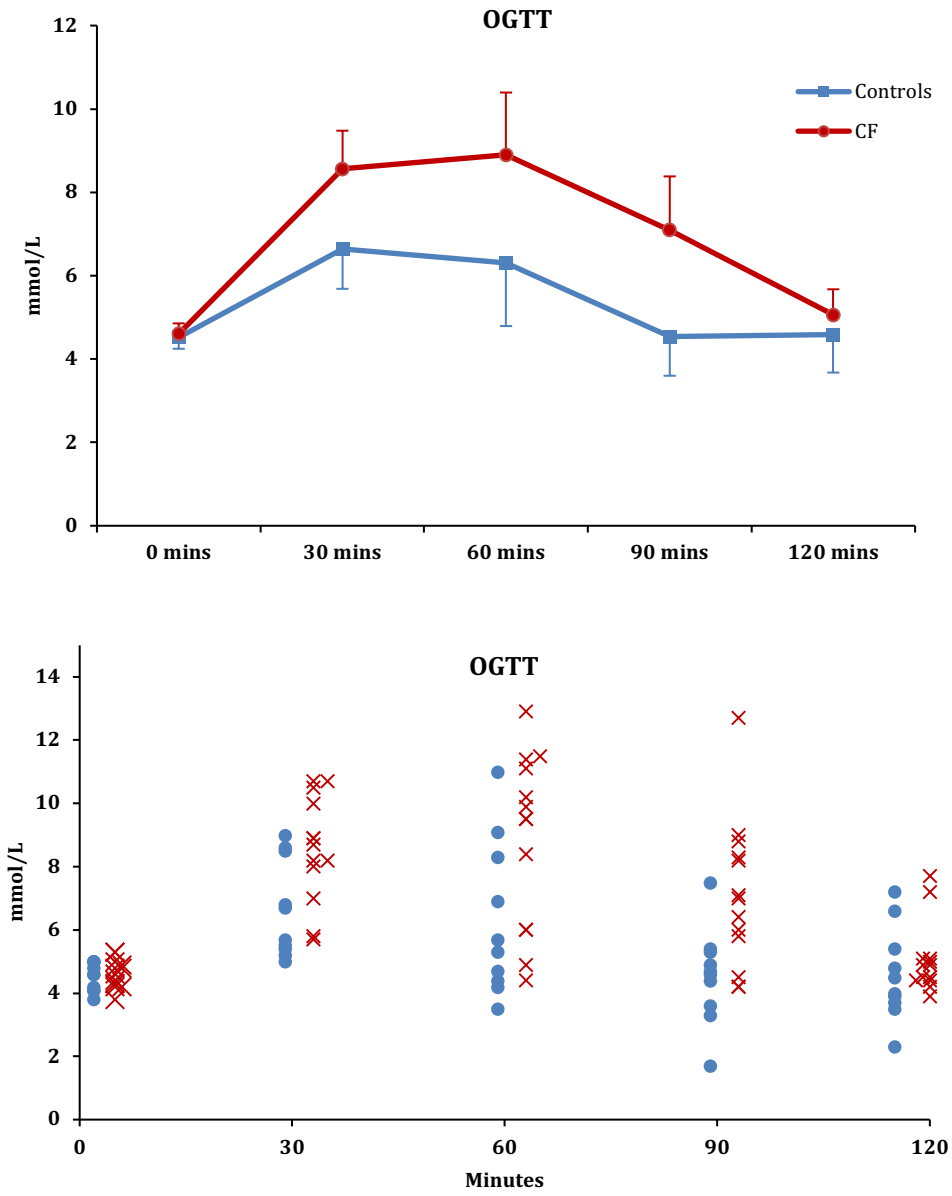
C. Controls v Cystic Fibrosis

Compared to healthy controls, in response to the OGTT, CF subjects had higher 30 (p=0.01), 60 (p=0.03) and 90 (p=0.008)-minute glucose levels.

Table 4-1: Glucose levels following the OGTT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	4.52 ± 0.44	6.64 ± 1.54	6.31 ± 2.45	4.54 ± 1.52	4.59 ± 1.48
CF	4.61 ± 0.46	8.56 ± 1.68	8.90 ± 2.75	7.09 ± 2.37	5.06 ± 1.13
p	0.65	0.01	0.03	0.008	0.39

Comparison of glucose (mmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the independent t-test.



(Top panel): Comparison of glucose (mmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual glucose levels over 120 minutes, following the liquid meal (OGTT). [• Controls x CF].

Figure 4-1: Plasma glucose responses to the OGTT

4.3.1.2 Insulin

See Table 4-2 on page 80 and Figure 4-2 on page 81

A. Controls

All control subjects had baseline insulin levels within the normal range (< 25 μIU/mL). However, at 120-minutes 4 control subjects had low insulin levels (normal range 16 - 166 μIU /L).

B. Cystic Fibrosis

All CF subjects had baseline insulin levels within the normal range (< 25 μIU/mL), but at 120-minutes, 7 CF subjects had low insulin levels.

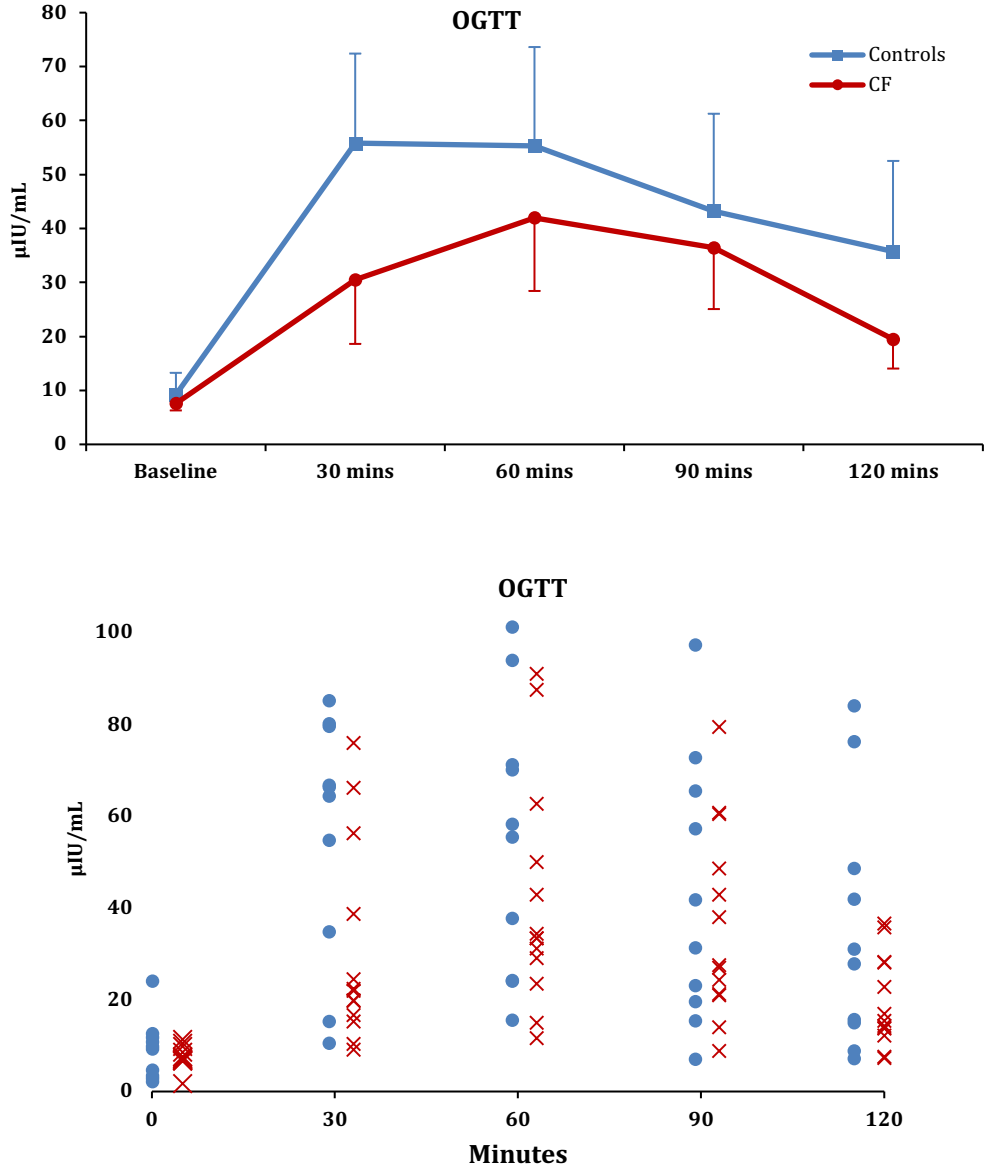
C. Controls v Cystic Fibrosis

When the 2 groups were compared, there was no difference in the baseline, 60, 90 and 120-minute values, but the mean 30-minute insulin level was lower in CF subjects (p=0.02).

Table 4-2: Insulin levels following the OGTT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	9.20 ± 6.55	55.82 ± 26.77	55.25 ± 29.65	43.16 ± 29.21	35.69 ± 27.15
CF	7.57 ± 2.35	30.52 ± 21.90	41.96 ± 24.90	36.46 ± 20.95	19.43 ± 9.90
p	0.47	0.02	0.26	0.53	0.1

Comparison of insulin (μIU/mL) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the independent t-test.



(Top panel): Comparison of insulin (µIU/mL) following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual insulin levels over 120 minutes, following the liquid meal (OGTT). [• Controls x CF].

Figure 4-2: Plasma insulin responses to the OGTT

4.3.1.3 C-peptide

See Table 4-3 below and Figure 4-3 on page 83

A. Controls

As a group, controls had normal C-peptide levels at baseline (4.38 ± 2.25 ng/mL) and at 120-minutes (8.43 ± 2.31). One subject had a high fasting c-peptide level (normal range 0.8 - 5.1 ng/mL)

B. Cystic Fibrosis

As a group, CF subjects had normal C-peptide levels at baseline (2.61 ± 2.03) and at 120-minutes (7.64 ± 2.67), but one subject had a high fasting c-peptide level.

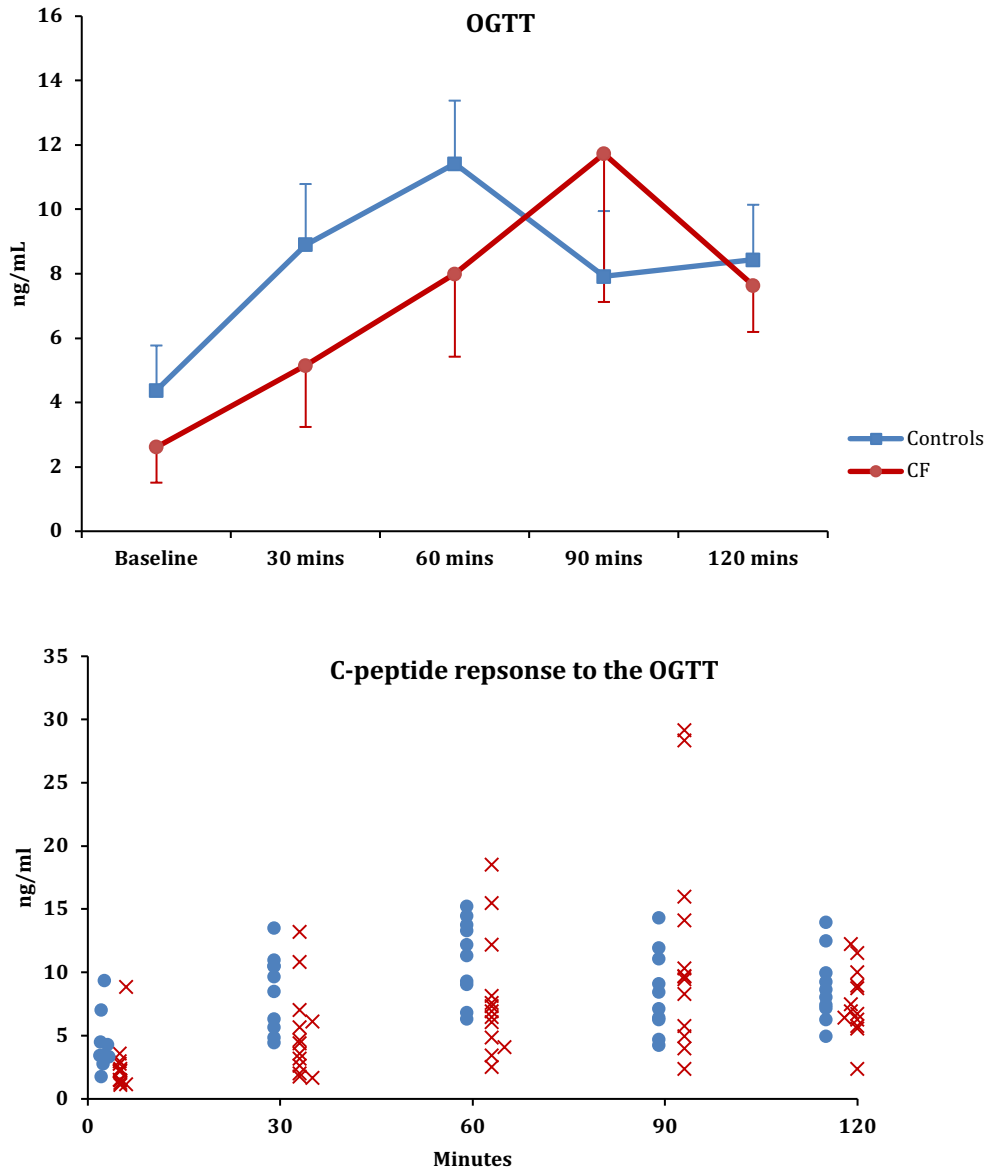
C. Controls v Cystic Fibrosis

Compared to healthy controls, CF subjects had lower 30 (p=0.02) and 60-minute (p=0.04) c-peptide levels (Table 4-3), and had lower baseline values although this did not reach statistical significance (p=0.06).

Table 4-3: C-peptide levels following the OGTT

	Baseline	30 mins	60 mins	90 mins	120 mins
Controls	4.4 ± 2.2	8.9 ± 3.0	11.4 ± 2.8	7.9 ± 2.8	8.4 ± 2.3
CF	2.6 ± 2.0	5.2 ± 3.5	8.0 ± 4.7	11.7 ± 8.5	7.6 ± 2.7
p	0.06	0.02	0.04	0.22	0.29

Comparison of c-peptide (ng/mL) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the independent t-test.



(Top panel): Comparison of c-peptide (ng/mL) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual c-peptide levels over 120 minutes, following the liquid meal (OGTT). [• Controls x CF]

Figure 4-3: C-peptide responses to the OGTT

4.3.1.4 Glucagon

See Table 4-4 below and Figure 4-4 on page 85

A. **Controls**

Glucagon analysis was not possible due to insufficient sampling for 1 control subject (subject 6) at baseline, 60, 90 and 120-minutes and another subject (subject 10) at 120-minutes. Data are reported for the remaining samples. Glucagon levels at baseline in healthy control subjects were 20.1 ± 7.9 pmol/L and at 120-minutes were 15.3 ± 5.1 pmol/L. 5 control subjects had glucagon levels above the normal range (Normal < 17.2 pmol/L).

B. **Cystic Fibrosis**

Glucagon levels at baseline were 11.0 ± 3.4 pmol/L and at 120-minutes 8.8 ± 4.3 pmol/L. 3 subjects had levels below the lower limit of detection (<5 pmol/L) – subject 1 (60-minutes), subject 12 (baseline, 90 and 120-minutes) and subject 18 (60-minutes). No CF subject had glucagon levels above the normal range (< 17.2 pmol/L).

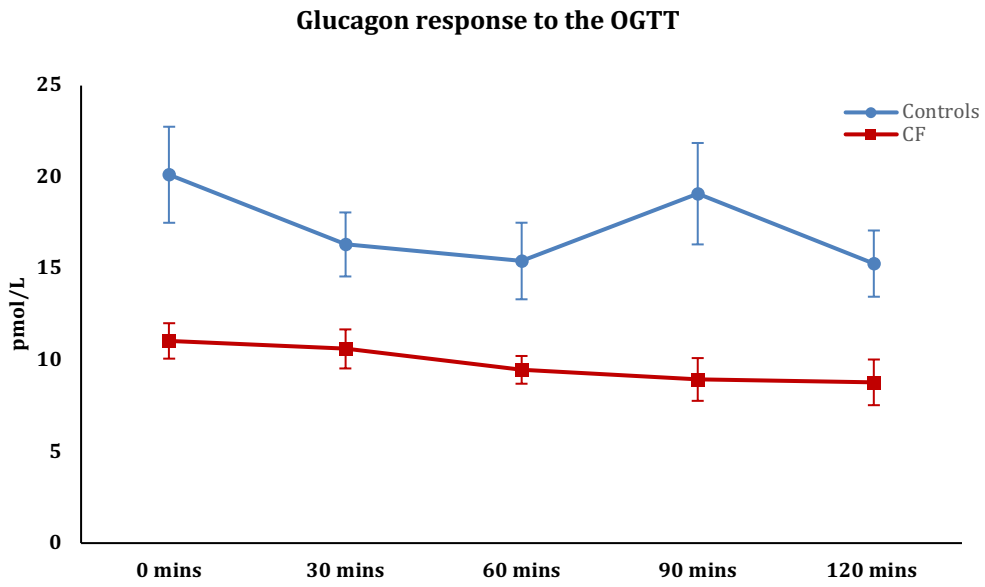
C. Controls v Cystic Fibrosis

When the 2 groups were compared, CF subjects had lower glucagon levels at all-time points.

Table 4-4: Glucagon responses to the OGTT

	Baseline	30 mins	60 mins	90 mins	120 mins
Controls	20.1 ± 7.9	16.3 ± 5.5	15.4 ± 6.3	19.1 ± 8.3	15.3 ± 5.1
CF	11.0 ± 3.4	10.6 ± 3.8	9.5 ± 2.5	8.9 ± 4.0	8.8 ± 4.3
p	0.009	0.008	0.02	0.006	0.007

Comparison of glucagon (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Mann-Whitney U test.



Comparison of glucagon (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI).

Figure 4-4: Glucagon responses to the OGTT

4.3.1.5 Time to peak response

See Table 4-5 below and Figure 4-5 on page 86

I then calculated the time to peak levels for insulin, c-peptide and glucagon following the OGTT and compared the differences between control and CF subjects.

A. Controls

Control subjects took 54 ± 24 minutes to peak insulin level, 60 ± 24 minutes to peak c-peptide level and 45 ± 47minutes to peak glucagon level.

B. Cystic Fibrosis

CF subjects took 65 ± 21 minutes to peak insulin level, 79 ± 23 minutes to peak c-peptide level and 24 ± 32minutes to peak glucagon level.

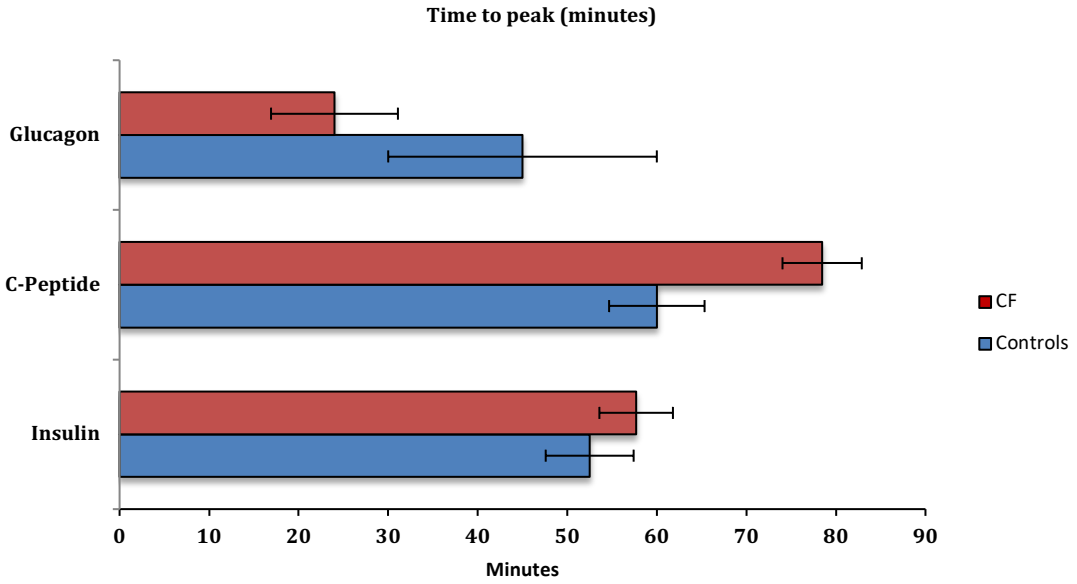
C. Controls v Cystic Fibrosis

When the 2 groups were compared, there were no differences in the time for insulin and glucagon to peak, but the time to c-peptide peak was longer in CF subjects (p=0.02).

Table 4-5: Time to reach a peak level following the OGTT

	Insulin	C-peptide	Glucagon
Controls	54 ± 24	60 ± 24	45 ± 47
CF	65 ± 21	79 ± 23	24 ± 32
p	0.14	0.02	0.37

*Comparison between control and CF subjects in the time (minutes) to reach a peak response for insulin, c-peptide and glucagon following the liquid meal (OGTT). [*Mean ± SD]



Comparison between control and CF subjects in the time (minutes) to reach a peak response for insulin, c-peptide and glucagon following the liquid meal (OGTT). [Mean ± SEM]

Figure 4-5: Time to peak response (OGTT)

4.3.1.6 Incremental changes in response to the OGTT

See Table 4-6 on page 89

I calculated the incremental and total response to the OGTT by evaluating the initial response at 30-minutes (AUC_{30}) and the total response at 120-minutes (AUC_{120}).

A. Glucose

CF subjects had higher Glucose AUC_{30} [controls: 166 ± 28, CF: 198 ± 29; p=0.02] and a higher AUC_{120} [controls: 661 ± 170, CF: 882 ± 179; p= 0.007].

B. Insulin

The insulin AUC_{30} was lower in CF subjects [controls: 975 ± 465, CF: 606 ± 356; p=0.04]. However, there was no statistical difference in the total insulin produced (AUC_{120}).

C. C-peptide

The c-peptide AUC_{30} [controls: 194 ± 75, CF: 116 ± 76; p=0.02;] was lower in CF subjects, but there was no difference in the AUC_{120}.

D. Glucagon

With regards to glucagon, the CF group had lower AUC_{30} [controls: 556 ± 152, CF: 332 ± 78; p=0.002] and AUC_{120} between the 2 groups [controls: 2004 ± 597, CF: 1201 ± 204; p=0.006].

Table 4-6: Incremental and total responses to the OGTT

		Glucose (mmol/L)	**Insulin** (μIU/mL)	**C-peptide** (ng/mL)	**Glucagon** (pmol/L)
AUC_{30}	**Controls**	166 ± 28	975 ± 465	194 ± 75	556 ± 152
	CF	198 ± 29	606 ± 356	116 ± 76	332 ± 78
	p	0.02	0.04	0.02	0.002
AUC_{120}	**Controls**	662 ± 170	4945 ± 1699	1044 ± 242	2004 ± 597
	CF	882 ± 179	3743 ± 1610	900 ± 433	1201 ± 204
	p	0.007	0.1	0.35	0.006

Comparison of the initial (AUC_{30}) and total (AUC_{120}) glucose (mmol/L), insulin (μIU/mL), c-peptide (ng/mL) and glucagon (pmol/L) levels following a liquid meal (OGTT) between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the independent t-test for glucose and c-peptide and the Mann-Whitney U test for insulin and glucagon.

4.3.1.7 Summary of OGTT Results

A. Glucose

- Although baseline and 120-minute levels were normal, CF subjects had higher blood glucose levels throughout the study.

B. Insulin

- Although insulin production in the CF subjects overall compared with the controls, early insulin production was deficient.
- A number of CF and control subjects had low insulin levels at the end of the study.

C. C-peptide

- CF subjects had lower early levels and the time to reach a peak c-peptide level was delayed.

D. Glucagon

- CF subjects had lower levels at all time-points.
- A number of controls had high glucagon levels.

4.3.2 Mixed Meal Test 1 (solid meal)

4.3.2.1 Glucose

See Table 4-7 below and Figure 4-6 on page 92.

A. Controls

The control group had normal baseline (4.6 ± 0.4 mmol/L) and 120-minute (4.6 ± 1.2) glucose levels, and all subjects had normal values (<7.8 mmol/L) at 120-minutes.

B. Cystic Fibrosis

Similarly, the CF group had normal baseline (4.8 ± 0.1 mmol/L) and 120-minute (5.4 ± 0.2) values, and all subjects had normal values (<7.8 mmol/L) at 120-minutes.

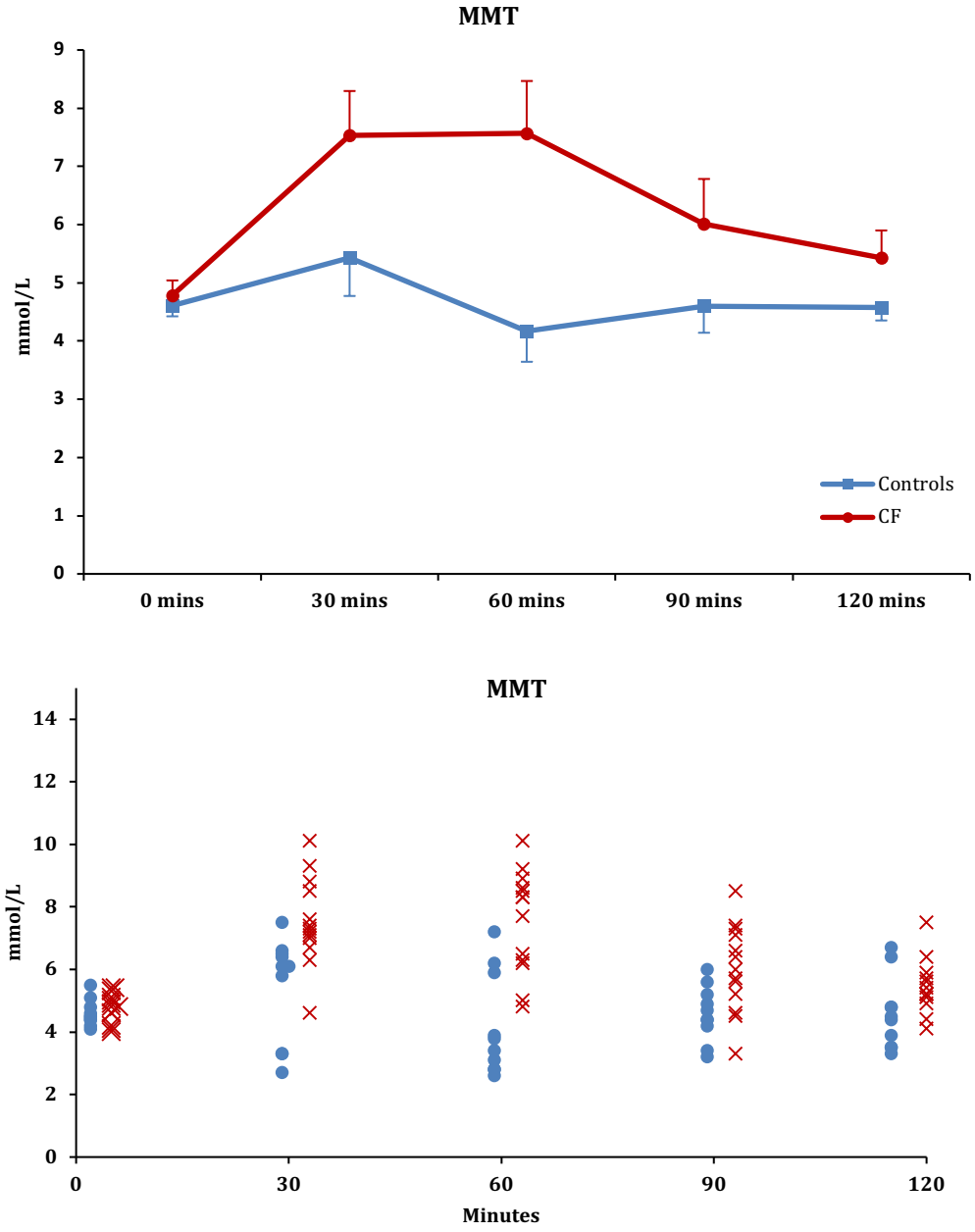
C. Controls v Cystic Fibrosis

Compared to the control group, CF subjects had higher 30 (p=0.004), 60 (p<0.0001) and 90 (p=0.01)-minute glucose levels. At 120-minutes CF subjects had higher levels, although this did not reach statistical significance (p=0.06).

Table 4-7: Glucose levels following the MMT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	4.61 ± 0.42	5.43 ± 1.68	4.17 ± 1.65	4.60 ± 0.89	4.58 ± 1.17
CF	4.78 ± 0.13	7.53 ± 0.39	7.57 ± 0.46	6.02 ± 0.39	5.43 ± 0.24
p	0.37	0.004	<0.0001	0.01	0.06

Comparison of glucose (mmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the independent t-test.



(Top panel): Comparison of glucose (mmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual glucose levels over 120 minutes, following the mixed meal (MMT). [• Controls x CF]

Figure 4-6: Glucose responses following the MMT

4.3.2.2 Insulin

See Table 4-8 on page 93 and Figure 4-7 on page 94

All control subjects had baseline insulin levels within the normal range (< 25 μIU/mL), (8.45 ± 4.75), and although the group were within the normal range (16 - 166 μU/mL) at 120-minutes (29.72 ± 23.24 μU/mL) 3 subjects had low insulin levels.

A. Cystic Fibrosis

Similarly, all CF subjects had baseline insulin levels within the normal range (7.75 ± 2.85) and although the group were within the normal range at 120-minutes (19.82 ± 8.49 μU/mL), 6 subjects had low insulin levels.

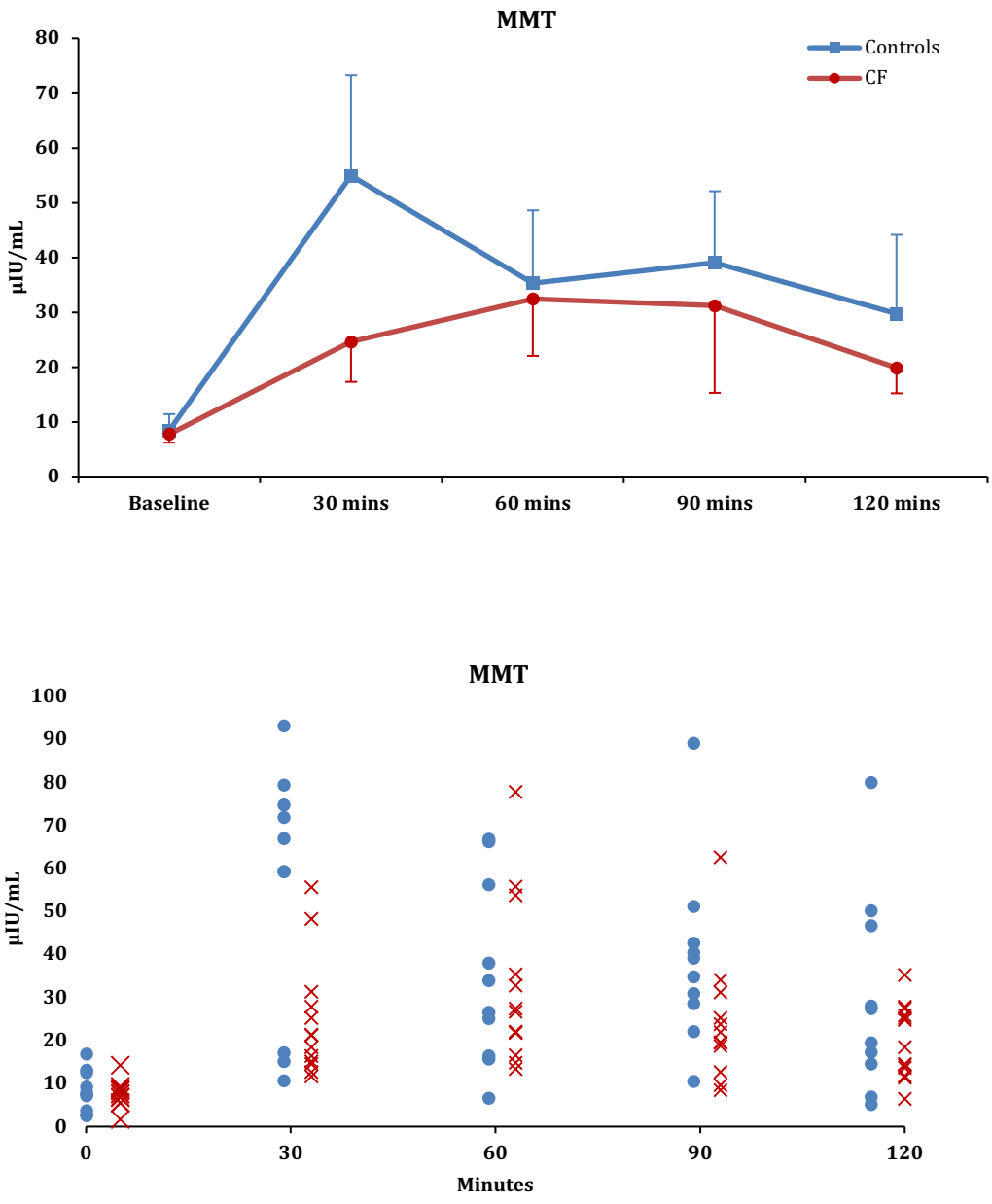
B. Controls v Cystic Fibrosis

When the 2 groups were compared, CF subjects had lower insulin levels at 30-minutes (p=0.01), but there was no difference at baseline levels or other times of the MMT.

Table 4-8: Insulin levels following the MMT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	8.45 ± 4.75	54.92 ± 29.61	35.31 ± 21.44	39.09 ± 20.97	29.72 ± 23.24
CF	7.75 ± 2.85	24.67 ± 13.56	32.43 ± 19.12	31.18 ± 29.23	19.82 ± 8.49
p	0.66	0.01	0.73	0.48	0.11

Comparison of insulin (μIU/mL) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Mann-Whitney U test.



(Top panel): Comparison of insulin (µIU/mL) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual insulin levels over 120 minutes, following the mixed meal (MMT). [• Controls x CF]

Figure 4-7: Insulin levels following the MMT

4.3.2.3 C-peptide

See summary data Table 4-9 on page 95 and Figure 4-8 on page 96

A. Controls

The group had normal C-peptide levels at baseline (3.9 ± 2.4) and 120-minutes (7.2 ± 2.4 ng/mL), but 2 subjects had high baseline levels and 1 a low baseline level. (normal range 0.8-5.1 ng/mL).

B. Cystic Fibrosis

The group had normal C-peptide levels at baseline (2.5 ± 1.7) and 120-minutes (5.6 ± 3.6 ng/mL), but 1 subject had a high and 1 a low baseline level (normal range 0.8-5.1 ng/mL).

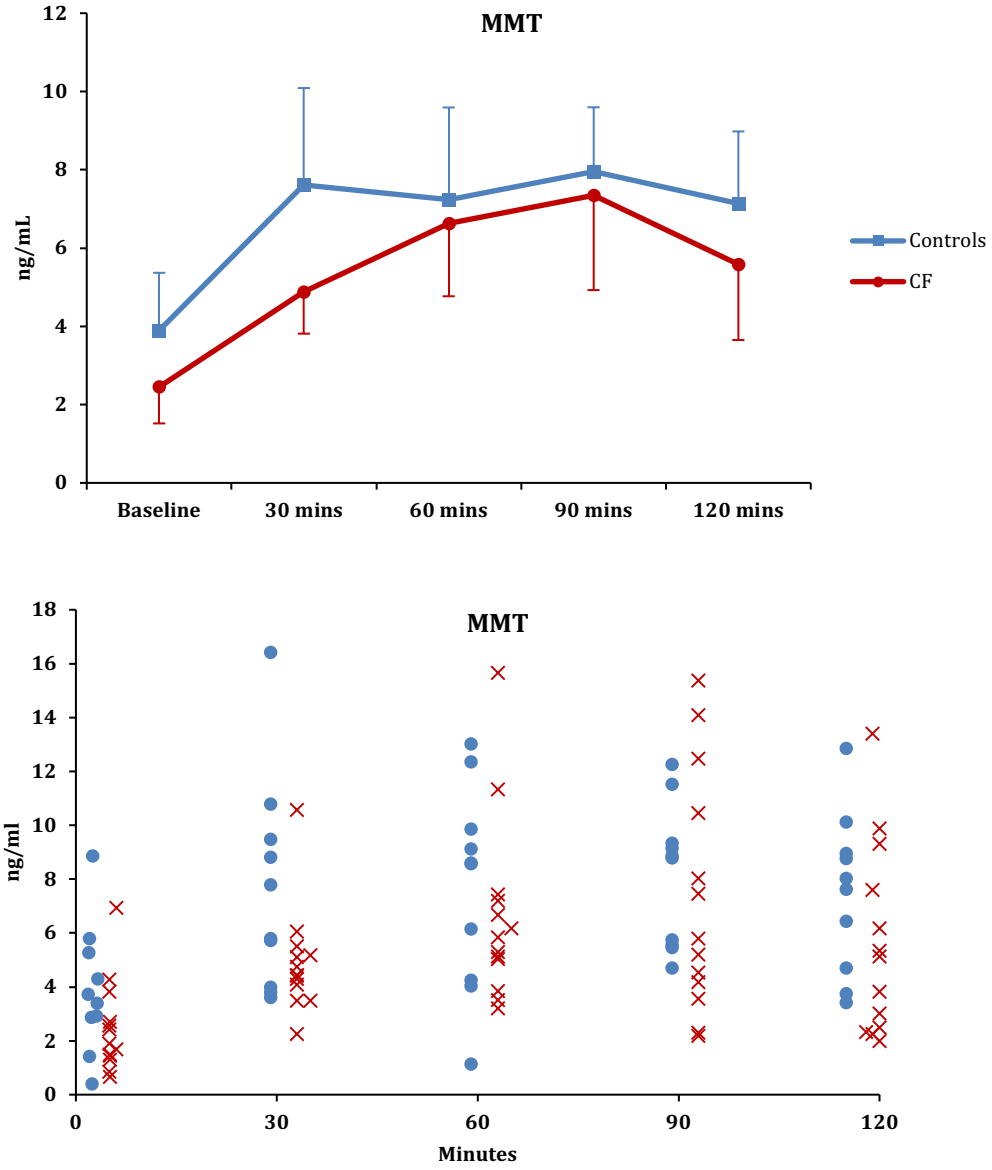
C. Controls v Cystic Fibrosis

There was no statistical difference between the 2 groups at any time point. At 30 minutes, CF subjects had lower levels; however, this difference did not achieve statistical significance.

Table 4-9: C-peptide levels following the MMT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	3.89 ± 2.38	7.61 ± 4.42	7.24 ± 3.72	7.95 ± 2.36	7.14 ± 2.44
CF	2.45 ± 1.71	4.88 ± 1.97	6.63 ± 3.43	7.35 ± 4.45	5.58 ± 3.56
p	0.1	0.07	0.48	0.62	0.19

Comparison of c-peptide (ng/mL) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the independent t-test.



(Top panel): Comparison of c-peptide (ng/mL) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual c-peptide levels over 120 minutes, following the mixed meal (MMT). [• Controls x CF]

Figure 4-8: C-peptide responses following the MMT

4.3.2.4 Glucagon

See Table 4-10 on page 98 and Figure 4-9 on page 99

A. Controls

Insufficient samples were available for glucagon analysis in subject 6 at baseline and 30-minutes, and for subject 10 at baseline, 60- and 120-minutes. Data are therefore reported for the remaining samples.

In this group glucagon levels at baseline (15.2 ± 4.8 pmol/L) and 120-minutes (21.2 ± 3.8 pmol/L) were within the normal range (< 17.2 pmol/L), but 2 subjects had baseline levels above the normal range.

B. Cystic Fibrosis

Insufficient samples were available for glucagon analysis in subject 16 at baseline and in subject 13 at 90-minutes: data are reported for the remaining samples.

As a group baseline and 120-minutes levels were within the normal range (11.4 ± 5.1 pmol/L and 15.5 ± 9.3 pmol/L respectively), but 2 subjects had levels below the lower limit of detection (<5 pmol/L) – (one at 30- and 60-minutes and another at 90-minutes). One subject had a glucagon level above the normal range (Normal < 17.2 pmol/L).

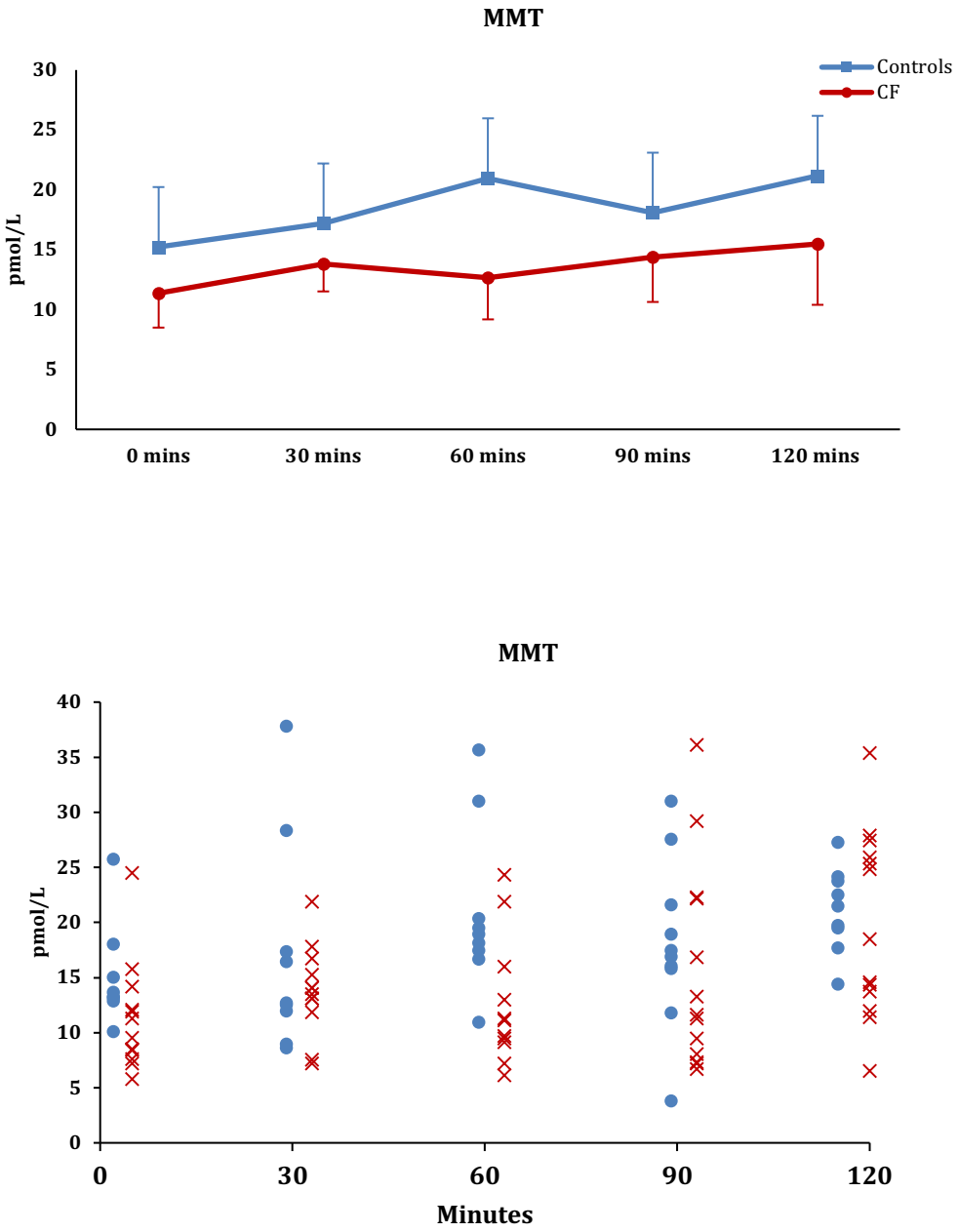
C. Controls v Cystic Fibrosis

When the 2 groups were compared, there was no statistical difference in baseline (p=0.1) or 120-minute (p=0.06) levels, but CF subjects at 60-minutes had lower levels (p=0.01).

Table 4-10: Glucagon levels following the MMT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	15.2 ± 4.8	17.2 ± 9.7	21.0 ± 7.6	18.1 ± 7.6	21.2 ± 3.8
CF	11.4 ± 5.1	13.8 ± 4.0	12.6 ± 5.8	14.4 ± 6.6	15.5 ± 9.3
p	0.1	0.35	0.01	0.23	0.06

Comparison of glucagon (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Mann-Whitney U test.



(Top panel): Comparison of glucagon (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual glucagon levels over 120 minutes, following the mixed meal (MMT). [• Controls x CF]

Figure 4-9: Glucagon responses following the MMT

4.3.2.5 Time to peak response

See Table 4-11 below and Figure 4-10 on page 101

As with the OGTT, I calculated the time for insulin, c-peptide and glucagon to reach a peak level, following the MMT and compared the differences between control and CF subjects.

A. Controls

Control subjects took 53 ± 27 minutes to peak insulin level, 60 ± 32 minutes to peak c-peptide level and 63 ± 52 minutes to peak glucagon level.

B. Cystic Fibrosis

CF subjects took 68 ± 29 minutes to peak insulin level, 83 ± 27 minutes to peak c-peptide level and 86 ± 44 minutes to peak glucagon level.

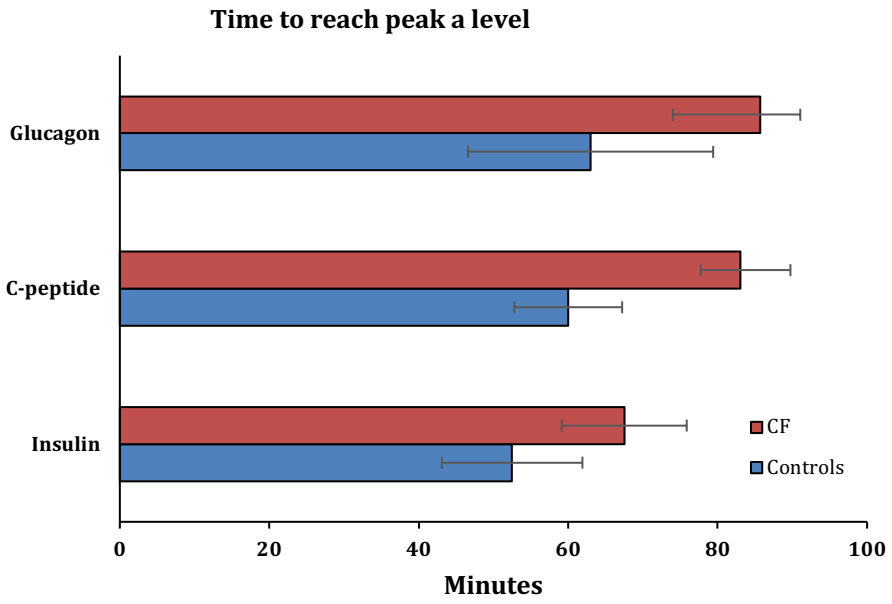
C. Controls v Cystic Fibrosis

When the two groups were compared there was no difference in the time to reach peak levels for insulin, c-peptide and glucagon.

Table 4-11: Time to reach a peak level following the MMT

	Insulin	C-peptide	Glucagon
Controls	53 ± 27	60 ± 32	63 ± 52
CF	68 ± 29	83 ± 27	86 ± 44
p	0.11	0.17	0.23

Comparison between control and CF subjects in the time (minutes) to reach a peak response for insulin, c-peptide and glucagon following the liquid meal (OGTT). [Mean ± SD]



Comparison between control and CF subjects in the time (minutes) to reach a peak response for insulin, c-peptide and glucagon following the liquid meal (OGTT). [Mean ± SEM]

Figure 4-10: Time to reach a peak level following the MMT

4.3.2.6 Incremental changes in response to the MMT

See Table 4-12 on page 103

I measured the incremental and total responses to the MMT in the morning for glucose, insulin, c-peptide and glucagon and compared the 2 groups.

A. Controls

The initial response (AUC_{30}) for glucose was 151 ± 29 mmol/L, insulin 951 ± 476 µIU/mL, c-peptide 173 ± 92 ng/ml and glucagon 173 ± 92 pmol/L, with a total response (AUC_{120}) of 564 ± 120 mmol/L, 4452 ± 2068 µIU/mL, 874 ± 354 ng/ml and 2219 ± 632pmol/L respectively.

B. Cystic Fibrosis

The initial response (AUC_{30}) for glucose was 185 ± 25 mmol/L, insulin 487 ± 195 µIU/mL, c-peptide 110 ± 54 ng/ml and glucagon 394 ± 121 pmol/L, with a total response (AUC_{120}) of 787 ± 119 mmol/L, 3067 ± 1427 µIU/mL, 686 ± 333 ng/ml and 1741 ± 660 pmol/L respectively.

C. Controls v Cystic Fibrosis

Compared to controls, the CF group had higher initial (AUC_{30} p= 0.006) and total (AUC_{120} p= 0.0003) glucose levels.

Both initial response insulin and c-peptide responses were greater in the control group (p=0.01 and p=0.048 respectively), but there was no difference in the total response.

There was no difference in glucagon levels at any stage throughout the study.

Table 4-12: Incremental and total responses to the MMT

		Glucose *(mmol/L)*	**Insulin** *(μIU/mL)*	**C-peptide** *(ng/mL)*	**Glucagon** *(pmol/L)*
AUC_{30}	**Controls**	151 ± 29	951 ± 476	173 ± 92	448 ± 166
	CF	185 ± 25	487 ± 195	110 ± 54	394 ± 121
	p	0.006	0.01	0.048	0.42
AUC_{120}	**Controls**	564 ± 120	4452 ± 2068	874 ± 354	2219 ± 632
	CF	787 ± 119	3067 ± 1427	686 ± 333	1741 ± 660
	p	0.0003	0.08	0.2	0.15

Comparison of the initial (AUC_{30}) and total (AUC_{120}) glucose (mmol/L), insulin (μIU/mL), c-peptide (ng/mL) and glucagon (pmol/L) levels following a mixed meal (MMT) between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the independent t-test.

4.3.3 Comparison between OGTT and MMT

4.3.3.1 Glucose

See Table 4-13 on page 105 and Figure 4-11, Figure 4-12 on page 107

I then evaluated the responses between the OGTT and the MMT in controls and CF subjects.

A. Controls

The OGTT glucose value at 60-minutes was higher (p=0.0009) compared to MMT, and although there was no difference in the initial (AUC_{30}) response, the total (AUC_{120}) response was higher following the OGTT (p=0.02) compared to the MMT.

B. Cystic Fibrosis

The OGTT glucose 30-minute (p=0.02) and 60-minute (p=0.01) levels were higher compared to the MMT. Although the AUC_{30} after the OGTT was higher, this difference was not statistically significant (p=0.07), but the total response (AUC_{120}) was higher after the OGTT (p=0.02).

Table 4-13: Glucose differences between OGTT and MMT

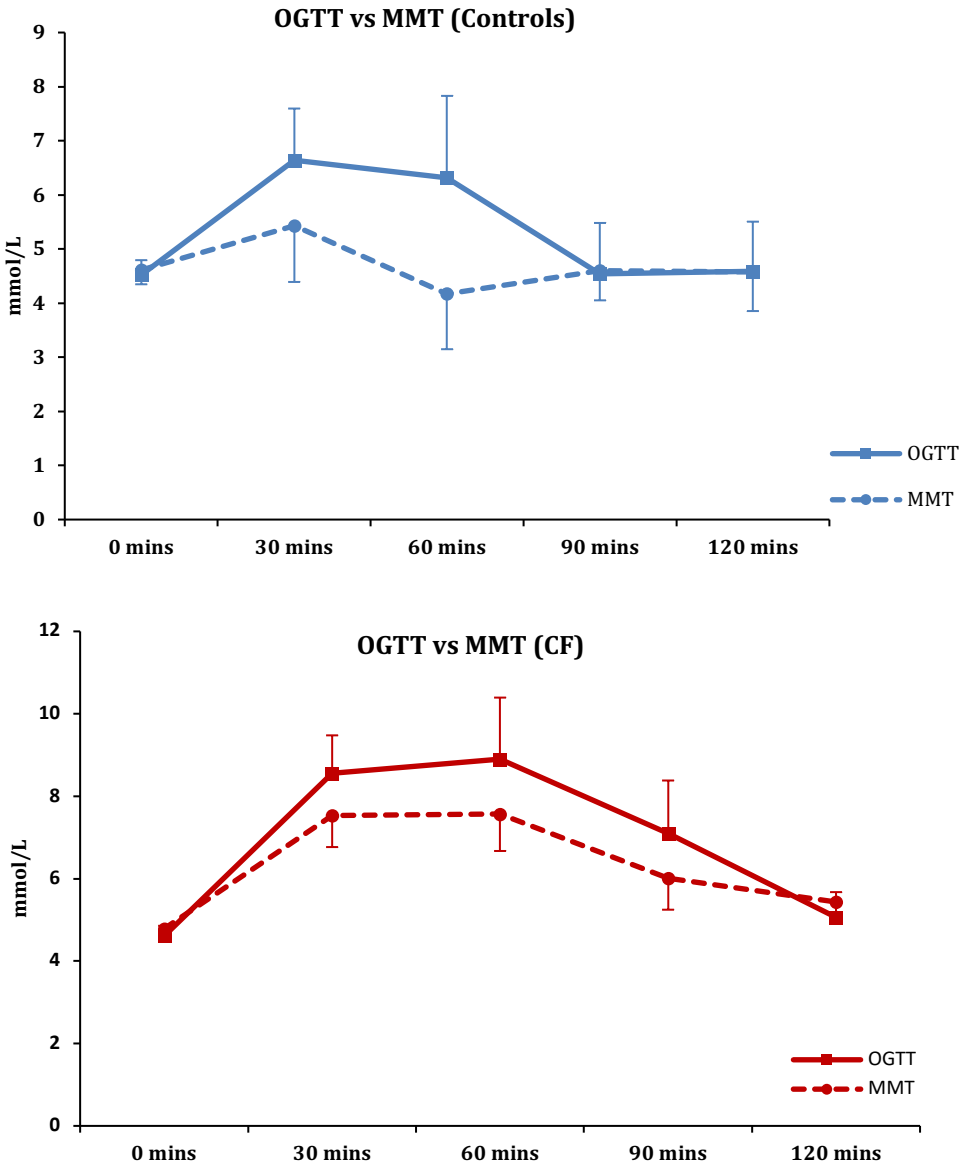
		0 mins	30 mins	60 mins	90 mins	120 mins
Controls	**OGTT**	4.52 ± 0.44	6.64 ± 1.54	6.31 ± 2.45	4.54 ± 1.52	4.59 ± 1.48
	MMT	4.61 ± 0.42	5.43 ±1.68	4.17 ± 1.65	4.60 ± 0.89	4.58 ±1.17
	p	0.41	0.05	0.0009	0.88	0.98
CF	**OGTT**	4.61 ± 0.46	8.56 ± 1.68	8.90 ± 2.75	7.09 ± 2.37	5.06 ± 1.13
	MMT	4.78 ± 0.13	7.53 ± 0.39	7.57 ± 0.46	6.02 ± 0.39	5.43 ±0.24
	p	0.26	0.02	0.01	0.11	0.38

Comparison of glucose (mmol/L) levels following a liquid (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) and CF (n=12) subjects (mean ± SD). Statistical comparisons between tests were carried out using the paired t-test.

Table 4-14: Glucose – Differences between the OGTT and MMT – incremental and total responses

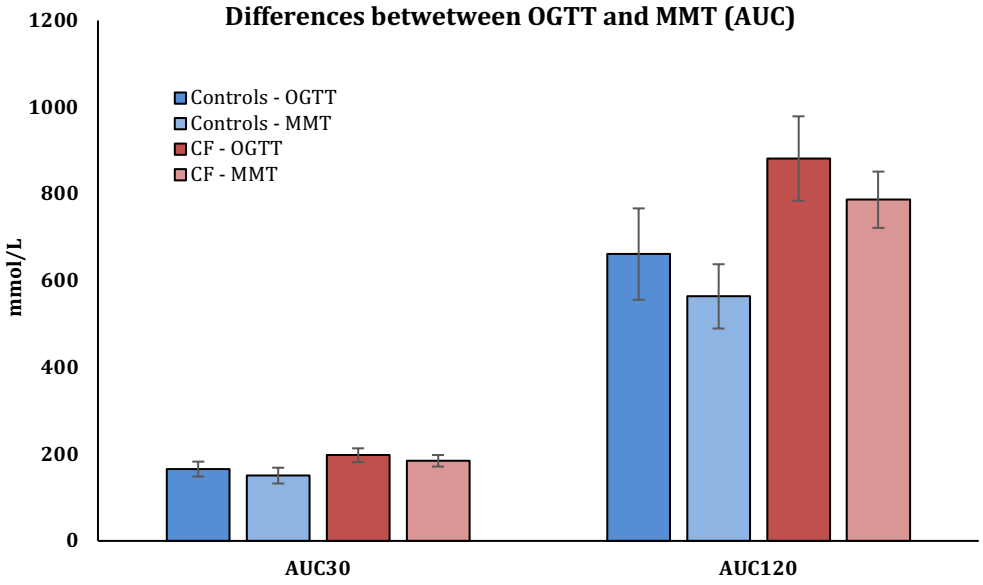
		AUC_{30}	AUC_{120}
Controls	**OGTT**	166 ± 28	661 ± 170
	MMT	151 ± 29	564 ± 120
	p	0.1	0.02
CF	**OGTT**	198 ± 29	882 ± 179
	MMT	185 ± 25	787 ± 119
	p	0.07	0.02

Comparison of the initial (AUC_{30}) and total (AUC_{120}) glucose (mmol/L) levels following a liquid meal (OGTT) and mixed meal (MMT) in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the paired t-test.



Comparison of glucose (mol/L) levels following a liquid meal (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10, top panel) and CF (n=13) subjects (bottom panel). [mean ± CI].

Figure 4-11: Glucose differences between OGTT and MMT



Comparison of the initial (AUC_{30}) and total (AUC_{120}) glucose (mmol/L) levels following a liquid meal (OGTT) and mixed meal (MMT) in control (n=10) and CF (n=13) subjects (mean ± CI).

Figure 4-12: Glucose differences between the OGTT and MMT – incremental and total responses

4.3.3.2 Insulin

See Table 4-15 below, Table 4-16 on page 109 and Figure 4-13 on page 110

Controls

Insulin value was higher on the OGTT (p=0.005) at 60-minutes, but there was no difference in the initial (AUC_{30}) or the total (AUC_{120}) response between the OGTT and MMT.

A. Cystic Fibrosis

The AUC_{30} in CF subjects was higher (p=0.0001) after the OGTT compared to the MMT.

Table 4-15: Insulin differences between OGTT and MMT

		0 mins	30 mins	60 mins	90 mins	120 mins
Controls	**OGTT**	9.2 ± 6.5	55.8 ± 26.8	55.3 ± 29.6	43.2 ± 29.2	35.7 ± 27.2
	MMT	8.5 ± 4.7	54.9 ± 29.6	35.3 ± 21.4	39.1 ± 21.0	29.7 ± 23.2
	p	0.76	0.9	0.005	0.65	0.39
CF	**OGTT**	7.6 ± 2.3	30.5 ± 21.9	42.0 ± 24.9	36.5 ± 20.9	19.4 ± 9.9
	MMT	7.7 ± 2.9	24.7 ± 13.6	32.4 ± 19.1	31.2 ± 29.2	19.8 ± 8.5
	p	0.8	0.19	0.15	0.4	0.89

Comparison of insulin (μIU/mL) levels following a liquid (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using the Wilcoxon signed-rank test.

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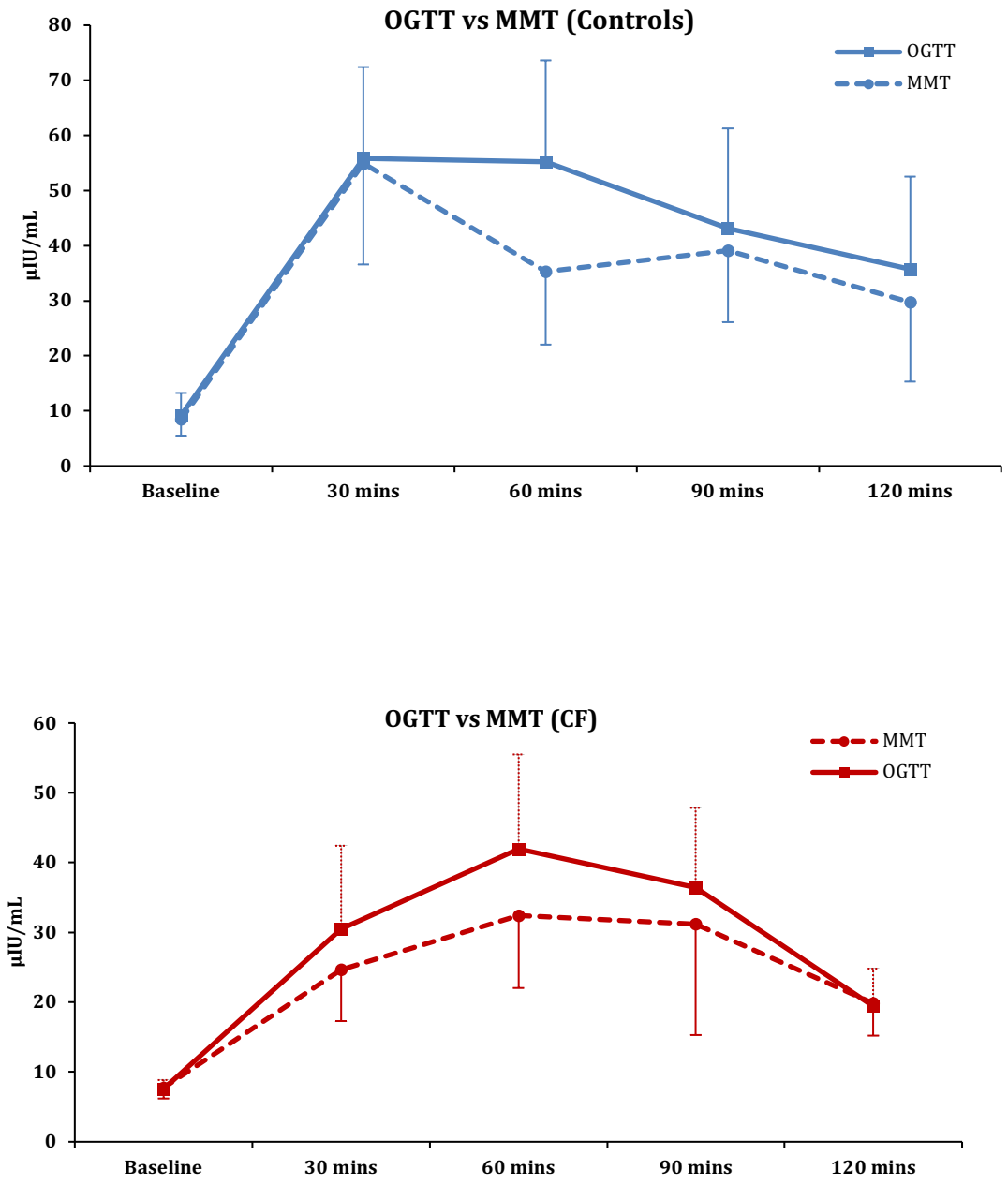
Table 4-16: Insulin - Differences between the OGTT and MMT – incremental and total responses

		AUC_{30}	AUC_{120}
Controls	**OGTT**	975 ± 465	4945 ± 1699
	MMT	951 ± 476	4452 ± 2068
	p	0.82	0.37
CF	**OGTT**	606 ± 356	3743 ± 1610
	MMT	487 ± 195	3067 ± 1427
	p	0.0001	0.13

Comparison of the initial (AUC_{30}) and total (AUC_{120}) insulin (μIU/mL) levels following a liquid meal (OGTT) and mixed meal (MMT) in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Wilcoxon signed-rank test.



Comparison of insulin (µIU/mL) levels following a liquid meal (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10, top panel) and CF (n=13) subjects (bottom panel). [mean ± CI].

Figure 4-13: Insulin differences between OGTT and MMT

4.3.3.3 C-peptide

See Table 4-17, Table 4-18 on page 112 and Figure 4-14, Figure 4-15 on page 114

A. Controls

C-peptide at 60-minutes was higher for the OGTT (p=0.02), but there was no difference at baseline, 30, 90 and 120-minutes.

Although there was no difference in the initial (AUC_{30}) response, the total (AUC_{120}) response was higher (p=0.03) after the OGTT.

B. Cystic Fibrosis

There were no statistically significant differences up to 90-minutes or the AUC_{30} but there were higher levels after the OGTT at 120-minutes (p=0.04). Although the total response (AUC_{120}) after the OGTT was higher, this did not reach statistical significance (p=0.07).

Table 4-17: C-peptide - differences between OGTT and MMT

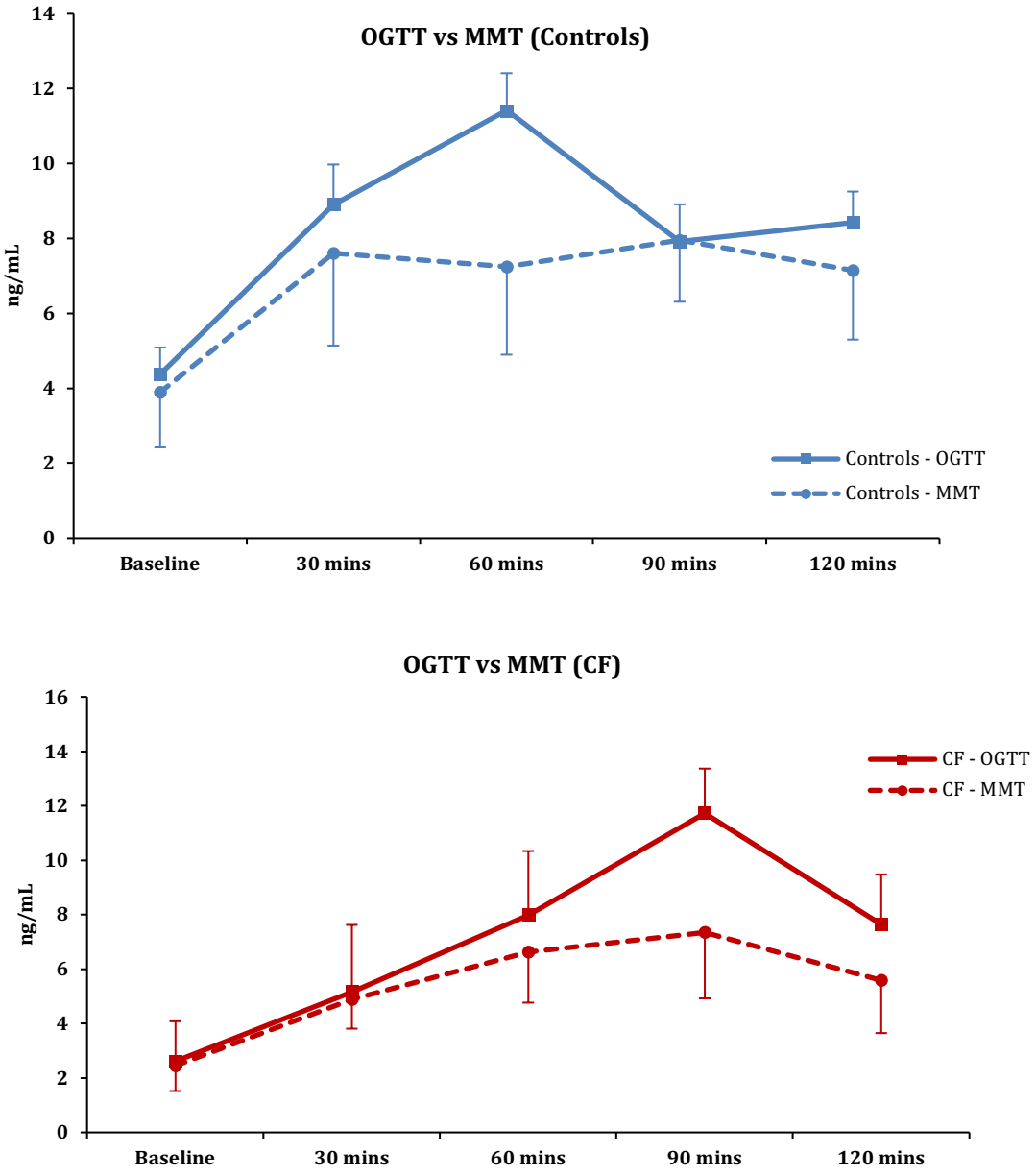
		0 mins	30 mins	60 mins	90 mins	120 mins
Controls	**OGTT**	4.4 ± 2.2	8.9 ± 3.0	11.4 ± 2.8	7.9 ± 2.8	8.4 ± 2.3
	MMT	3.89 ± 2.38	7.61 ± 4.42	7.24 ± 3.72	7.95 ± 2.36	7.14 ± 2.44
	p	0.23	0.31	0.02	0.76	0.11
CF	**OGTT**	2.6 ± 2.0	5.2 ± 3.5	8.0 ± 4.7	11.7 ± 8.5	7.6 ± 2.7
	MMT	2.45 ± 1.71	4.88 ± 1.97	6.63 ± 3.43	7.35 ± 4.45	5.58 ± 3.56
	p	0.62	0.72	0.21	0.06	0.04

Comparison of c-peptide (ng/ml) levels following a liquid (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using the paired t-test.

Table 4-18: C-peptide- Differences between the OGTT and MMT – incremental and total responses

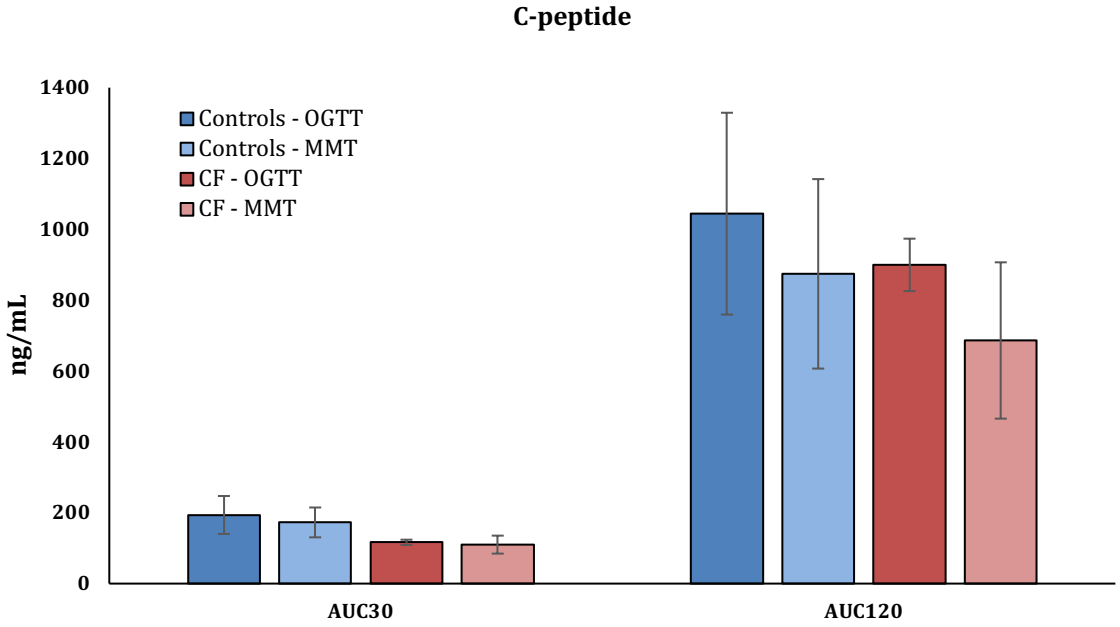
		AUC_{30}	AUC_{120}
Controls	**OGTT**	193.7 ± 74.9	1044.4 ± 242.1
	MMT	172.7 ± 91.8	874.5 ± 353.6
	p	0.19	0.03
CF	**OGTT**	116.4 ± 76.1	899.8 ± 432.5
	MMT	110.0 ± 54.0	686.3 ± 333.1
	p	0.64	0.07

Comparison of the initial (AUC_{30}) and total (AUC_{120}) c-peptide (ng/ml) level following a liquid meal (OGTT) and mixed meal (MMT) in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the paired t-test.



Comparison of c-peptide (ng/mL) levels following a liquid meal (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10, top panel) and CF (n=13) subjects (bottom panel). [mean ± CI].

Figure 4-14: C-peptide - Differences between OGTT and MMT



Comparison of the initial (AUC_{30}) and total (AUC_{120}) c-peptide (ng/ml) levels following a liquid meal (OGTT) and mixed meal (MMT) in control (n=10) and CF (n=13) subjects (mean ± CI).

Figure 4-15: C-peptide - Differences between the OGTT and MMT – incremental and total responses

4.3.3.4 Glucagon

See Table 4-19, Table 4-20 on page 116 and Figure 4-16 and Figure 4-17 on page 118

A. Controls

The glucagon value at 60-minutes (p=0.05) and 120-minutes (p=0.03) was higher for the MMT, but there was no difference at baseline, 30 and 90-minutes.

There was no difference in the initial (AUC_{30}) or total (AUC_{120}) response.

B. Cystic Fibrosis

CF subjects had higher glucagon levels after the MMT at 90 (p=0.002) and 120 (p=0.02) minutes, and the total glucagon response (AUC_{120}) was higher for the MMT compared to the OGTT.

Table 4-19: Glucagon differences between OGTT and MMT

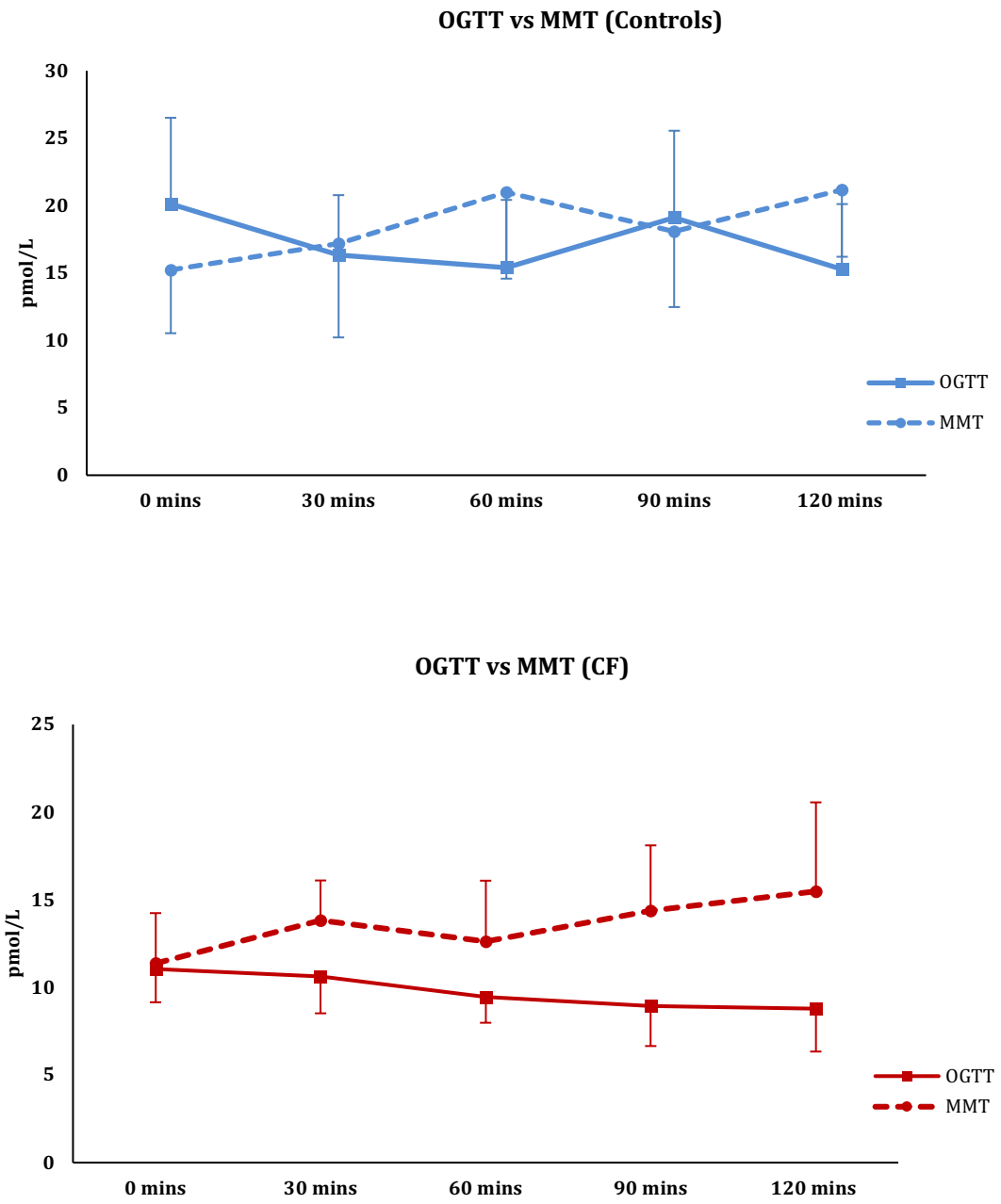
		0 mins	30 mins	60 mins	90 mins	120 mins
Controls	**OGTT**	20.1 ± 7.9	16.3 ± 5.5	15.4 ± 6.3	19.1 ± 8.3	15.3 ± 5.1
	MMT	15.2 ± 4.8	17.2 ± 9.7	21.0 ± 7.6	18.1 ± 7.6	21.2 ± 3.8
	p	0.21	0.94	0.05	0.87	0.03
CF	**OGTT**	11.0 ± 3.4	10.6 ± 3.8	9.5 ± 2.5	8.9 ± 4.0	8.8 ± 4.3
	MMT	11.4 ± 5.1	13.8 ± 4.0	12.6 ± 5.8	14.4 ± 6.6	15.5 ± 9.3
	p	0.86	0.17	0.18	0.002	0.02

Comparison of glucagon (pmol/L) levels following a liquid (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using the Wilcoxon signed-rank test.

Table 4-20: Glucagon - Differences between the OGTT and MMT – incremental and total responses

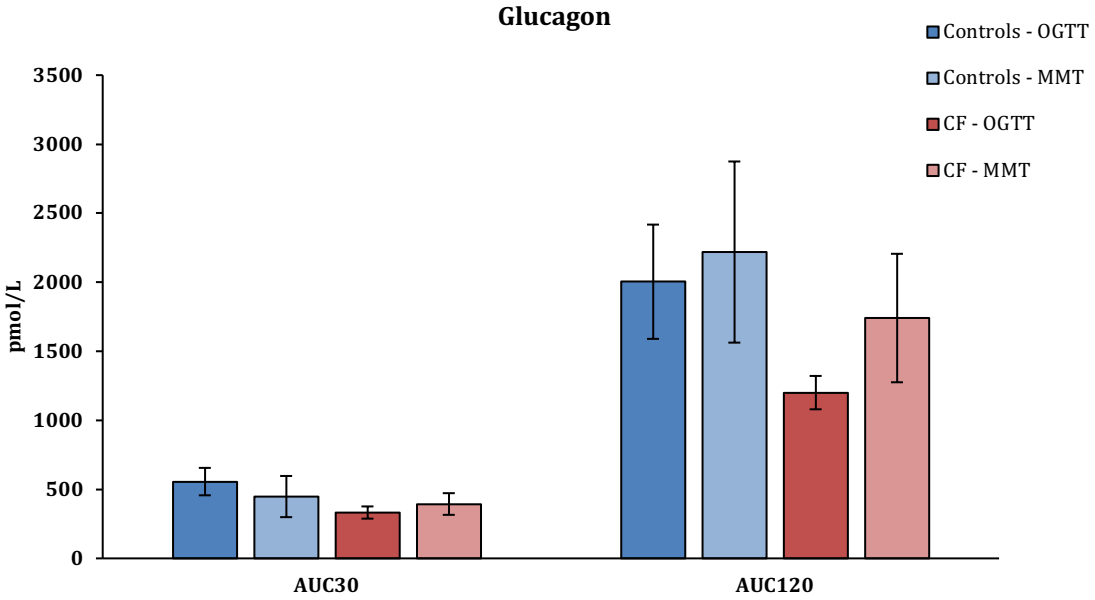
		AUC_{30}	AUC_{120}
Controls	**OGTT**	556 ± 51	2004 ± 597
	MMT	448 ± 59	2219 ± 223
	p	0.23	0.45
CF	**OGTT**	332 ± 23	1201 ± 62
	MMT	394 ± 37	1741 ± 220
	p	0.19	0.04

Comparison of the initial (AUC_{30}) and total (AUC_{120}) glucagon (pmol/L) level following a liquid meal (OGTT) and mixed meal (MMT) in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Wilcoxon signed ranks test.



Comparison of glucagon (pmol/L) levels following a liquid meal (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) (top panel) and CF (n=13) subjects (bottom panel). [mean ± CI].

Figure 4-16: Glucagon - Differences between OGTT and MMT



Comparison of the initial (AUC_{30}) and total (AUC_{120}) glucagon (pmol/L) levels following a liquid meal (OGTT) and mixed meal (MMT) in control (n=10) and CF (n=13) subjects (mean ± CI).

Figure 4-17: Glucagon - Differences between the OGTT and MMT – incremental and total responses

4.3.4 Diurnal Studies (MMT2 and MMT3)

As with gastric motility, I then compared changes in blood glucose, insulin, c-peptide and glucagon through the day and evaluated the early response [30-minutes (AUC_{30})] and the total response [120-minutes (AUC_{120})], to evaluate the concept of pancreatic fatigue.

4.3.4.1 Glucose

See Table 4-21, Table 4-22 on page 121 and Figure 4-18 and Figure 4-19 **Error! Reference source not found.**on pages 122 and 123

A. Controls:

In the afternoon (MMT2), all had normal baseline (4.6 ± 0.3 mmol/L) and 120-minute values (5.0 ± 0.4 mmol/L). In the evening (MMT3), all subjects again had normal baseline (4.6 ± 0.3 mmol/L) and 120-minute values (5.03 ± 0.4 mmol/L).

In the healthy control subjects, there was no difference between times of the day with the corresponding time during the test.

When the incremental initial (AUC_{30}) and total (AUC_{120}) responses were evaluated, there was no difference within the control group between the various tests.

B. Cystic Fibrosis

In the afternoon (MMT2), CF subjects had a normal baseline (mean 4.3 ± 0.7 mmol/L) and 120-minute values (5.3 ± 0.5 mmol/L).

However, at the evening study (MMT3), two subjects had impaired baseline levels (6.8, 5.7 mmol/L) and at 120-minutes 6 had impaired glucose values (mean 6.11 ± 1.07 mmol/L) and two had glucose levels within the diabetic range (7.3 and 8.6 mmol/L).

A diurnal variation existed for the 60-minute glucose value ($p=0.03$) within the CF group with the afternoon level less than the (morning ($p=0.01$) and the evening ($p=0.01$). Although

the 120-minute did show diurnal variation (p=0.04) with the evening level the highest, on the post-hoc analysis this did not achieve statistical significance.

There was a diurnal variation in the AUC_{30} (p=0.04) and AUC_{120} (p=0.02). The AUC_{30} was lower in the afternoon (166 ± 20 mmol/L) compared to the morning (185 ± 25 mmol/L; p=0.004) and evening (180 ± 16 mmol/L, p=0.001). The AUC_{120} was lower in the afternoon (692 ± 81 mmol/L) compared to the morning [787 ± 119 mmol/L; p=0.002] and the evening (792 ± 124 mmol/L, p=0.012). This was not seen in control subjects.

C. Controls v Cystic Fibrosis

When the 2 groups were compared, after the MMT2, CF subjects had higher blood glucose levels at 30 (p=0.04) and 60-minutes (p=0.009). For the MMT3, CF subjects had higher 30 (p=0.03), 60 (p=0.008), 90 (p=0.009) and 120-minute (p=0.01) values compared to control subjects.

Furthermore, CF subjects had higher glucose levels (AUC_{30}) in the morning (p=0.006) and evening (p=0.01) and the AUC_{120} was higher throughout the day compared to controls.

Table 4-21: Glucose levels through the day

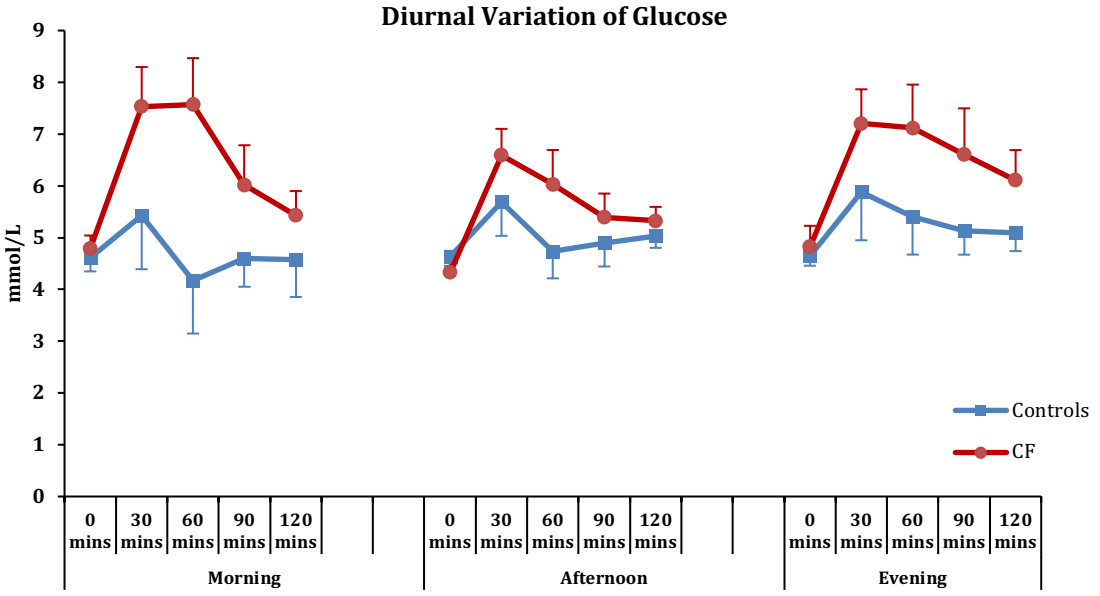
	Morning			Afternoon			Evening		
	Controls	**CF**	**p**	**Controls**	**CF**		**Controls**	**CF**	
0 mins	4.61± 0.42	4.78 ± 0.47	0.37	4.64 ± 0.30	4.33 ± 0.71	0.20	4.65 ± 0.31	4.82 ± 0.75	0.46
30 mins	5.43 ± 1.68	7.53 ± 1.41	0.004	5.69 ± 1.06	6.59 ± 0.94	0.04	5.88 ± 1.50	7.21 ± 1.21	0.03
60 mins	4.17 ± 1.65	7.57 ± 1.65	<0.0001	4.74 ± 0.85	6.03 ± 1.22*^	0.009	5.40 ± 1.17	7.12 ±1.55	0.008
90 mins	4.60 ± 0.89	6.02 ± 1.41	0.01	4.90 ± 0.74	5.39 ± 0.85	0.15	5.13 ± 0.74	6.61 ±1.64	0.009
120 mins	4.58 ±1.17	5.43 ± 0.86	0.06	5.03 ± 0.37	5.32 ± 0.50	0.13	5.09 ± 0.56	6.11 ± 1.07	0.01

*Comparison between glucose (mmol/L) levels over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-tests and a repeated measures ANOVA was performed to study differences within the group through the day [*p=0.01 (compared to morning); ^p=0.02 (compared to evening)].*

Table 4-22: Glucose - Incremental and total responses through the day

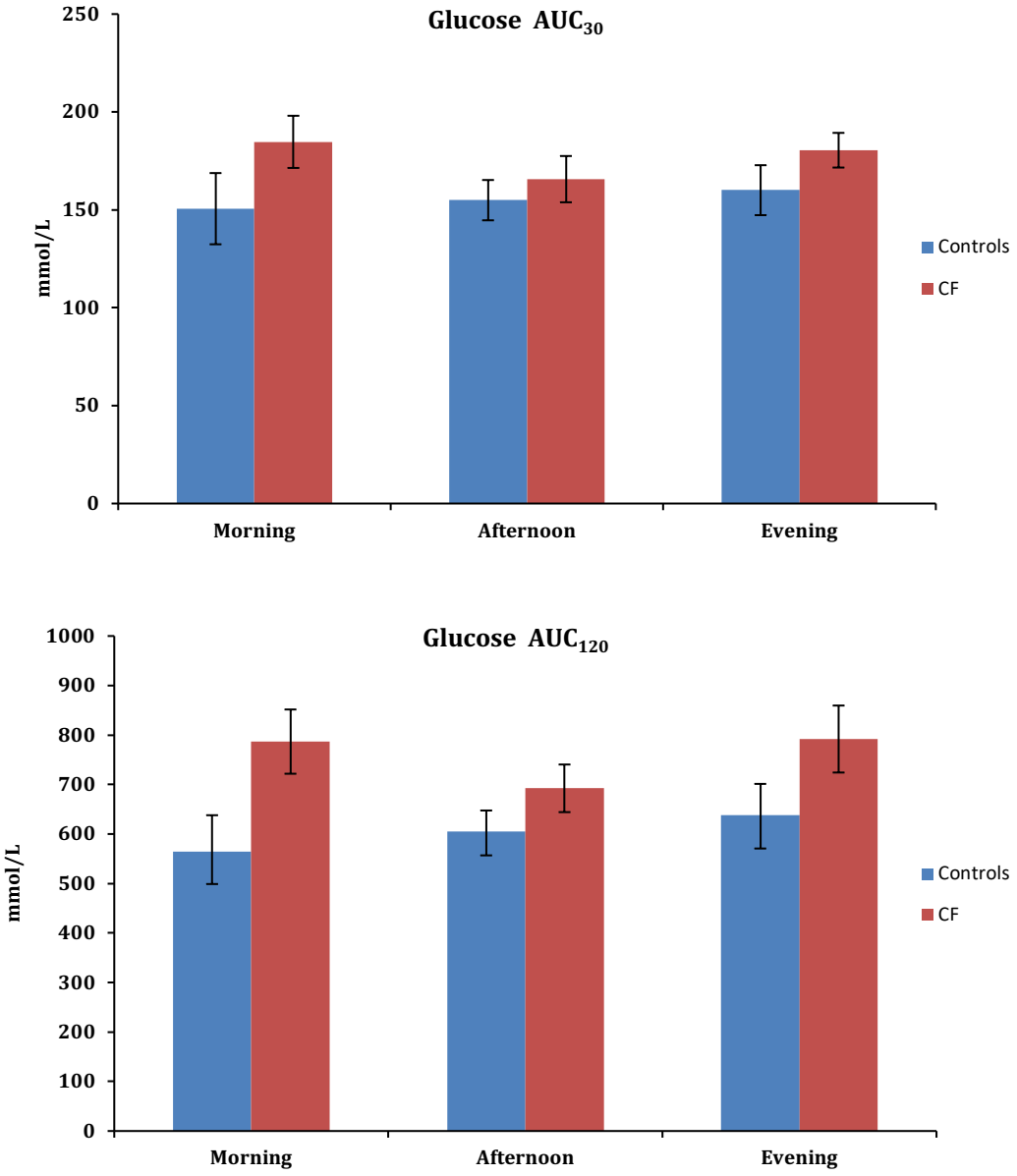
		Morning	Afternoon	Evening
AUC_{30}	**Controls**	151 ± 29	155 ±17	160 ± 21
	CF	185 ± 25 *	166 ± 20	180 ±16 Ω
	p	0.006	0.2	0.01
	AUC_{30}: Within Group: vs afternoon: *p=0.004; Ω p=0.001			
		Morning	**Afternoon**	**Evening**
AUC_{120}	**Controls**	564 ± 120	605 ± 69	638 ±101
	CF	787 ± 119 Ω	692 ± 81	792 ± 124 ^
	p	0.0003	0.02	0.004
	AUC_{120}: Within Group: vs afternoon: Ωp=0.002; ^p=0.01			

Comparison between the initial (AUC_{30}) and total (AUC_{120}) glucose (mmol/L) levels, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-test and a repeated measures ANOVA was performed to study differences within the group through the day.



Comparison of glucose (mmol/L) levels in response to the mixed meal (mean ± CI) over 120 minutes between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening. [For p-values see Table 4-21]

Figure 4-18: Glucose handling through the day



Comparison of initial (AUC_{30}) and total (AUC_{120}) glucose (mmol/L) levels following the mixed meal (mean ± CI) between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening.

Figure 4-19: Glucose - Incremental and Total responses through the day

4.3.4.2 Insulin

See Table 4-23, Table 4-24 on pages 126 and Figure 4-20, Figure 4-21 on page 128

A. Controls

In the afternoon (MMT2), all but one subject (41.9 μIU/mL) had normal (< 25 μIU/mL) baseline (12.1 ± 11.4 μIU/mL) levels and all but 3 subjects (<16 μIU/mL) had normal (16-166 μIU/mL) 120-minute values (26.5 ± 18.5 μIU/mL).

In the evening (MMT3), all subjects had normal baseline levels (mean 9.1 ± 6.0 μIU/mL) but 3 subjects had low insulin levels at 120-minutes (group mean 28.7 ± 20.6 μIU/mL).

Analysis of insulin release throughout the day, showed no differences in control subjects at any time point.

There was no difference within the group for MMT1, MMT2 and MMT3 in incremental initial (AUC_{30}) and total (AUC_{120}) responses.

B. Cystic Fibrosis

At the afternoon study (MMT2), two baseline samples and one 120-minute sample were unable to be analysed due to haemolysis.

In the remaining samples, there was a normal baseline (mean 8.5 ± 3.1 μIU/mL) but at 120-minutes, 7 subjects had low 120-minute levels.

In the evening (MMT3), there was a normal baseline (mean 9.7 ± 6.2 μIU/mL) but at 120-minutes, (mean 21.9 ± 12.3 μIU/mL), five had low insulin levels (<16 μIU/mL).

Analysis of insulin release throughout the day showed no difference in control subjects at any time point.

Total (AUC_{120}) insulin responses were, were lower (p=0.003) in the afternoon (2393 ± 919 μIU/mL) compared to the morning (3067 ± 1427 μIU/mL), but there was no difference in the AUC_{30} between the 3 tests.

C. Controls v Cystic Fibrosis

Following MMT2 and MMT3, CF subjects had lower insulin levels at 30-minutes (p=0.01), with diminished AUC_{30} MMT2 (p=0.03) and MMT3 (p=0.02). There was no difference at other time points.

The total insulin response (AUC_{120}) was less in CF subjects (p=0.05) in the afternoon (p=0.04). In the evening, although the CF group had lower levels, this was not statistically significant (p=0.08).

Table 4-23: Insulin levels through the day

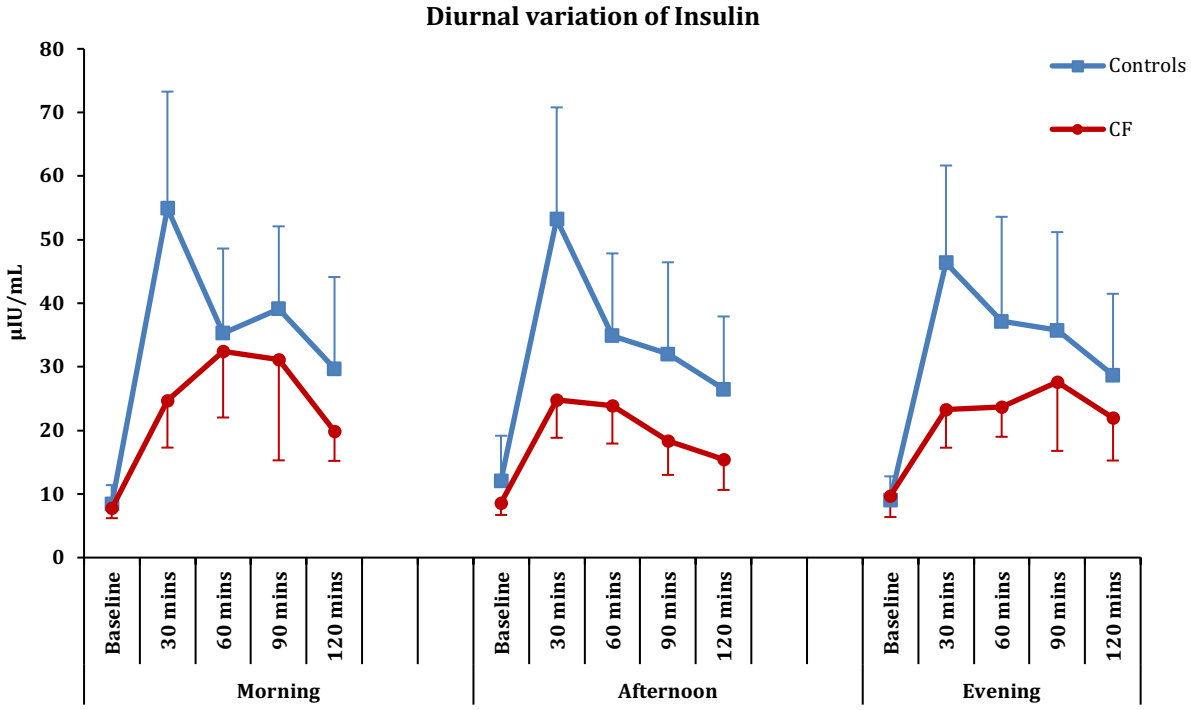
	Morning			Afternoon			Evening		
	Controls	**CF**	**p**	**Controls**	**CF**	**p**	**Controls**	**CF**	**p**
0 mins	8.5 ± 4.7	7.7 ± 2.9	0.66	12.1 ± 11.4	8.5 ± 3.1	0.36	9.1 ± 6.0	9.7 ± 6.2	0.8
30 mins	54.9 ± 29.6	24.7 ± 13.6	0.01	53.2 ± 28.3	24.8 ± 10.9	0.01	46.4 ± 24.7	23.3 ± 11.1	0.02
60 mins	35.3 ± 21.4	32.4 ± 19.1	0.73	34.9 ± 20.9	23.9 ± 11.0	0.16	37.2 ± 26.5	23.7 ± 8.7	0.15
90 mins	39.1 ± 21.0	31.2 ± 29.2	0.48	32.0 ± 23.2	18.3 ± 9.7	0.1	35.8 ± 24.9	27.6 ± 19.9	0.41
120 mins	29.7 ± 23.2	19.8 ± 8.5	0.11	26.5 ± 18.5	15.4 ± 8.4	0.11	28.7 ± 20.6	21.9 ± 12.3	0.37

Comparison between insulin (μIU/mL) levels over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using the Mann-Whitney U test and a repeated measures ANOVA was performed to study differences within the group through the day [No significant differences].

Table 4-24: Insulin - Incremental and total responses through the day

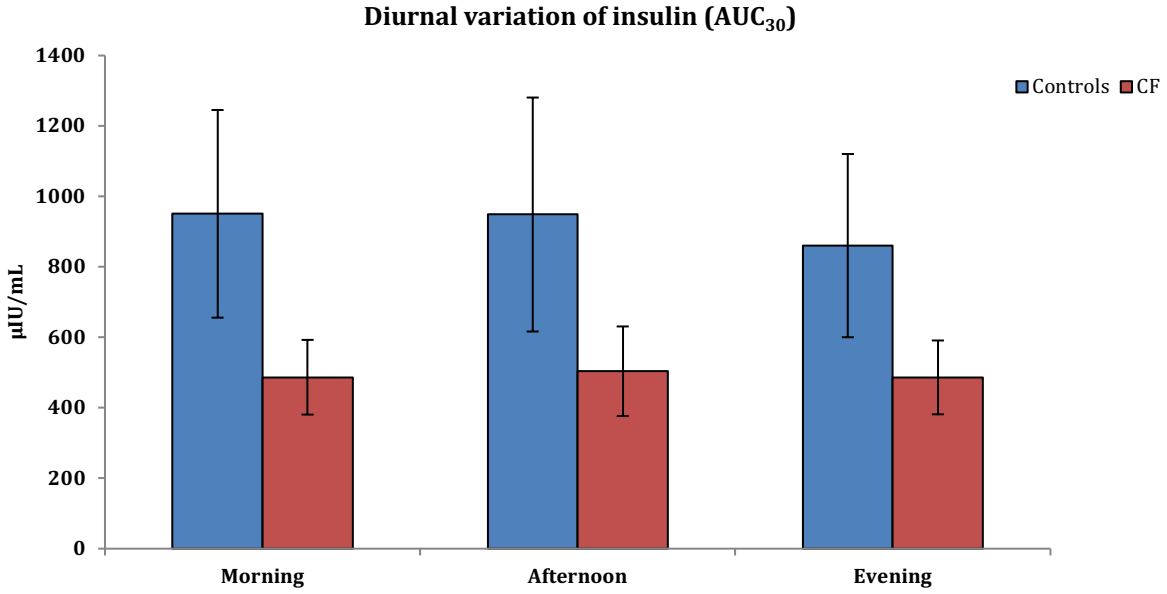
		Morning	Afternoon	Evening
AUC_{30}	**Controls**	951 ± 476	949 ± 536	860 ± 420
	CF	487 ± 195	504 ± 205	486 ± 193
	p	0.01	0.03	0.02
AUC_{120}		**Morning**	**Afternoon**	**Evening**
	Controls	4452 ± 2068	4054 ± 2256	4048 ± 2236
	CF	3067 ± 142	2393 ± 919*	2703 ±1213
	p	0.08	0.04	0.08

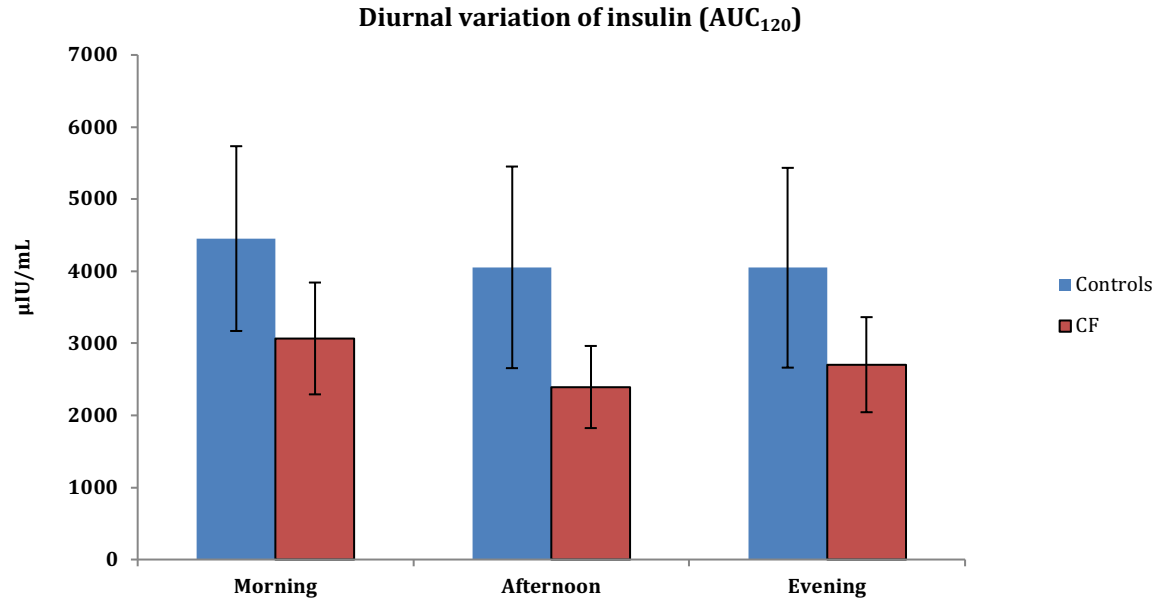
*Comparison between the initial (AUC_{30}) and total (AUC_{120}) insulin (μIU/mL) levels, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using the Mann-Whitney U test and a repeated measures ANOVA was performed to study differences within the group through the day [Within CF group: comparison with morning: * p=0.003].*



Comparison of insulin (µIU/mL) levels in response to the mixed meal (mean ± CI) over 120 minutes between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening. [For p-values see Table 4-23*]*

Figure 4-20: Diurnal variation in insulin levels





Comparison of initial (AUC_{30}) and total (AUC_{120}) insulin (µIU/mL) levels following the mixed meal (mean ± CI) between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening.

Figure 4-21: Insulin - Incremental and Total responses through the day

4.3.4.3 C-peptide

See Table 4-25, Table 4-26 on page 130 and Figure 4-22 Figure 4-23 on pages 131 and 132

A. Controls

In the afternoon (MMT2), c-peptide levels in healthy controls at baseline were 5.2 ± 2.1 and at 120-minutes were 6.5 ± 1.9 ng/mL. 3 subjects had high baseline levels (normal 0.8-5.1 ng/mL).

In the evening (MMT3), c-peptide levels in healthy controls at baseline were 5.2 ± 1.7 and at 120-minutes were 8.2 ± 2.4 ng/mL. 5 subjects had high baseline levels (normal 0.8-5.1 ng/mL).

Analysis of c-peptide release through the day, showed no difference in healthy control subjects during the day at any time point with the corresponding time point between MMT1, MMT2 and MMT3.

There was no difference the incremental initial (AUC_{30}) and total (AUC_{120}) responses within the control group throughout the day (MMT1, MMT2 and MMT3).

B. Cystic Fibrosis

Two baseline and one 120-minute c-peptide samples were not analysed (haemolysis and insufficient sample).

In the afternoon (MMT2), one subject had a high baseline level (group mean 3.5 ± 1.8 ng/mL) but all had normal 120-minute level (group mean 5.7 ± 2.6 ng/mL).

In the evening (MMT3), 3 subjects had high baseline levels (group mean 3.5 ± 2.4 ng/mL) and all had normal 120-minutes levels (group mean 7.9 ± 3.8 ng/mL).

There was no difference the incremental initial (AUC_{30}) and total (AUC_{120}) responses within the CF group throughout the day (MMT1, MMT2 and MMT3).

C. Controls v Cystic Fibrosis

CF subjects had lower c-peptide levels at 30-minutes during the MMT2 (p=0.02) and MMT3 (p=0.04). Although they had lower baseline levels in the afternoon and evening, the difference was not statistically significant (p=0.06)

There was a lower initial (AUC_{30}) response in the CF group in the morning (p=0.048) and evening (p=0.04), but no difference in the total (AUC_{120}) c-peptide levels between the 2 groups throughout the day.

Table 4-25: C-peptide levels through the day

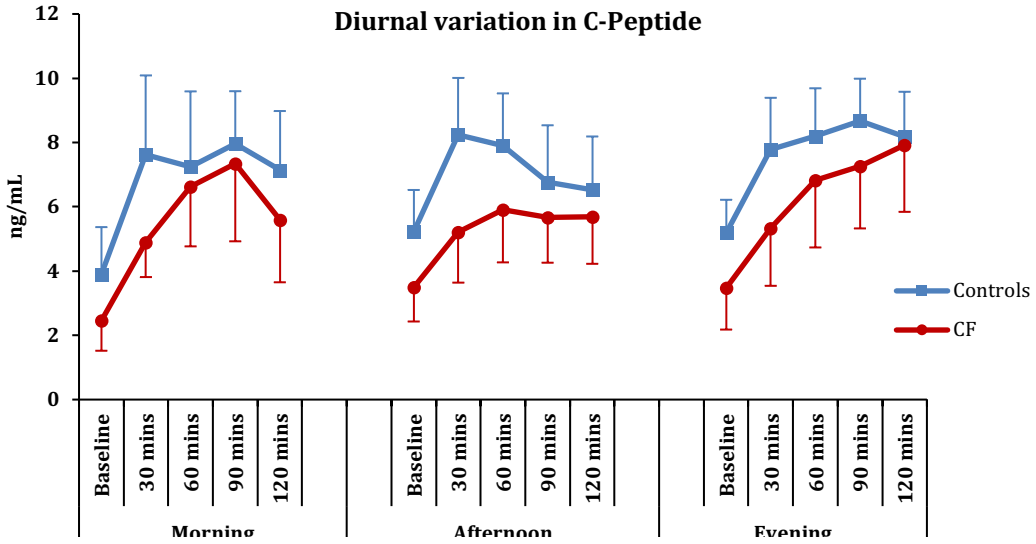
	Morning			Afternoon			Evening		
	Controls	**CF**	**p**	**Controls**	**CF**	**p**	**Controls**	**CF**	**p**
0 mins	3.9 ± 2.4	2.5 ± 1.7	0.1	5.2 ± 2.1	3.5 ± 1.8	0.06	5.2 ± 1.7	3.5 ± 2.4	0.06
30 mins	7.6 ± 4.4	4.9 ± 2.0	0.07	8.2 ± 2.9	5.2 ± 2.9	0.02	7.8 ± 2.6	5.3 ± 3.3	0.04
60 mins	7.2 ± 3.7	6.6 ± 3.4	0.48	7.9 ± 2.4	5.9 ± 3.0	0.06	8.2 ± 2.1	6.8 ± 3.8	0.24
90 mins	8.0 ± 2.4	7.3 ± 4.5	0.62	6.8 ± 2.5	5.7 ± 2.6	0.19	8.7 ± 2.0	7.3 ± 3.6	0.19
120 mins	7.1 ± 2.4	5.6 ± 3.6	0.19	6.5 ± 1.9	5.7 ± 2.6	0.23	8.2 ± 2.4	7.9 ± 3.8	0.82

Comparison between c-peptide (ng/mL) levels over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using the independent test and a repeated measures ANOVA was performed to study differences within the group through the day [No significant differences].

Table 4-26: C-peptide - Incremental and Total responses through the day

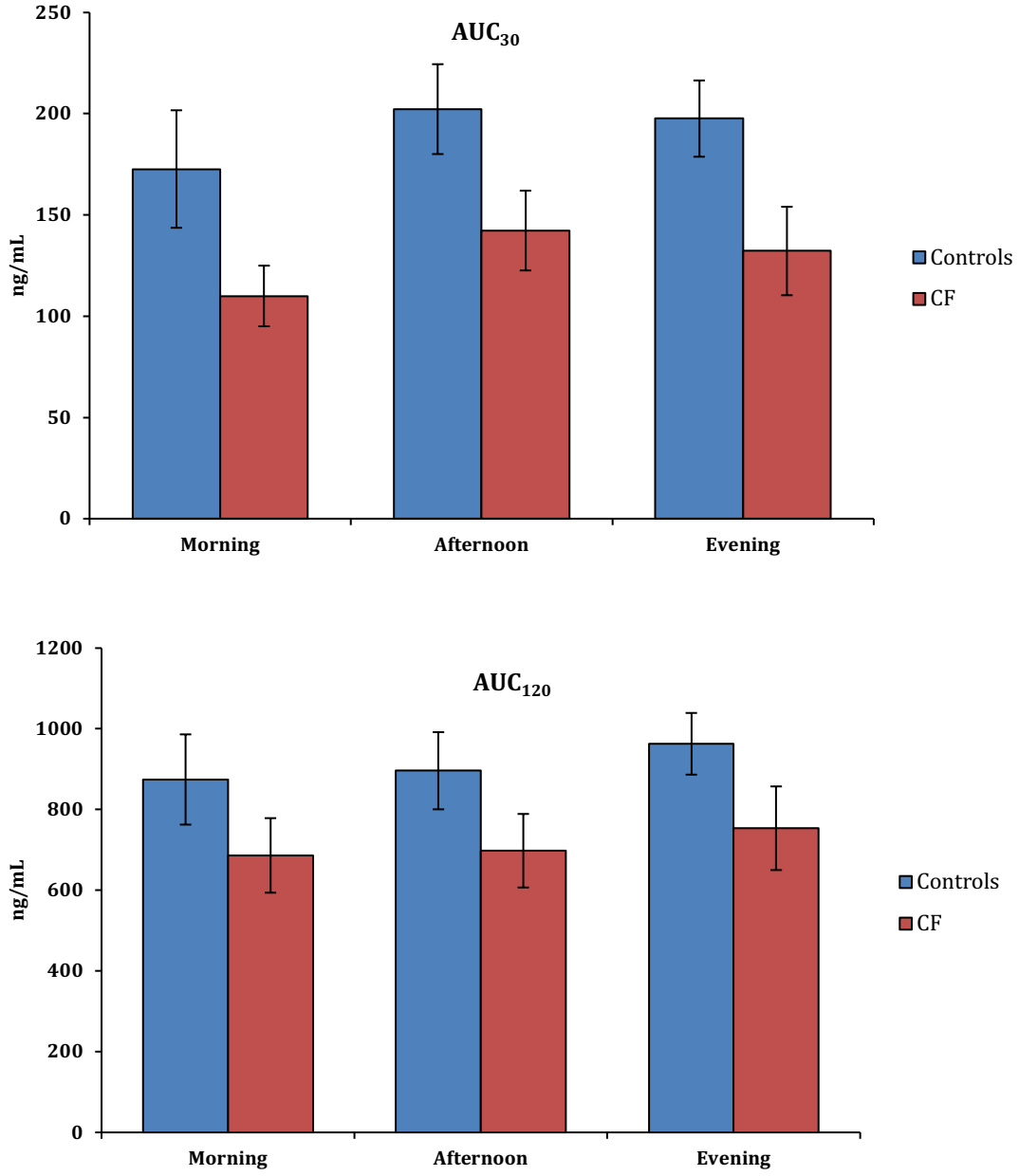
		Morning	Afternoon	Evening
AUC_{30}	**Controls**	173 ± 92	202 ± 70	198 ± 60
	CF	110 ± 54	142 ± 62	132 ± 79
	p	0.048	0.06	0.04
		Morning	**Afternoon**	**Evening**
AUC_{120}	**Controls**	874 ± 354	896 ± 302	963 ± 242
	CF	686 ± 333	698 ± 288	754 ± 374
	p	0.2	0.15	0.11

Comparison between the initial (AUC_{30}) and total (AUC_{120}) c-peptide (ng/mL) levels, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using the independent test and a repeated measures ANOVA was performed to study differences within the group through the day [No significant differences].



Comparison of c-peptide (ng/mL) *levels in response to the mixed meal (mean ± CI) over 120 minutes between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening. [For p-values* see Table 4-25*]*

Figure 4-22: Diurnal variation in C-peptide



Comparison of initial (AUC_{30}) and total (AUC_{120}) c-peptide (ng/mL) levels following the mixed meal (mean ± CI) between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening.

Figure 4-23: C-peptide - Incremental and Total responses through the day

4.3.4.4 Glucagon

See Table 4-27, *Comparison between glucagon (pmol/L) levels over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using the independent test and a repeated measures ANOVA was performed to study differences within the group through the day [No significant differences].*

Table 4-28 on page 135 and Figure 4-24 and Figure 4-25 on pages 136 and 137

A. Controls

In the afternoon (MMT2), 3 subjects had high baseline levels (normal < 17.2 pmol/L), 1 had a level below the assay detection range at baseline, 60, 90 and 120-minutes, and another at 30, 60 and 90-minutes. Glucagon levels in the remainder were 18.6 ± 9.3 at baseline and 23.6 ± 12.4 pmol/L at 120-minutes.

In the evening (MMT3), 1 subject had glucagon levels below the lower level of assay detection at baseline, 60 and 90-minutes, and levels could not be analysed in a further subject at 90 and 120-minutes due to insufficient sampling. In the remainder, 3 subjects had high baseline levels (group mean 16.7 ± 4.6) and the group mean at 120-minutes was 19.2 ± 6.3 pmol/L.

There was no difference the incremental initial (AUC_{30}) and total (AUC_{120}) responses within the control group throughout the day (MMT1, MMT2 and MMT3).

B. Cystic Fibrosis

In the afternoon (MMT2) insufficient samples were taken for baseline analysis in 2 subjects. In the remainder, 2 had high baseline levels (group mean 12.2 ± 4.3) and at 120-minutes (17.4 ± 6.6 pmol/L).

In the evening (MMT3) insufficient sampling prevented analysis in one subject at 60-minutes, but 5 subjects had high baseline levels (group mean 16.4 ± 4.2) with a group mean of 13.1 ± 4.5 pmol/L at 120-minutes.

There was no difference in the incremental initial (AUC_{30}) and total (AUC_{120}) responses within the CF group throughout the day (MMT1, MMT2 and MMT3).

C. Controls v Cystic Fibrosis

There was no difference between control and CF subjects at any time point during the MMT2, but CF subjects had lower glucagon levels at 120-minutes (p=0.01) at MMT3.

There was no difference in the initial (AUC_{30}) response between the groups in the morning and evening, but in the afternoon, CF subjects had a lower incremental response (p=0.03). There was no difference in the total (AUC_{120}) glucagon levels between the 2 groups throughout the day.

Table 4-27: Glucagon levels through the day

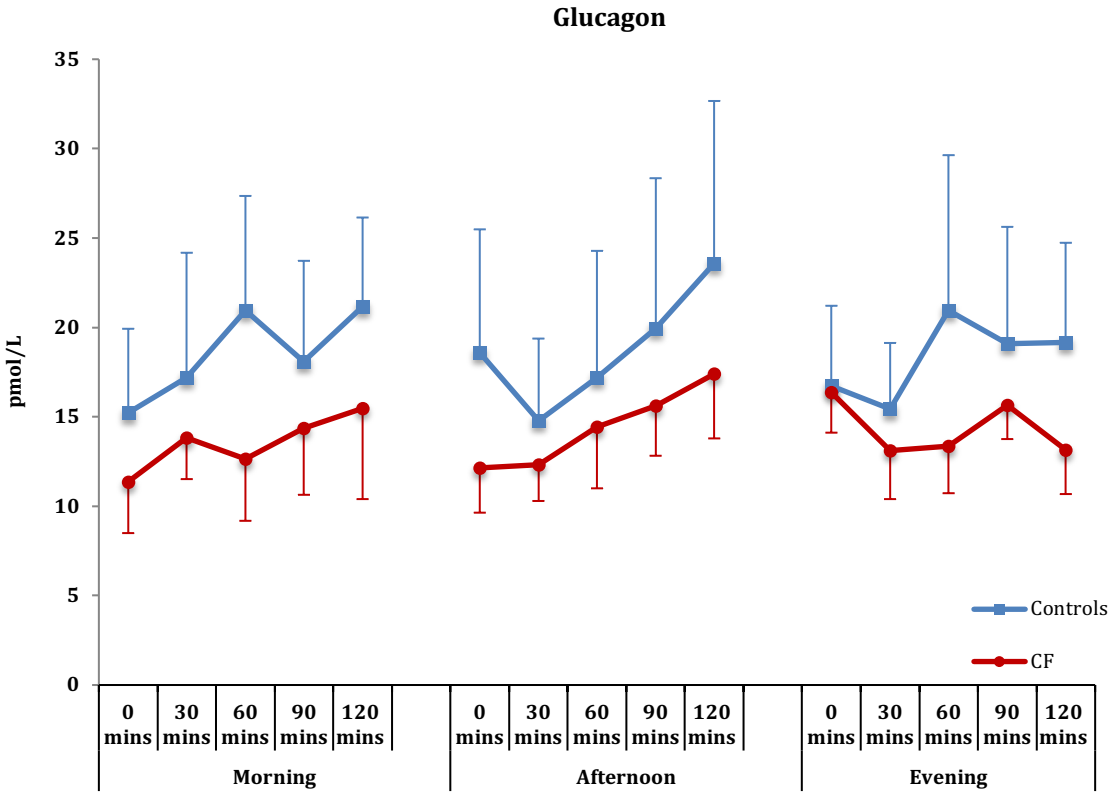
	Morning			Afternoon			Evening		
	Controls	**CF**	**p**	**Controls**	**CF**	**p**	**Controls**	**CF**	
0 mins	15.2 ± 4.8	11.4 ± 5.1	0.1	18.6 ± 9.3	12.2 ± 4.3	0.08	16.7 ± 4.6	16.4 ± 4.2	0.85
30 mins	17.2 ± 9.7	13.8 ± 4.0	0.35	14.8 ± 4.8	12.3 ± 3.7	0.2	15.5 ± 3.9	13.1 ± 5.0	0.23
60 mins	21.0 ± 7.6	12.6 ± 5.8	0.01	17.2 ± 9.1	14.4 ± 6.0	0.42	21.0 ± 12.2	13.4 ± 4.7	0.11
90 mins	18.1 ± 7.6	14.4 ± 6.6	0.23	19.9 ± 11.9	15.6 ± 5.1	0.33	19.1 ± 7.4	15.7 ± 3.5	0.25
120 mins	21.2 ± 3.8	15.5 ± 9.3	0.06	23.6 ± 12.4	17.4 ± 6.6	0.2	19.2 ± 6.3	13.1 ± 4.5	0.01

Comparison between glucagon (pmol/L) levels over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using the independent test and a repeated measures ANOVA was performed to study differences within the group through the day [No significant differences].

Table 4-28: Glucagon - Incremental and Total responses through the day

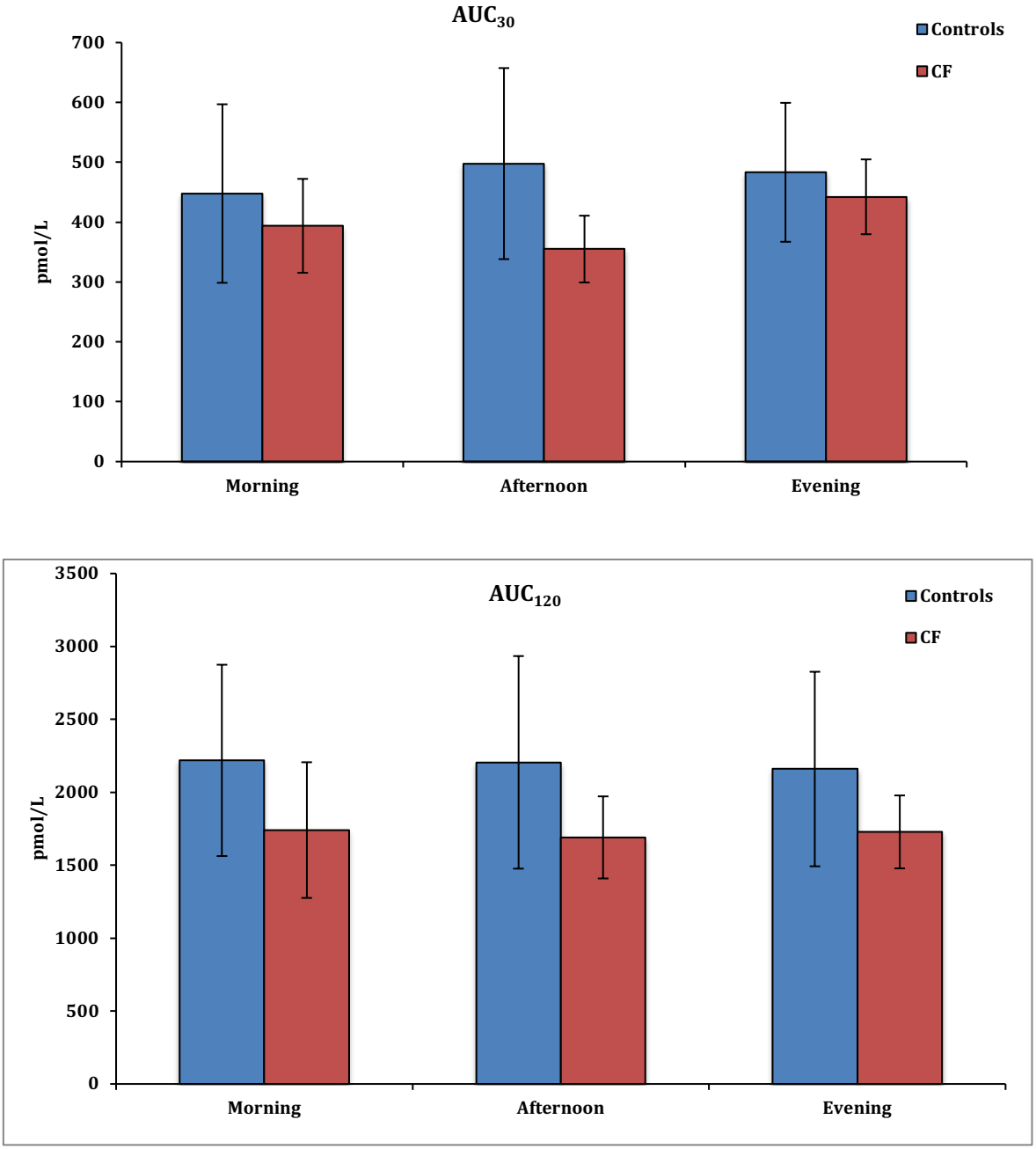
		Morning	Afternoon	Evening
AUC_{30}	**Controls**	448 ± 166	498 ± 171	483 ± 96
	CF	394 ± 121	355 ± 94	442 ± 110
	p	0.42	0.03	0.38
AUC_{120}				
	Controls	2219 ± 632	2205 ± 804	2159 ± 683
	CF	1741 ± 660	1691 ± 454	1728 ± 408
	p	0.14	0.1	0.1

Comparison between the initial (AUC_{30}) and total (AUC_{120}) glucagon (pmol/L) levels, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using the independent test and a repeated measures ANOVA was performed to study differences within the group through the day [No significant differences].



Comparison of glucagon (pmol/L) levels in response to the mixed meal (mean ± CI) over 120 minutes between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening. [For p-values see Table 4-27]

Figure 4-24: Glucagon responses through the day



Comparison of initial (AUC_{30}) and total (AUC_{120}) glucagon (pmol/L) *levels following the mixed meal (mean ± CI) between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening.*

Figure 4-25: Glucagon - Incremental and Total responses through the day

4.3.4.5 Time to peak response

See Table 4-29 on page 139

I also evaluated the time taken to reach a peak level for insulin, c-peptide and glucagon at the different times of the day.

A. Insulin

There was no difference within the control group or the CF group in the time taken to reach a peak level of insulin. Comparing the two groups, the time taken to reach a peak insulin level was longest in CF subjects in the evening compared to control subjects (78 ± 31 vs 51 ± 5-minutes, p=0.03).

B. C-peptide

There was no difference within the control group or the CF group in the time taken to reach a peak level of insulin. Comparing the two groups, the time taken to reach a peak c-peptide level was greater in CF subjects in the afternoon and evening compared to control subjects (afternoon 65 ± 36 vs 83 ± 27-minutes, p=0.04; evening 72 ± 35 vs 90 ± 35 p=0.04).

C. Glucagon

There were no differences within the control group or the CF group in the time taken to reach a peak level of glucagon. Comparing the two groups, there was no difference in the time taken to reach a peak level through the day.

Table 4-29: Time to reach a peak level through the day

		Insulin	C-peptide	Glucagon
Morning	**Controls**	52.5 ± 26.6	60.00 ± 32.28	63 ± 51.87
	CF	67.50 ± 28.96	83.08 ± 27.24	85.71 ± 43.80
	p	0.11	0.18	0.35
Afternoon	**Controls**	45.0 ± 22.7	64.5 ± 35.5	84.0 ± 44.3
	CF	55.4 ± 24.0	83.1 ± 27.2	68.6 ± 44.7
	p	0.17	0.04	> 0.99
Evening	**Controls**	48.8 ± 22.3	72.0 ± 35.2	72.0 ± 42.9
	CF	78.5 ± 31.3	90.0 ± 34.6	81.4 ± 43.1
	p	0.03	0.04	0.06

Mean ± SD (minutes)

4.3.5 Insulin Sensitivity

See Table 4-30 below

As glucose handling appeared better in the afternoon compared to the other times of the day in CF subjects, I went on to evaluate β cell indices looking at insulin sensitivity and disposition.

Within CF subjects, the Matsuda Index (MI) was highest in the afternoon compared to control subjects (p=0.04) suggesting greater insulin sensitivity in the afternoon in the CF group. The Disposition Index (DI) was consistently lower throughout the day compared to control subjects. There were no statistical differences between the DI at various times of the day in both groups.

Table 4-30: Indices of Insulin sensitivity

		Morning	Afternoon	Evening
Matsuda Index (MI)	**Controls**	9.6 ± 6.6	8.4 ± 5.3	9.6 ± 6.6
	CF	8.1 ± 2.8	10.5 ± 5.3^	8.5 ± 4.1
Disposition Index (DI)	**Controls**	11.3 ± 5.7	21.6 ± 21.9	16.9 ± 16.3
	CF	2.1 ± 1.6 *	4.5 ± 2.9 ^	3 ± 3.2 *

Mean ± SD; Comparison with controls: * p=0.02; ^ p=0.04

4.4 Discussion

In this chapter I have shown that in CF subjects without frank diabetes, there is deficient glucose handling throughout the OGTT and early morning MMT. This confirms the results of other workers [256] and therefore validates my methodology.

Clinical decline in CF precedes the detection of diabetes by current OGTT criteria and its early treatment confers a clinical advantage, with improvement in lung function and body mass index (BMI) within 3 to 6 months of introducing insulin replacement therapy [40, 46, 257]. This "CFRD" is characterised by post-prandial hyperglycaemia rather than fasting hyperglycaemia and is frequently evident in CF patients with a normal OGTT.

The factors contributing to this early decline in CF are not fully understood. The patho-physiology of diabetes has been studied using a variety of oral glucose tolerance tests, but the OGTT is un-physiological, unpalatable, time consuming and poorly reproducible [258, 259]. Furthermore, in CF it has poor sensitivity and specificity [23, 75] and might give misleading results.

Hence, the administration of a standard mixed meal, considered a more physiological test, both at the time of diagnosis and during follow-up has been proposed for clinical practice.

I have also shown that fasting and total insulin secreted are similar in CF and healthy subjects, again in keeping with previous studies [260, 261]. However, healthy subjects achieved an earlier peak level (30-minutes). A number of studies have shown this 'insulin lag' in CF and a decreased first-phase insulin secretion in CFRD. Data from our unit showed that the time taken to achieve peak insulin was delayed in individuals with impaired glucose tolerance (IGT) and CFRD patients [101]. This first phase insulin secretion and consequent increase in plasma insulin after a glucose load is crucial in limiting post-prandial hyperglycaemia [262] and CFRD is best thought of as an insulinopaenic condition, as a result of β cell failure [36, 52, 263] resulting in impaired and delayed insulin release. It is plausible that the well described rapid hepatic uptake of glucose, contributed to no difference observed in insulin secretion at 90-minutes between the 2 groups, in this study.

To evaluate pancreatic β cell function further I studied C-peptide, a 31 amino acid peptide, a good marker of pancreatic β cell function as it is released from the β cells during cleavage of insulin from proinsulin, is mainly excreted by the kidney and has a half-life 3-4 times longer than that of insulin. I have shown by this study, lower 30 and 60-minute c-peptide levels and a longer time to reach a peak level in CF subjects. This reflects a similar response to insulin release and reiterates a β cell defect.

This suggests that altered glucose handling is related to decreased insulin and c-peptide levels, a reflection of decreased β cell activity. This is in keeping with the previous study by Battezzati et al [264] who showed that β cell function is impaired in CF patients with NGT, specifically with regard to insulin secretion increments for a given glucose concentration increment. This group also described the impairment of the early insulin secretion index with the reduced ability to increase insulin secretion, in response to the speed of glucose increment after glucose ingestion. In addition, CF patients with NGT may compensate for a decreased β cell function with an intact or increased insulin sensitivity [264].

What my study adds to the existing literature is the contribution of glucagon, an α cell hormone, to glucose handling. Glucagon is the most important of the counter-regulatory hormones. In those with type 2 diabetes, defects in insulin secretion and loss of β cells, contribute in part to glucose imbalance. Another major pathway is the counter-regulation by glucagon to decreased insulin levels. Raised glucagon levels have been shown to contribute to T2DM. Lanng et al [36] demonstrated that following an oral glucose load, glucagon suppression is increasingly impaired. Lippe [138] and Moran [52] demonstrated that CF patients are not able to appropriately increase glucagon secretion in response to arginine or hypoglycaemia using clamp studies, consistent with a reduced α cell mass. By evaluating α cell activity, I have shown that glucagon does not contribute to the increased glucose levels in CF patients with NGT. This lack of glucagon may explain why the occurrence of abnormal fasting plasma glucose is a late occurrence in CF and why the OGTT is a poor test in CF.

In humans, the diurnal variation in glucose tolerance is well established, yet the current diagnostic criteria for diabetes are based on plasma glucose levels recorded in the morning after an overnight fast of at least 8 hours. However, many in the CF population have an

uncertain duration of fasting and usually have their largest meal later in the day - thereby making an early morning assessment of blood glucose un-physiological.

Despite this, the diurnal variation of glucose handling in CF has not been previously studied. This experiment has evaluated glucose tolerance through the day and sets out to describe insulin, c-peptide, glucagon responses.

I chose a standard mixed meal in this study, as it provides a more physiological alternative to the OGTT, as dietary constituents other than glucose affect insulin secretion. The addition of protein, amino acids or fat to carbohydrate is known to enhance insulin secretion, via the incretin system [265]. The main incretins - GIP and GLP_1 are secreted from the intestinal L and K cells respectively, with the primary physiological stimulus being fat [266] and the insulin secretory response of incretins, called the incretin effect, accounting for two-thirds of the insulin response to an oral glucose load.

I have shown that glucose handling in CF patients is variable through the day. Although the 120-minute glucose value failed to differentiate between healthy and CF subjects with abnormal glucose handling based on the standard early morning OGTT, its value in the evening deserves further attention and this may be a better indication of glucose intolerance later in the day.

Early morning rises in fasting glucose levels and insulin requirements known as the 'dawn phenomenon' have been seen in patients with diabetes [218] and in some patients with non-diabetes [219, 220]. Nocturnal elevations in growth hormone and early morning increases in cortisol secretion have been explored as contributors to this phenomenon [221-223]. However, a number of studies have shown that blood glucose levels in response to a mixed meal are markedly higher in the late afternoon or evening compared to the morning and both the size of responses and diurnal variation are more pronounced when the meal has a high carbohydrate content [224, 225] and could be partially mediated by circadian variations in circulating concentrations of cortisol, a counter-regulatory hormone [226].

Interestingly, I found that in the CF group glucose handling improved in the afternoon. This has not been evaluated or described in the CF population before and is unexpected. The main

difference between studies in the healthy population showing glucose intolerance increasing throughout the day [209-215] and my subjects (both healthy controls and CF patients) was the younger group that I studied and this might possibly account for the differences in responses compared to other studies.

This study also shows CF subjects are insulinopaenic at different times of the day, with the lowest insulin level in the afternoon. The initial insulin response, a reflection of the first-phase insulin response, was lower than control subjects through the day. Importantly the time taken to reach a peak insulin level in the evening was the longest. This would suggest the β cells take longer to produce insulin at this time of the day – a concept I refer to as 'CF pancreatic fatigue'.

It is possible to attribute the loss of glucose tolerance to a diminished pancreatic response to a glycaemic stimulus. Freinkel et al [228] observed a diurnal variation in plasma insulin levels during periods of total fasting, with mean levels in the morning exceeding those in the afternoon. Lambert and Hoet [229] also noted a diurnal pattern in insulin levels. They found high levels during the night, even though daytime meals were presumably stimulating insulin release from the pancreas. It is also plausible that these observations in our CF group may be explained by the existence of a periodicity that would regulate insulin secretion. It is also possible that insulin liberated post-prandially in the morning conserves a certain activity at the moment of the next meal, and still intervenes in maintaining glucose homeostasis. Later in the day, however, glucose homeostasis would necessitate a new synthesis of insulin, which would explain the plasma insulin response to the evening meal.

Importantly with a lower blood glucose level in the afternoon and a decreased insulin level, other factors might be contributing to this response in the CF group. In healthy subjects, ~80% of endogenous insulin secretion is extracted during the first liver passage, insulin clearance is pulsatile, wherein the rate of pre-hepatic insulin secretion primarily dictates time-varying clearance of endogenously secreted insulin. Moreover, the pattern of insulin delivery by the pancreas is the predominant determinant of momentary hepatic insulin clearance [267]. It is possible that hepatic extraction of insulin was highest in the afternoon

in the CF group in this study. In addition, this might be partly explained by the similar diurnal variation in the sensitivity to endogenous insulin.

Changes in glucose handling can also be explained by circadian rhythms in insulin sensitivity which is higher in the afternoon (1200-1800) compared to the night [268]. Moreover, insulin sensitivity is impacted by diet and fat, healthy people are more insulin-sensitive, suggesting that changes would be exaggerated in a more insulin-resistant population [269].

I have shown that insulin sensitivity as measured by the "Matsuda Index" (MI) was highest in the afternoon in the CF group possibly explaining the improved level of glucose control at this time.

β cell indices determined by insulin secretion and insulin sensitivity have been validated in CF [270]. The MI has been designed to indicate values which are comparable to the rate of disappearance of plasma glucose and is a surrogate measure of insulin secretion and insulin sensitivity. Such indices have been developed and validated for the OGTT [117] and the MMT [271, 272]. The product of insulin secretion and sensitivity derived from OGTT also termed disposition index (DI) is a useful marker of integrated islet β cell function. β cell function as measured by the DI is reduced in CF patients compared to non-CF controls [121] - even in CF patients with normal glucose tolerance and is further decreased in CF patients with diabetes.

The low DI in our sample of CF patients is due primarily to an insulin secretory defect despite stable pulmonary disease and adequate nutritional status. Our findings indicate that in CF patients with normal glucose tolerance, the underlying insulin secretory defect might be unmasked during times of pancreatic stress such as a response to a meal, possibly resulting in hyperglycaemia.

The DI has the potential to be used as a screening tool and predictor of the development of pre-diabetes and CFRD in larger studies and allows for the identification of CF patients who are at particularly high risk. This would allow early intervention aimed to preserve their β cells and help prevent or delay the development of CFRD and its associated morbidity and mortality.

In summary, these data from a representative sample of non-diabetic CF adults demonstrate that there are clinically significant differences in subjects examined in the afternoon compared to the morning and evening. The 120-minute result on a test of glucose handling is of no-significance when done in the morning in CF patients and the 120-minute value on an evening test and the concept of 'pancreatic fatigue' deserves further attention. This study re-emphasises the need for tests of serial glucose handling with tests that provide 'glucose profiles' and Continuous Glucose Monitoring (CGM) provides this and I encourage its use.

Moreover, dietary constituents other than glucose affect insulin secretion. The addition of protein, amino acids or fat to carbohydrate is known to enhance insulin secretion, via the incretin system [265] The main incretins – GIP and GLP_1 are secreted from the intestinal L and K cells respectively, with the primary physiological stimulus being fat [266] and the insulin secretory response of incretins, called the incretin effect, accounts for two-thirds of the insulin response to an oral glucose load. The incretin effect is severely impaired or absent in patients with Type 2 Diabetes and has fuelled the interest in the development of therapies that target the incretin system.

To explain the changes I observed in this experiment, I set out to evaluate the GLP1 and GIP and pancreatic polypeptide and determine their impact on glucose handling through the day.

5 Experiment 3: Pancreatic endocrine and incretin responses to mixed versus liquid meal tests in non-diabetic CF subjects

The experiment was carried out as in the study protocol described in the previous chapter.

5.1 GLP_1 analysis (RIA)

Analysis was carried out at the Clinical Biochemistry Laboratory, Department of Genomics of Common Disease, Hammersmith Hospital, Imperial College London, Burlington-Danes Building, Du Cane Road, London, W12 0NN, UK. [Dr Paul Bech]

Total GLP_1 was measured using established in-house RIAs [253, 254]. All samples were assayed in duplicate. GLP_1 was purchased from Bachem Ltd (Switzerland). All other reagents and materials were supplied by Sigma (Poole, Dorset, UK). The GLP_1 labels were prepared by Professor M. Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) who iodinated the peptide using the iodogen method [255] and this was purified by reverse-phase HPLC.

Assays were performed in veronal buffer (1 litre distilled water containing 10.3g sodium barbitone, 0.3g sodium azide), at pH 8.0 with 0.02% tween for the GLP_1 assay (VWR International, UK). Standard curves were prepared in assay buffer at 0.25, added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 µl.

The GLP_1 antibody was 149, raised in rabbits against the N terminal of GLP_1 (7-36) and binds to all amidated forms of GLP_1 (1-36, 7-36, 9-36). Experimental samples of 50 µl, 100 µl GLP_1 antibody solution and 100 µl of GLP_1 label solution were used and all tubes were buffered to a total volume of 700 µl with assay buffer. The assays were incubated for 96 hours at 4°C. Free peptide was separated from bound using charcoal adsorption. To each tube, 4 mg of charcoal, suspended in 0.06 M phosphate buffer with gelatine was added immediately prior to

centrifugation. The samples were then centrifuged at 1500 rpm at 4° C, for 20 minutes. Bound and free label were separated and both the pellet and supernatant counted for 180 seconds in a γ-counter (model NE1600, Thermo Electron Corporation). GLP_1 concentrations in the samples were calculated using a non-linear plot (RIA Software, Thermo Electron Corporation) and results calculated in terms of the standard.

5.2 GIP analysis (RIA)

Analysis was carried out at the Clinical Biochemistry Laboratory, Department of Genomics of Common Disease, Hammersmith Hospital, Imperial College London, Burlington-Danes Building, Du Cane Road, London, W12 0NN, UK. [Dr Paul Bech]

Plasma GIP concentration was measured by gel-permeation chromatography radioimmunoassay [273]. This method is summarised below:

^{125}I-Labelled GIP was prepared using a modification of an enzymatic iodination method [274] carried out at room temperature. Two nmol GIP were dissolved in 50 µL 0.05 mol sodium acetate/L, pH 5.0 followed by rapid serial additions of approximately 0.5 nmol $Na^{125}I$ (37 MBq; IMS 30,555 MBq/ µgL; The Radiochemical Centre, Amersham), 22 pmol lacto-peroxidase derived from milk (Sigma Chemical Co) and 1.8 nmol H_2O_2/L, the latter two in 10 µL acetate buffer. The reagents were mixed by bubbling and then allowed to incubate for 15 minutes. The reaction was stopped by dilution with 250 µL of a solution of 100 µL 0.1 mol formic acid/L, 200 µL human serum albumin (HAS; Lister Blood Products Laboratory, Elstree, Herts), 100 µL 2000 Kallikrein inhibitor units (KIU) aprotinin/ml (Trasylol; Bayer Co West Germany), 30 mmol cysteine hydrochloride and 0.05 mol potassium iodide/L. The reaction mixture was immediately loaded onto a 60 x 0.9 cm column containing Sephadex G-50 superfine gel (Pharmacia Co, Uppsala, Sweden) and eluted overnight at 4 °C with 0.1 mol formic acid/L containing 10 µmol HSA/L, 1000 KIU aprotinin/ml and 0.02 mol potassium iodide/L. Flow rate was maintained at 4 ml/h. Fractions containing radioactivity were tested under normal assay conditions for adsorption to charcoal and for binding to antiserum at the normal assay concentrations and also in excess. Fractions demonstrating the highest

antibody binding and the lowest charcoal blank were pooled, diluted in a special solution for freeze-drying, lyophilized to 1.33 Pa, sealed in vacuo and stored at -20 °C in portions each sufficient for one assay of 500 tubes.

Vials of standard hormone were prepared by weighing desiccated pure porcine GIP directly on a Cahn electrostatic balance. This was then dissolved in a solution designed to inhibit losses by surface adsorption or oxidation and which was easily solubilized after lyophilization. The constituents of this solution were 0-14 mol lactose/L, 0.04 mol BSA/L, 11 nmol citric acid/1.6 nmol cysteine hydrochloride/L and 1600 KIU aprotinin/ml in 0.1 mol formic acid/L. Vials containing 1.5 pmol GIP were freeze-dried to 1.33 Pa, sealed in vacuo and stored at -20 °C. The stability of this preparation was regularly assessed by assaying against standard.

Samples were assayed in duplicate in 2ml polystyrene tubes. The plasma sample (200 μL) was added to 600 μL 0.05 mol sodium barbitone/L buffer, pH 8.0, containing 1% HSA and 1.5 fmol ^{125}I-labelled GIP (approx. 100counts/s). Antiserum was added to make a final dilution of 1:96 000. Tubes for calculating the standard curve and other control tubes contained 200 μL subject plasma. The GIP was extracted to constant value by specific immunoadsorption, using a high avidity GIP antiserum covalently bound to Sepharose beads [275] .

To demonstrate that the process completely and specifically removed GIP, samples of plasma were taken and exogenous GIP was added. These samples were incubated with GIP antibody-linked beads, with vasoactive intestinal polypeptide antibody-linked beads and with beads, which were in the unlinked state. Beads linked to GIP antibody, which were incubated with all the other available gut peptides showed negligible cross-reactivity. Standard curves were derived by the addition to this plasma of pure porcine GIP in the range 0-50 fmol/tube. The assay constituents were incubated for 96 h at 4 °C before separation. The effect of pre-incubation with antibody at 4 °C was also assessed by delaying the addition of label for 24 h and by incubation after the addition of label for a further 96 h. Separation of antibody-bound from free ^{125}I-labelled GIP was achieved using dextran-coated (clinical grade; approximate average mol. wt 60-90 000; Sigma Chemical Co.) activated charcoal (Norit GSX, Hopkin and Williams, Chadwell Heath, Essex). This was added as a 500 μL slurry in 0.05 M-barbitone

buffer, pH 8.0, to each assay tube. The assay tubes were then mixed, incubated for 10 min and centrifuged at 2000 g for 15 min at 4 °C.

The supernatant fraction (containing antibody bound fraction) was aspirated and both this and the charcoal precipitate were counted sequentially in the same well of a multi-detector counter (NE 1600, Nuclear Enterprises, Edinburgh). The dose-response meta-meter used was percentage antibody bound.

5.3 Pancreatic polypeptide (PYY) analysis

Analysis was carried out at the Clinical Biochemistry Laboratory, Department of Genomics of Common Disease, Hammersmith Hospital, Imperial College London, Burlington-Danes Building, Du Cane Road, London, W12 0NN, UK. [Dr Paul Bech] [276]

Preparation of ^{125}I-PYY Tracer

Peptides were iodinated using the iodogen method [277] and purified by HPLC using a C_{18} column (Waters, Milford, CT, USA). In brief, the iodogen method adds a ^{125}I molecule onto a tyrosine residue in an oxidative reaction. Iodogen reagent (1,3,4,6,-tetrachloro-3α,6α-diphenylglycoluril; Pierce Chemical Co., Rockford, IL, USA) is reacted with Na^{125}I to oxidise the ^{125}I to the reactive iodine state, which subsequently incorporates into available tyrosine residues. The iodogen method requires the reaction (peptide in 0.2 M phosphate buffer, 37 MBq Na^{125}I, 23 nmol iodogen reagent) to be incubated at 22 °C before the reaction products are purified by reverse-phase HPLC using a NovaPak C_{18} column (Millipore, Milford, MA, USA). The column was equilibrated for 10 minutes with 15% (*v*/*v*) acetonitrile (AcN) solution in water containing 0.05% (*v*/*v*) trifluoroacetic acid (TFA), at a flow rate of 1 ml/min. The concentration of AcN was then gradually increased to 20-45% over the following 80 minutes and maintained at the final concentration of 45% for the next 10 minutes. Fractions were collected every 1.5 minutes into tubes containing 1 ml 20 mM HEPES at pH 11.0 (to neutralise the acidity of the collected fractions) and 0.3% (v/v) bovine serum albumin (BSA) (ICN Biochemicals Inc, Costa Mesa, CA, USA). The fractions were tested in an RIA and used at 1500 cpm/tube.

PYY3-36 antibody

Plasma PYY3-36 was measured using a specific and sensitive RIA. The assay was performed using 0.06 M phosphate buffer with 0.3% BSA at a total volume of 700µl per tube and a sample volume of 1–100µl. The antiserum (Y21) was raised in rabbits against synthetic porcine PYY1-36 (Bachem Ltd. U.K), coupled to BSA, and used at a final dilution of 1:50,000. The Y21 antibody is specific for the C-terminal of PYY and reacts fully with human PYY1-36 and PYY3-36 and does not cross-react with PP, NPY or other known gut hormones.

The assays were incubated for 3 days at 4ºC before separation of free and antibody-bound label by secondary antibody (sheep anti-rabbit antibody). The intra- and inter-assay coefficients of variation were 6% and 10% respectively.

PYY3-36 assays were performed using 0.06M phosphate buffer and a standard curve was made for each assay using a solution of unlabelled antigen at a known concentration and made up to varying concentrations in the assay by adding in duplicate at volumes of 1, 2, 3, 5, 10, 20, 30, 50 and 100 µl to the assay buffer. All samples were assayed in duplicate. To measure for assay drift in antibody-antigen equilibrium, tubes with no sample ('zero' tubes) were placed at regular intervals throughout the assay and standard curves were performed at the beginning and end of each assay. Similar binding should be observed in the zero tubes and in both standard curves.

After incubation of RIAs for 72 hours at 4°C, free and bound peptides were separated using secondary antibody. For secondary antibody separation, 100µl sheep anti-rabbit solid phase secondary antibody (Pharmacia Diagnostics, Uppsala, Sweden) was added to the samples and allowed to incubate for at least one hour. Immediately prior to centrifugation, 500µl 0.01% Triton-X-100 solution was added to each tube. The samples were then immediately centrifuged at 1500*g*, 4°C for 20 minutes. Bound and free label were separated and both the pellet and the supernatant counted for 180 seconds in a γ counter (model NE1600, Thermo Electro Corporation, Reading, Berks, UK). Peptide concentrations in the samples were calculated using a non-linear plot (RIA Software, Thermo Electron Corporation) and results calculated in terms of the standard.

5.4 Results

As outlined in the subject demographics and selection, subjects with a 120-minute value >11.1 mmol/L were excluded from analysis. Data are reported on 10 controls and 13 CF subjects.

5.4.1 Oral Glucose Tolerance Test (liquid meal)

5.4.1.1 GLP_1

See Table 5-1 on page 153 and Figure 5-1 on page 154.

A. Controls

In response to the OGTT, control subjects had a mean baseline of 6.0 ± 2.2 pmol/L and 120-minute value of 5.9 ± 2.5 pmol/L.

Seven control subjects had low GLP_1 levels at baseline and 8 had low 120-minute levels [Normal levels 7-36 pmol/L] [254] .

B. Cystic Fibrosis

CF subjects had a baseline of 4.9 ± 1.5 pmol/L and 120-minute value of 4.8 ± 1.5 pmol/L. 12 CF subjects had low GLP_1 levels at baseline and all had low 120-minute levels [Normal levels 7 - 36 pmol/L] [254].

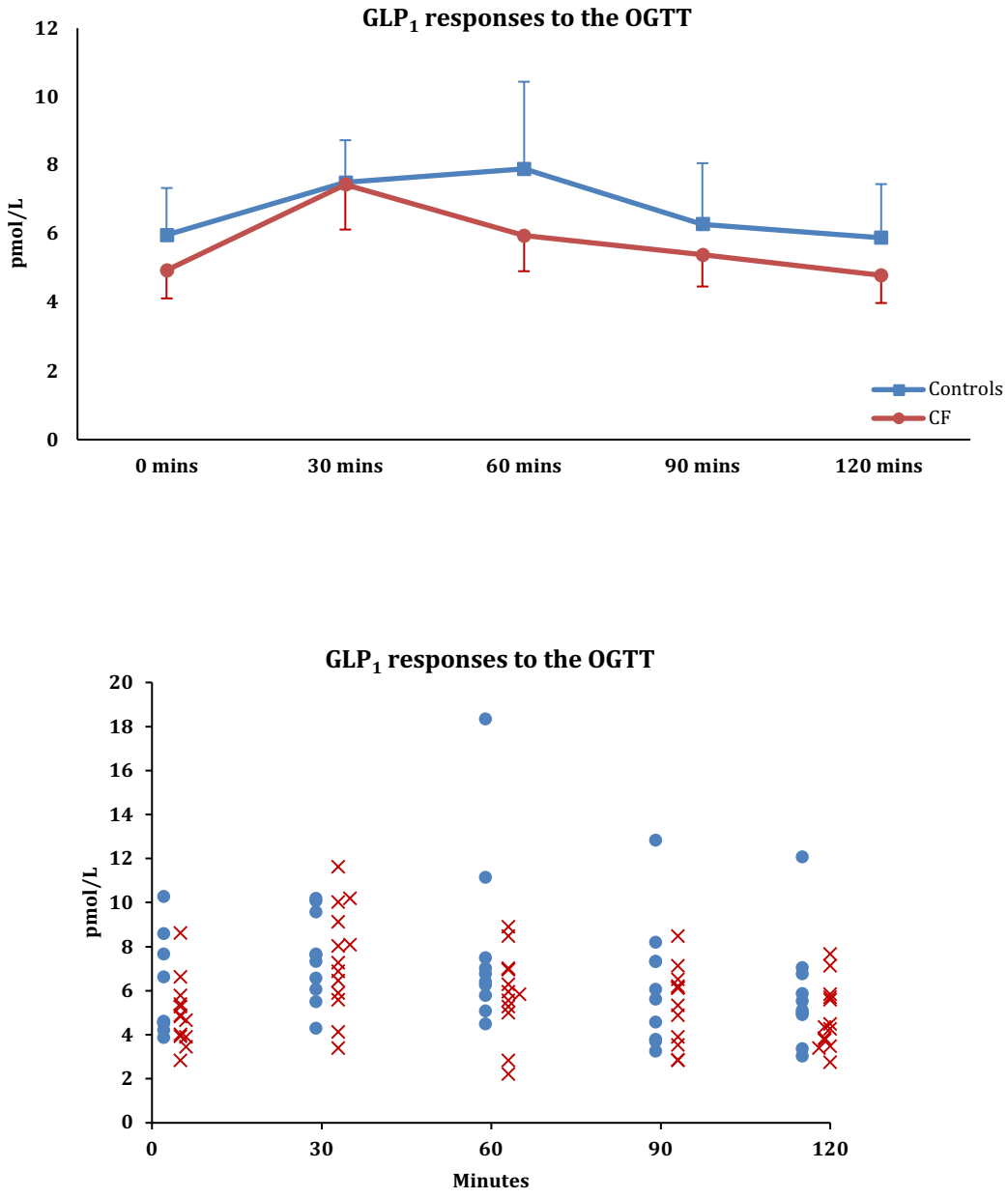
C. Controls v Cystic Fibrosis

Compared to healthy controls, in response to the OGTT there was no difference in CF subjects at any time points.

Table 5-1: GLP_1 levels following the OGTT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	6.0 ± 2.2	7.5 ± 2.0	7.9 ± 4.1	6.3 ± 2.9	5.9 ± 2.5
CF	4.9 ± 1.5	7.4 ± 2.4	5.9 ± 1.9	5.4 ± 1.7	4.8 ± 1.5
p	0.2	0.95	0.19	0.4	0.21

Comparison of GLP_1 (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using independent t-tests.



(Top panel): Comparison of GLP$_1$ (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual GLP$_1$ levels over 120 minutes, following the liquid meal (OGTT). [• Controls x CF]

Figure 5-1: Plasma GLP$_1$ response to the OGTT

5.4.1.2 GIP

See Table 5-2 on page 156 and Figure 5-2 on page 157

The assay limit of detection (LOD) for GIP was 32 pmol/L. Some subjects had concentrations < LOD and have been indicated below. These data were censored for analysis and substituted with a constant value(LOD/√2) as described by Helsel [278] and Boeckel [279].

A. Controls

Eight subjects had concentrations <LOD at baseline. Two subjects had concentrations < LOD at 30, 60, 90 and 120-minutes.

In response to the OGTT, these subjects had mean baseline of 36.9 ± 36.1 pmol/L and 120-minute value of 59.0 ± 31.6 pmol/L.

B. Cystic Fibrosis

Six CF subjects had concentrations <LOD (32 pmol/L) at baseline. One subject had concentrations < the LOD at 30, 60, 90 and 120-minutes and one had concentration < the LOD at 90 minutes.

CF subjects had a baseline of 41.8 ± 25.7 pmol/L and 120-minute value of 73.7 ± 39.9 pmol/L.

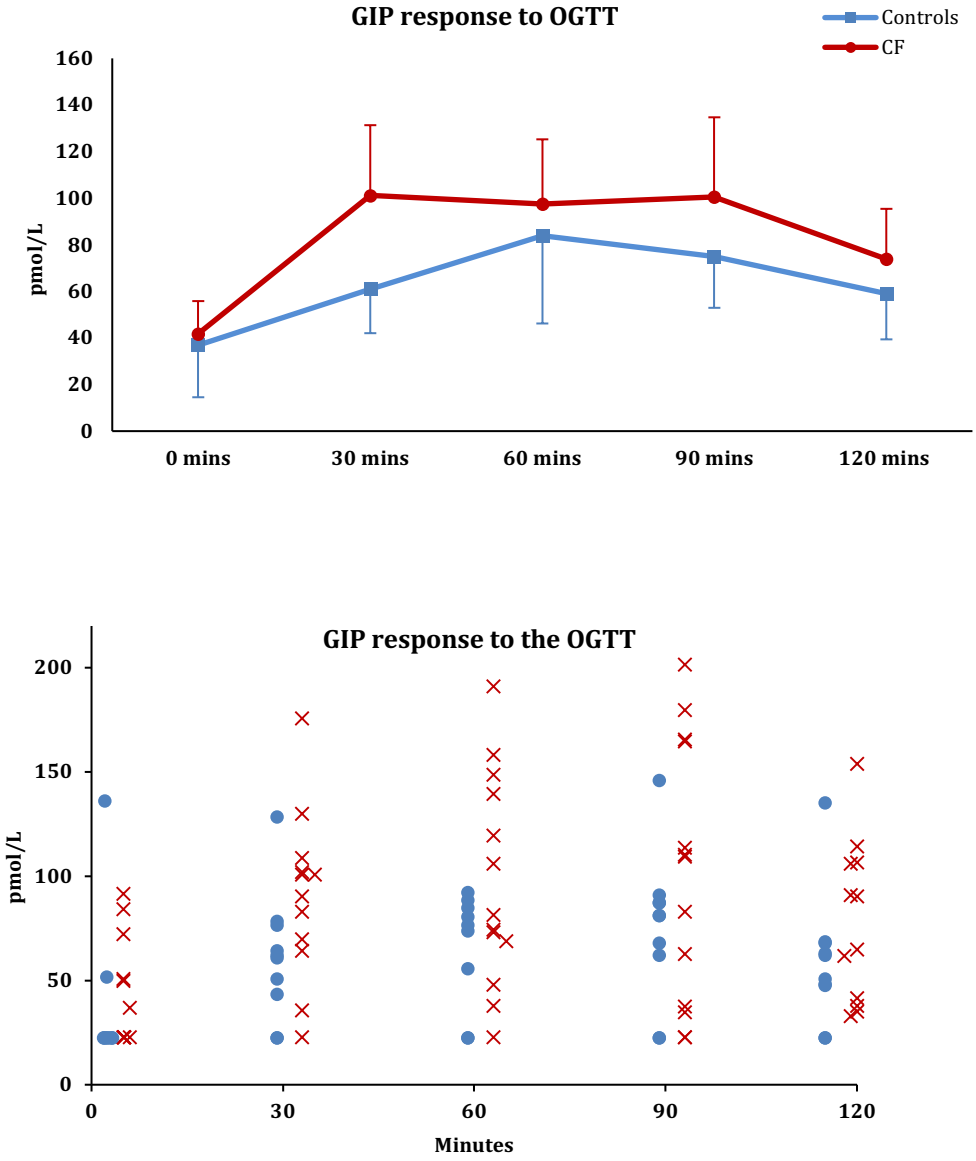
C. Controls v Cystic Fibrosis

Compared to healthy controls, in response to the OGTT there was no difference in CF subjects at baseline (p=0.71), 60 (p=0.57), 90 (p=0.26) and 120 (p=0.34) minutes. At 30 minutes, CF subjects had higher GIP [Controls: 61.0 ± 30.6, CF: 101.2 ± 55.4 (p=0.04)].

Table 5-2: GIP levels following the OGTT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	36.9 ± 36.1	61.0 ± 30.6	83.9 ± 60.8	75.0 ± 35.6	59.0 ± 31.6
CF	41.8 ± 25.7	101.2 ± 55.4	97.5 ± 51.0	100.6 ± 62.8	73.7 ± 39.9
p	0.71	0.04	0.57	0.26	0.34

Comparison of GIP (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using independent t-tests.



(Top panel): Comparison of GIP (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual GIP levels over 120 minutes, following the liquid meal (OGTT). [• Controls x CF]

Figure 5-2: Plasma GIP response to the OGTT

5.4.1.3 PYY

See Table 5-3 on page 159 and Figure 5-3 on page 160

The assay detection limit (LOD) for PYY was 10 pmol/L. Some subjects had concentrations <LOD and have been indicated below. These data were censored for analysis and substituted with a constant value (LOD/√2).

A. Controls

No control subjects had levels below the assay detection limit.

In response to the OGTT, subjects had a mean baseline level of 29.6 ± 11.1 pmol/L and 120-minute value of 35.9 ± 17.3 pmol/L.

B. Cystic Fibrosis

Seven CF subjects had concentrations <LOD, at baseline. Two subjects had concentrations < LOD at baseline, 30, 60 and 120-minutes, one at 60 and 90 minutes, two at all time points during the test, two at 90 and 120-minutes, one at 30 and 90 minutes and for one subject there was insufficient sample available at 60 minutes to assay.

CF subjects had a baseline of 10.3 ± 4.0 pmol/L and 120-minute value of 10.9 ± 4.6 pmol/L.

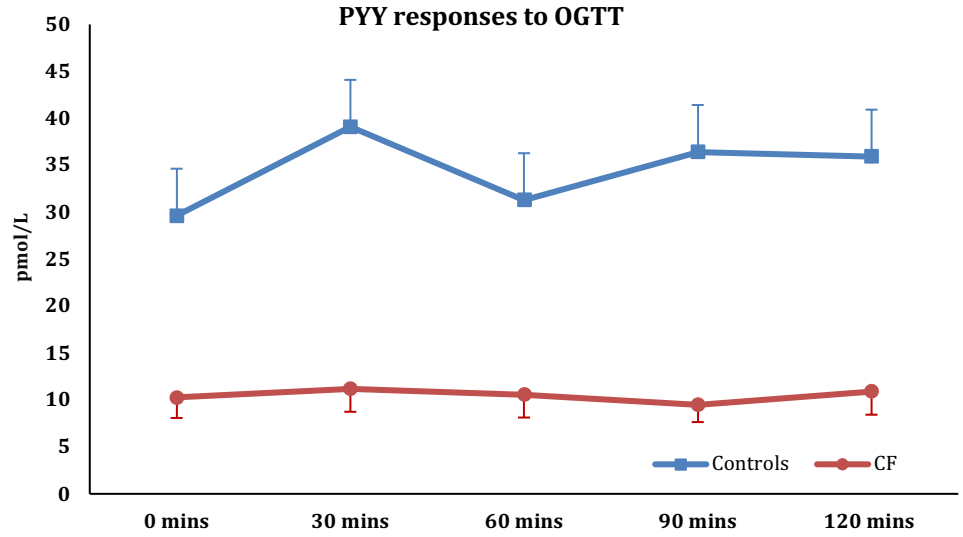
C. Controls v Cystic Fibrosis

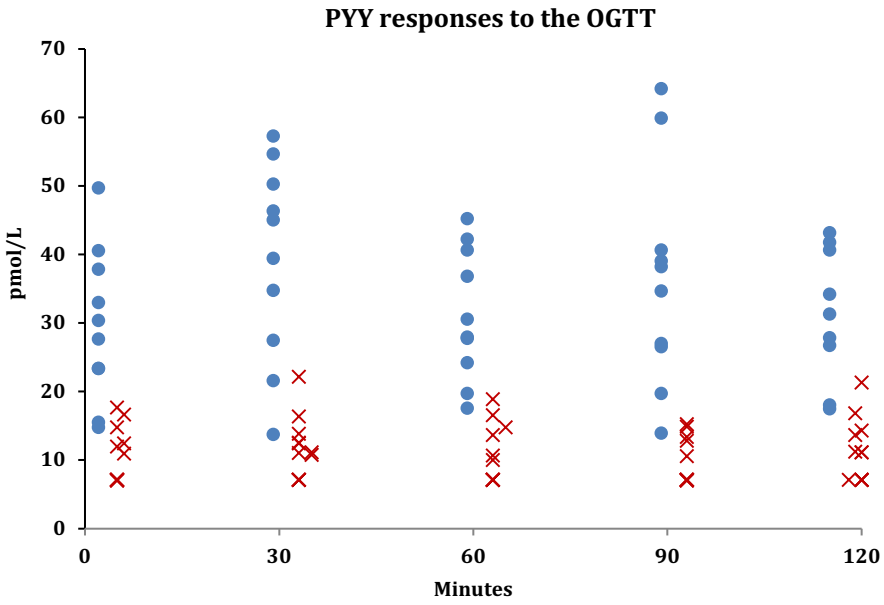
Compared to healthy controls (Table 5-3) in response to the OGTT, CF subjects had lower PYY levels at baseline ($p=0.0003$), 30 ($p=0.0001$), 60 ($p<0.0001$), 90 ($p=0.0004$) and 120 ($p=0.001$) minutes.

Table 5-3: PYY levels following the OGTT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	29.6 ± 11.1	39.1 ± 14.5	31.3 ± 9.6	36.4 ± 16.1	35.9 ± 17.3
CF	10.3 ± 4.0	11.2 ± 4.5	10.6 ± 4.3	9.5 ± 3.4	10.9 ± 4.6
p	0.0003	0.0001	<0.0001	0.0004	0.001

Comparison of PYY (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using the Mann-Whitney U test.





(Top panel): Comparison of PYY (pmol/L) levels following a liquid meal (OGTT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual PYY levels over 120 minutes, following the liquid meal (OGTT). [• Controls x CF]

Figure 5-3: PYY responses to the OGTT

5.4.1.4 Incremental changes in response to the OGTT

See Table 5-4 below

I studied the incremental and total response to the OGTT by evaluating the initial response at 30 minutes (AUC_{30}) and the total response at 120-minutes (AUC_{120}).

A. GLP_1:

CF subjects had comparable AUC_{30} [controls: 202 ± 51, CF: 174 ± 45 pmol/L p=0.43] and AUC_{120} [controls: 828 ± 259, CF: 712 ± 244 pmol/L, p= 0.20].

B. GIP

Again, CF subjects had comparable AUC_{30} [controls: 1611 ± 845, CF: 1837 ± 869 pmol/L p=0.54] and AUC_{120} [controls: 10680 ± 4699, CF: 10638 ± 6074 pmol/L, p= 0.98].

C. PYY

CF subjects had lower AUC_{30} [controls: 1031 ± 326, CF: 322 ± 122; p<0.0001] and AUC_{120} [controls: 4187 ± 141, CF: 1213 ± 364; p<0.0001].

Table 5-4: Incremental and Total responses to the OGTT

	AUC_{30}			AUC_{120}		
	GLP_1	**GIP**	**PYY**	**GLP1**	**GIP**	**PYY**
Controls	202 ± 51	1611± 845	1031 ± 326	828 ± 259	8035 ± 4628	4187 ± 1417
CF	174 ± 45	1837 ± 869	322 ± 122	712 ± 244	10713 ± 5413	1256 ± 382
p	0.43	0.11	<0.0001	0.2	0.26	<0.0001

Comparison of the initial (AUC_{30}) and total (AUC_{120}) responses to the liquid meal (OGTT) for GLP_1 (pmol/L), GIP (pmol/L) and PYY (pmol/L) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using independent t- tests.

5.4.2 Mixed meal test (MMT1)

5.4.2.1 GLP_1

See Table 5-5 below and Figure 5-4 on page 163

A. Controls

In response to the MMT, subjects had a mean baseline level of 5.2 ± 1.6 pmol/L and 120-minute value of 7.7 ± 4.7 pmol/L.

Eight control subjects had low levels at baseline and 5 had low 120-minute levels [Normal levels 7-36 pmol/L] [254] . No subjects had high GLP_1 levels.

B. Cystic Fibrosis

CF subjects had a baseline of 4.7 ± 0.9 pmol/L and 120-minute value of 6.1 ± 1.7 pmol/L.

All CF subjects had low levels at baseline and only three had normal 120-minute levels [Normal levels 7-36 pmol/L] [254].

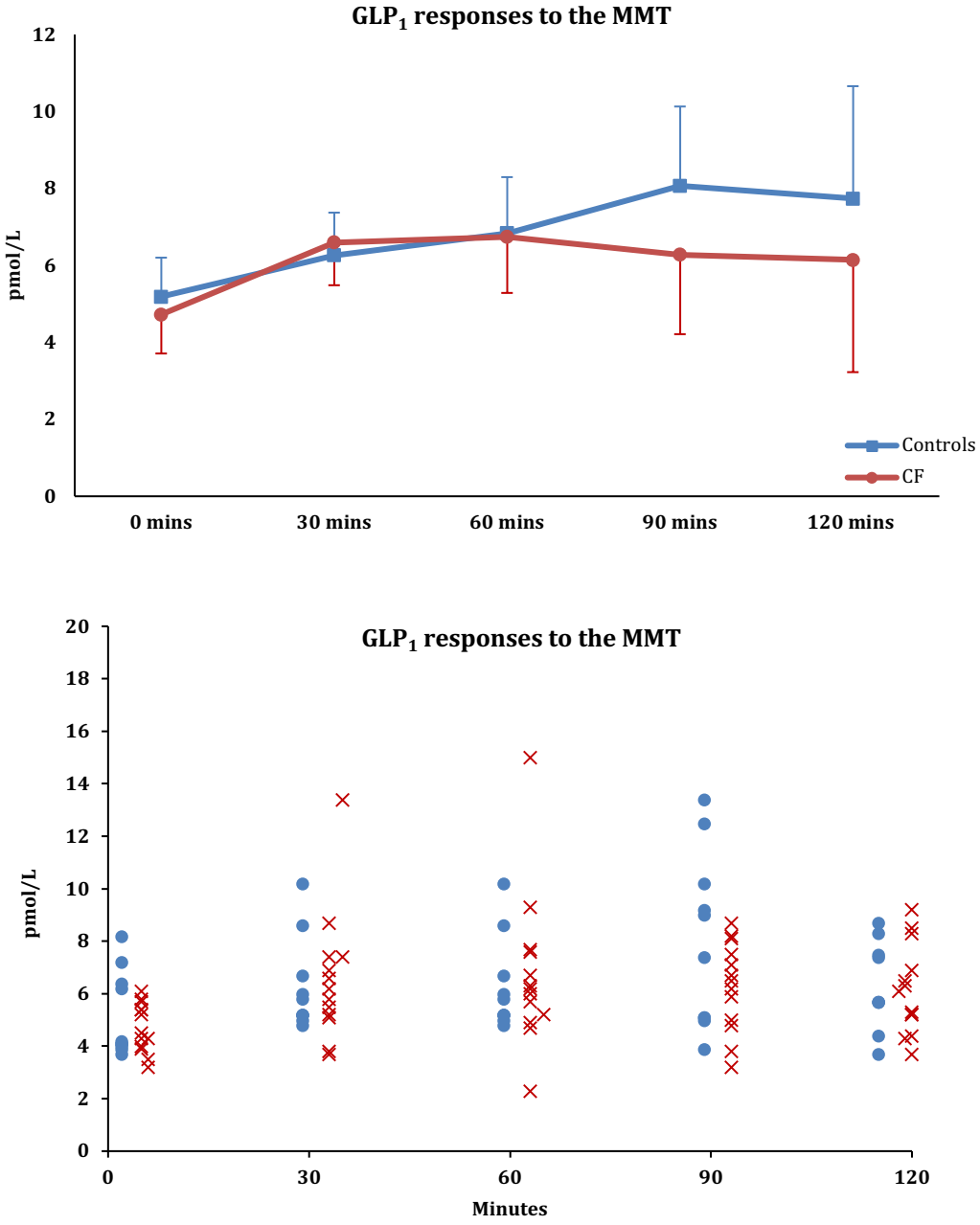
C. Controls v Cystic Fibrosis

Compared to healthy controls (Table 5-5), in response to the MMT there was no difference in CF subjects at any time points.

Table 5-5: GLP_1 levels following the MMT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	5.2 ± 1.6	6.3 ± 1.8	6.8 ± 2.3	8.1 ± 3.3	7.7 ± 4.7
CF	4.7 ± 0.9	6.6 ± 2.5	6.7 ± 3.0	6.3 ± 1.7	6.1 ± 1.7
p	0.38	0.73	0.93	0.14	0.33

Comparison of GLP_1 (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using independent t-tests.



(Top panel): Comparison of GLP$_1$ (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual GLP$_1$ levels over 120 minutes, following the mixed meal (MMT). [• Controls x CF]

Figure 5-4: Plasma GLP$_1$ response to the MMT

5.4.2.2 GIP

See Table 5-6 on page 165 and Figure 5-5 on page 166

The number of subjects with concentrations < LOD (32 pmol/L) have been indicated below. Data at these points were censored for analysis and substituted with a constant value (LOD/√2).

A. Controls

Six control subjects had concentrations <LOD. Of these, one subject had concentrations < LOD at 30 minutes, one at 30 and 60 minutes and one at 30, 60 and 120-minutes.

In response to the MMT, control subjects had mean baseline levels of 35.6 ± 20.9 pmol/L and 120- value of 108.2 ± 50.1 pmol/L.

B. Cystic Fibrosis

Five CF subjects had concentrations <LOD at baseline. One subject had concentrations < LOD at all time points during the test, one at baseline, 30, 90 and 120-minutes, one at 90 and 120-minutes and one at 120-minutes.

CF subjects had a baseline of 47.2 ± 24.4 pmol/L and 120-minute value of 87.1 ± 52.1 pmol/L.

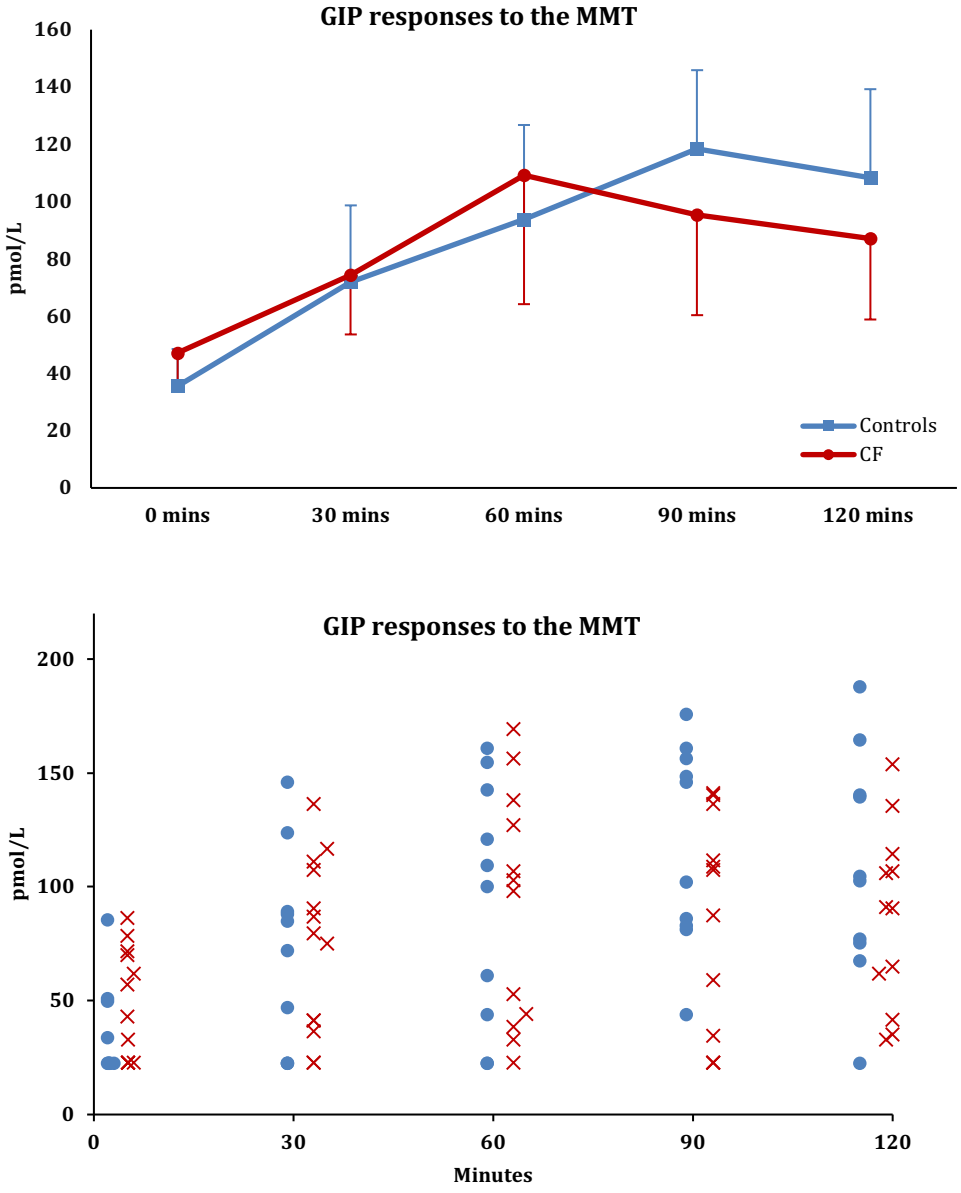
C. Controls v Cystic Fibrosis

In response to the MMT, compared to healthy controls, CF subjects had no difference in the GIP levels at any time points.

Table 5-6: GIP levels following the MMT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	35.6 ± 20.9	71.9 ± 43.2	93.9 ± 53.0	118.4 ± 44.3	108.2 ± 50.1
CF	47.2 ± 24.4	74.3 ± 38.1	109.2 ± 82.8	95.3 ± 64.4	87.1 ± 52.1
p	0.24	0.9	0.61	0.34	0.34

Comparison of GIP (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using independent t-tests.



(Top panel): Comparison of GIP (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual GIP levels over 120 minutes, following the mixed meal (MMT). [• Controls x CF]

Figure 5-5: Plasma GIP response to the MMT

5.4.2.3 PYY

See Table 5-7 on page 168 and Figure 5-6 on page 169

The assay detection limit (LOD) for PYY was 10 pmol/L. Some subjects had concentrations < LOD and have been indicated below. These data were censored for analysis and substituted with a constant value (LOD/√2).

A. Controls

One control subject had levels < LOD at all time points.

In response to the OGTT, the control subjects had mean baseline of 25.5 ± 9.5 pmol/L and 120-minute value of 43.4 ± 21.7 pmol/L.

B. Cystic Fibrosis

Four CF subjects had concentrations below the assay detection limit (10 pmol/L), at baseline. Two subjects had concentrations <LOD at baseline, 60 and 90-minutes, one at 30, 60, 90 and 120-minutes, one at 30-minutes, one had no detectable levels at any time point during the test, two at 90 and 120-minutes, one at baseline and 90-minutes, one at 90, one at 120-minutes and one at baseline, 30 and 90-minutes. There was insufficient sample for assay for one subject at baseline and for another at 120-minutes.

CF subjects had a baseline of 12.1 ± 7.1 pmol/L and 120-minute value of 16.8 ± 15.7 pmol/L.

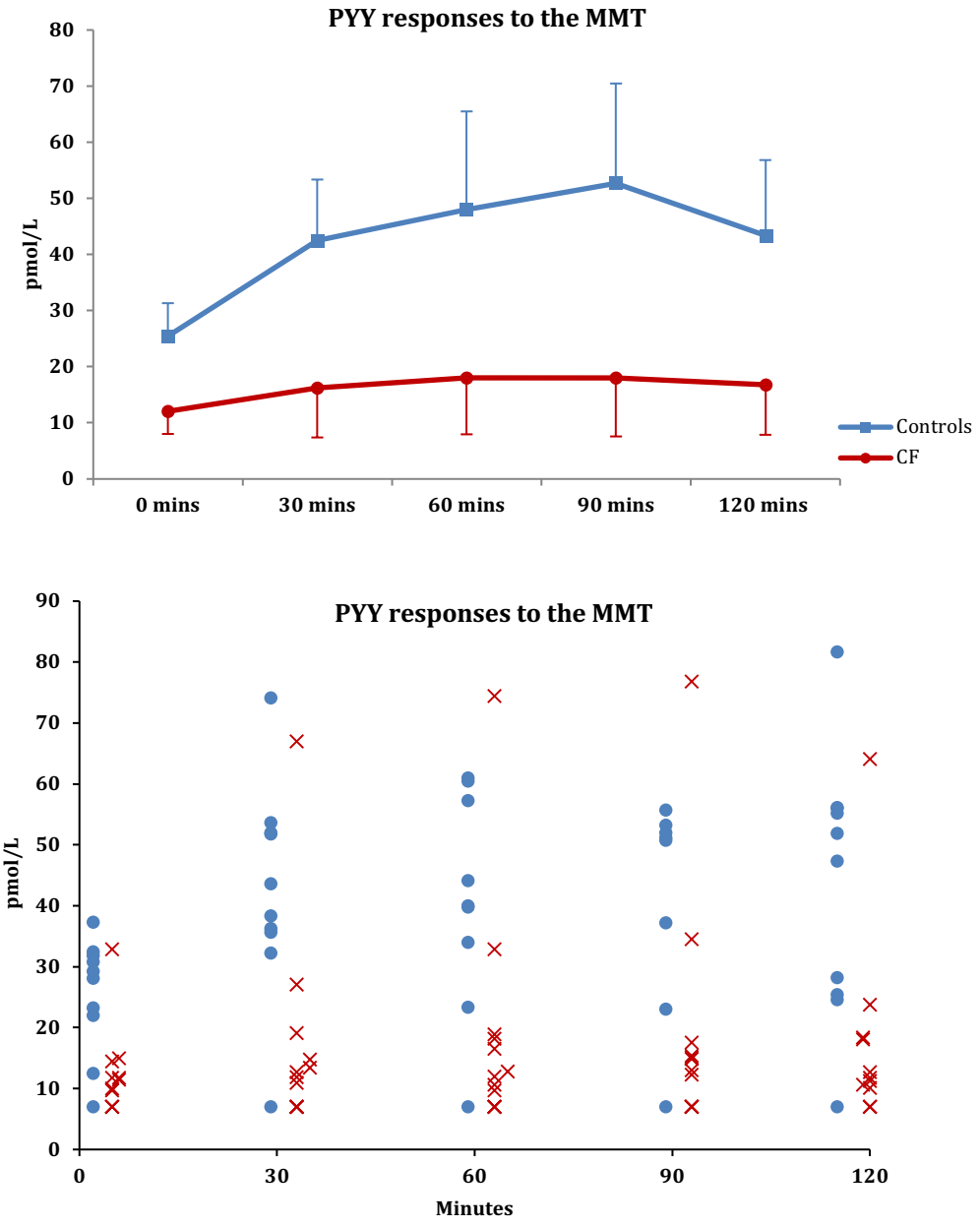
C. Controls v Cystic Fibrosis

Compared to healthy controls in response to the MMT, CF subjects had lower PYY levels at baseline (p=0.001), 30 (p=0.001), 60 (p=0.01), 90 (p=0.005) and 120 (p=0.006) minutes.

Table 5-7: PYY levels following the MMT

	0 mins	30 mins	60 mins	90 mins	120 mins
Controls	25.5 ± 9.5	42.5 ± 17.5	48.0 ± 28.3	52.7 ± 28.7	43.4 ± 21.7
CF	12.1 ± 7.1	16.3 ± 16.3	18.0 ± 18.4	18.0 ± 19.2	16.8 ± 15.7
p	0.001	0.001	0.01	0.005	0.006

Comparison of PYY (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using independent t- tests.



(Top panel): Comparison of PYY (pmol/L) levels following a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± CI). (Bottom panel): Comparison of the individual PYY levels over 120 minutes, following the mixed meal (MMT). [• Controls x CF]

Figure 5-6: Plasma PYY response to the MMT

5.4.2.4 Incremental changes in response to the MMT

See Table 5-8 below

As with the other hormones, I studied the incremental and total response to the MMT by evaluating the initial response at 30 minutes (AUC_{30}) and the total response at 120-minutes (AUC_{120}).

A. GLP_1

CF subjects had comparable AUC_{30} [controls: 172 ± 45, CF: 139 ± 17 pmol/L, p=0.98] and AUC_{120} [controls: 829 ± 280, CF: 649 ± 100 pmol/L, p= 0.52].

B. GIP

CF subjects had comparable AUC_{30} [controls: 1611 ± 845, CF: 1837 ± 869 pmol/L, p=0.54] and AUC_{120} [controls: 10680 ± 4699, CF: 10638 ± 6074 pmol/L, p= 0.99].

C. PYY

CF subjects had lower AUC_{30} [controls: 1019 ± 374, CF: 411 ± 267 pmol/L, p=0.0002] and AUC_{120} [controls: 5325 ± 2435, CF: 2037 ± 1720 pmol/L, p=0.0002].

Table 5-8: Incremental responses to the MMT

	AUC_{30}			AUC_{120}		
	GLP_1	**GIP**	**PYY**	**GLP_1**	**GIP**	**PYY**
Controls	172 ± 45	1611 ± 845	1019 ± 374	829 ± 280	10680 ± 4699	5325 ± 2435
CF	139 ± 17	1837 ± 869	411 ± 267	649 ± 100	10638 ± 6074	2037 ± 1720
p	0.98	0.54	0.0002	0.52	0. 99	0.0002

Comparison of the initial (AUC_{30}) and total (AUC_{120}) responses to the mixed meal (MMT) for GLP_1 (pmol/L), GIP (pmol/L) and PYY (pmol/L) over 120 minutes between control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between groups were carried out using independent t- tests.

5.4.3 Comparison between the OGTT and MMT

5.4.3.1 GLP_1

See Table 5-9, Table 5-10 on page 172 and Figure 5-7, Figure 5-8 on 174.

I then evaluated the responses between the OGTT and the MMT in both healthy controls and in CF subjects.

A. Controls

With the control group, when the OGTT and MMT were compared, there were no statistical differences between the two tests at any time point. This was also the case for the AUC_{30} and AUC_{120}.

B. Cystic Fibrosis

The responses were similar in the CF group, where there were no statistical differences between the two tests at any time point when the OGTT and MMT were compared. This was also the case for the AUC_{30} and AUC_{120}.

Table 5-9: GLP_1 differences between OGTT and MMT

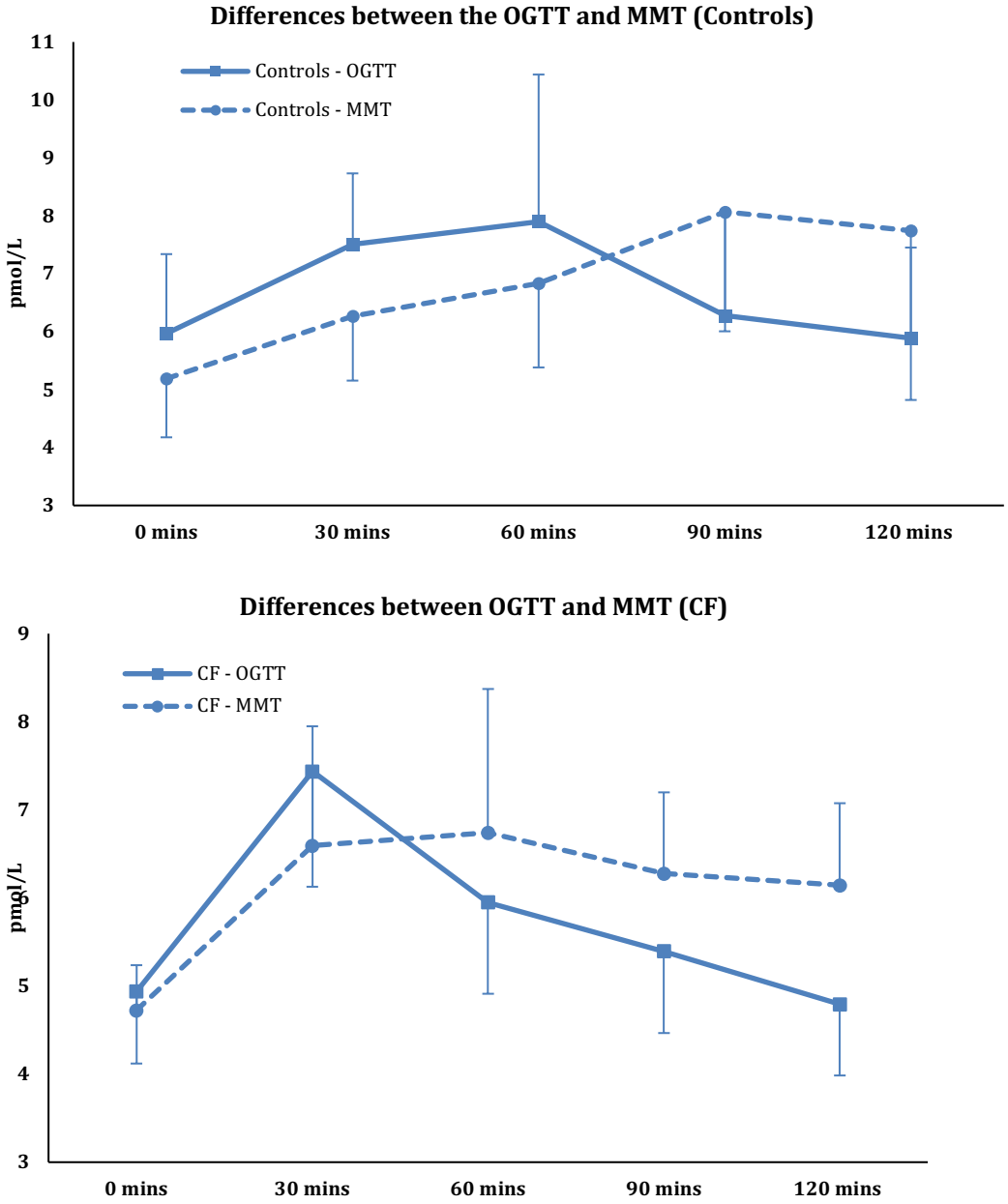
		0 mins	30 mins	60 mins	90 mins	120 mins
Controls	**OGTT**	6.0 ± 2.2	7.5 ± 2.0	7.9 ± 4.1	6.3 ± 2.9	5.9 ± 2.5
	MMT	5.2 ± 1.6	6.3 ± 1.8	6.8 ± 2.3	8.1 ± 3.3	7.7 ± 4.7
	p	0.13	0.1	0.51	0.06	0.29
CF	**OGTT**	4.9 ± 1.5	7.4 ± 2.4	5.9 ± 1.9	5.4 ± 1.7	4.8 ± 1.5
	MMT	4.7 ± 0.9	6.6 ± 2.5	6.7 ± 3.0	6.3 ± 1.7	6.1 ± 1.7
	p	0.54	0.27	0.48	0.27	0.08

Comparison of GLP_1 (pmol/L) responses to a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using the paired t-test.

Table 5-10: GLP_1 – Incremental and total responses

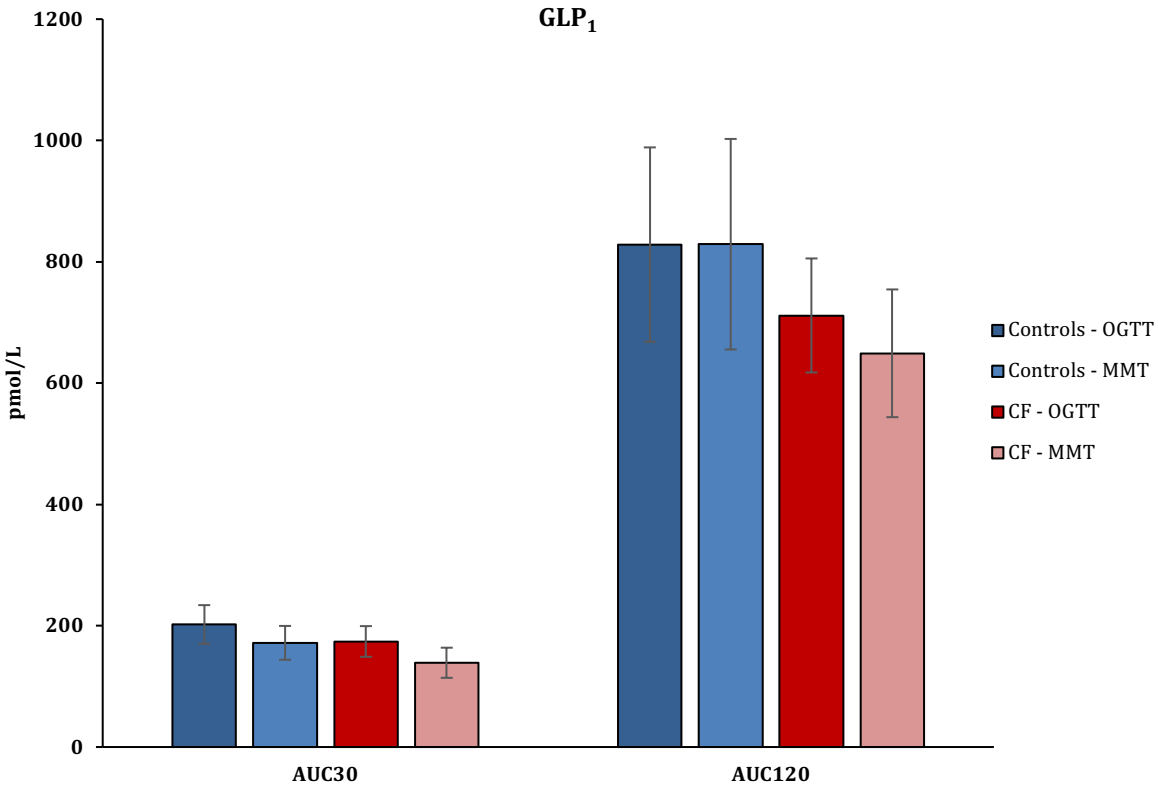
		AUC_{30}	AUC_{120}
Controls	**OGTT**	202 ± 51	828 ± 259
	MMT	172 ± 45	829 ± 280
	p	0.06	0.99
CF	**OGTT**	174 ± 45	712 ± 244
	MMT	139 ± 17	649 ± 100
	p	0.28	0.42

Comparison of the initial (AUC_{30}) and total (AUC_{120}) responses in GLP_1 (pmol/L) of a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using the paired t-test.



Comparison of GLP_1 (pmol/L) responses to a liquid meal (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) (top panel) and CF (n=13) subjects (bottom panel). [mean ± CI].

Figure 5-7: GLP_1 differences between OGTT and MMT



Comparison of the initial (AUC_{30}) and total (AUC_{120}) responses in GLP_1 (pmol/L) to a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± CI).

Figure 5-8: GLP_1 – Incremental and total responses

5.4.3.2 GIP

See Table 5-11, Table 5-12 on page 176 and Figure 5-9, Figure 5-10 on pages 177 and 178

A. Controls

With the control group, when the OGTT and MMT were compared, there were higher GIP levels after the MMT at 90 minutes [OGTT: 75.0 ± 35.6; MMT: 118.4 ± 44.3 pmol/L; p=0.02)] and 120-minutes [OGTT: 59.0 ± 31.6; MMT: 108.2 ± 50.1 pmol/L; p=0.005)]. There was no difference observed at the other time points.

When the incremental and total responses were compared, within the control group, there were no difference between the 2 tests for the AUC_{30} (p=0.52) and AUC_{120} (p=0.10).

B. Cystic Fibrosis

With the CF group, when the two tests were compared, there was lower GIP at 30 minutes after the MMT [OGTT: 101.2 ± 55.4; MMT 74.3 ± 38.1 pmol/L; p=0.04]. There were no differences observed at the other time points.

When the incremental and total responses were compared, within the CF group, there were no differences between the OGTT and MMT for both AUC_{30} (p=0.18) and AUC_{120} (p=0.96).

Table 5-11: GIP differences between OGTT and MMT

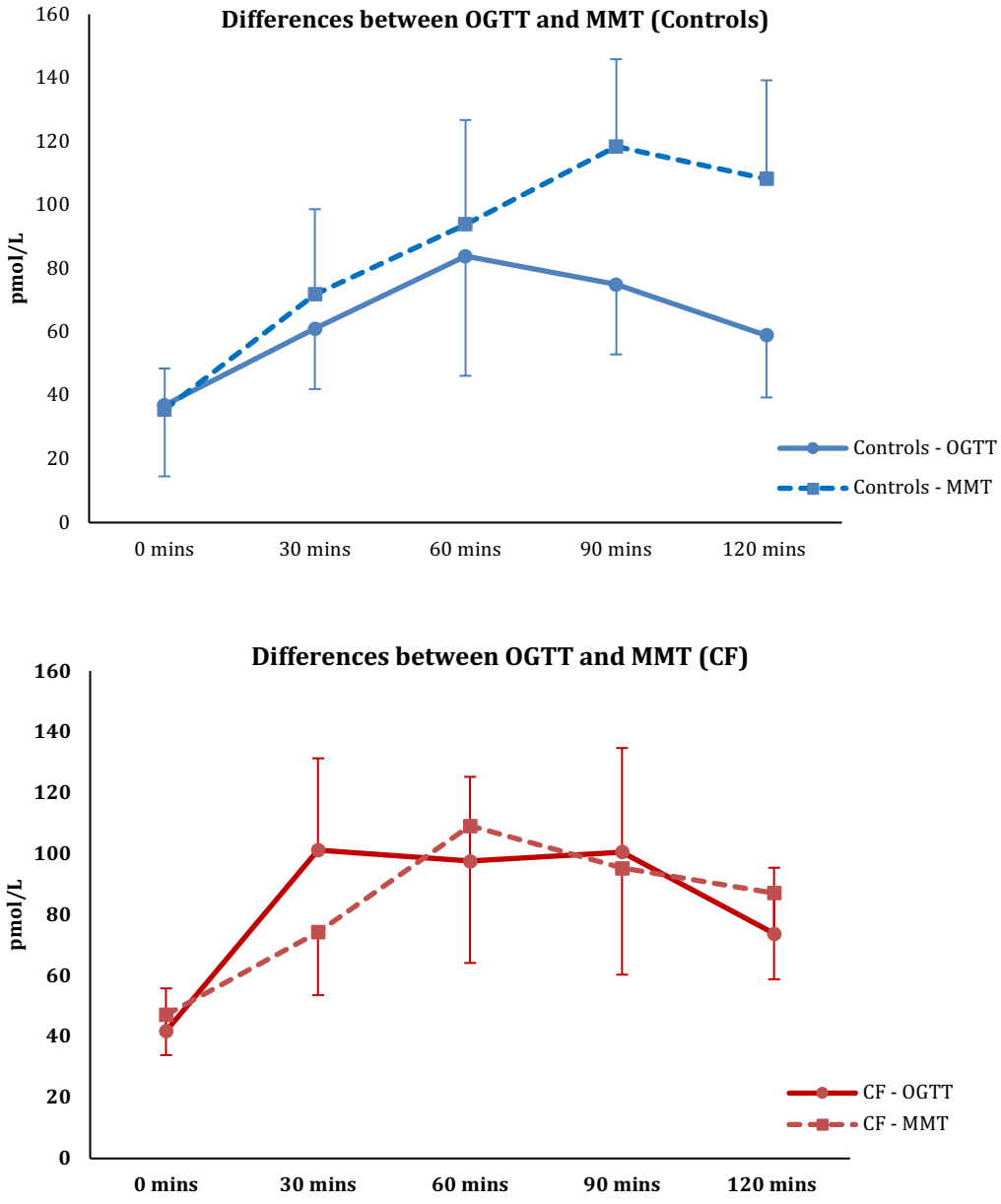
		0 mins	30 mins	60 mins	90 mins	120 mins
Controls	**OGTT**	36.9 ± 36.1	61.0 ± 30.6	83.9 ± 60.8	75.0 ± 35.6	59.0 ± 31.6
	MMT	35.6 ± 20.9	71.9 ± 43.2	93.9 ± 53.0	118.4 ± 44.3	108.2 ± 50.1
	p	0.82	0.37	0.61	0.02	0.005
CF	**OGTT**	41.8 ± 25.7	101.2 ± 55.4	97.5 ± 51.0	100.6 ± 62.8	73.7 ± 39.9
	MMT	47.2 ± 24.4	74.3 ± 38.1	109.2 ± 82.8	95.3 ± 64.4	87.1 ± 52.1
	p	0.38	0.04	0.55	0.78	0.31

Comparison of GIP (pmol/L) responses to a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using the paired t-test.

Table 5-12: GIP – Incremental and Total responses

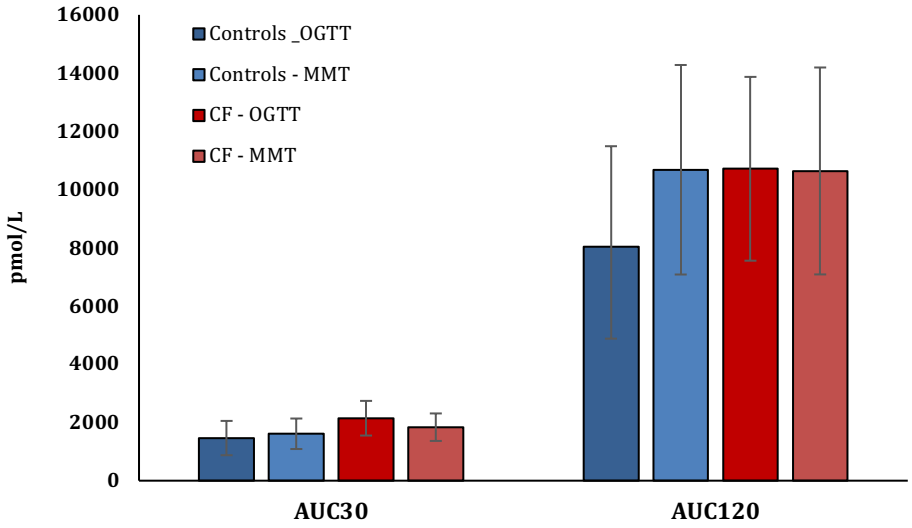
		AUC_{30}	AUC_{120}
Controls	**OGTT**	1469 ± 942	8035 ± 4628
	MMT	1611 ± 845	10680 ± 4699
	p	0.52	0.1
CF	**OGTT**	2145 ± 1095	10713 ± 5413
	MMT	1837 ± 869	110638 ± 6074
	p	0.18	0.96

Comparison of the initial (AUC_{30}) and total (AUC_{120}) GIP response to a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using the paired t-test.



Comparison of GIP (pmol/L) responses to a liquid meal (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) (top panel) and CF (n=13) subjects (bottom panel). [mean ± CI].

Figure 5-9: GIP differences between OGTT and MMT



Comparison of the initial (AUC_{30}) and total (AUC_{120}) responses in GIP (pmol/L) to a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± CI). For p-values see Table 5-12.

Figure 5-10: GIP – Incremental and total responses

5.4.3.3 PYY

See summary Table 5-13, Table 5-14 on page 180 and Figure 5-11, Figure 5-12 on pages 181 and 182

A. Controls

With the control group, when the OGTT and MMT were compared, there were no differences observed at all time points.

When the incremental and total responses were compared, within the control group, there were no difference between the 2 tests for the AUC_{30} ($p=0.59$) and AUC_{120} ($p=0.19$).

B. Cystic Fibrosis

As with the control group, in the CF group, when the OGTT and MMT were compared, there were no differences observed at all time points.

When the incremental and total responses were compared, within the CF group, there were no differences between the OGTT and MMT for both AUC_{30} ($p=0.23$) and AUC_{120} ($p=0.13$).

Table 5-13: PYY differences between OGTT and MMT

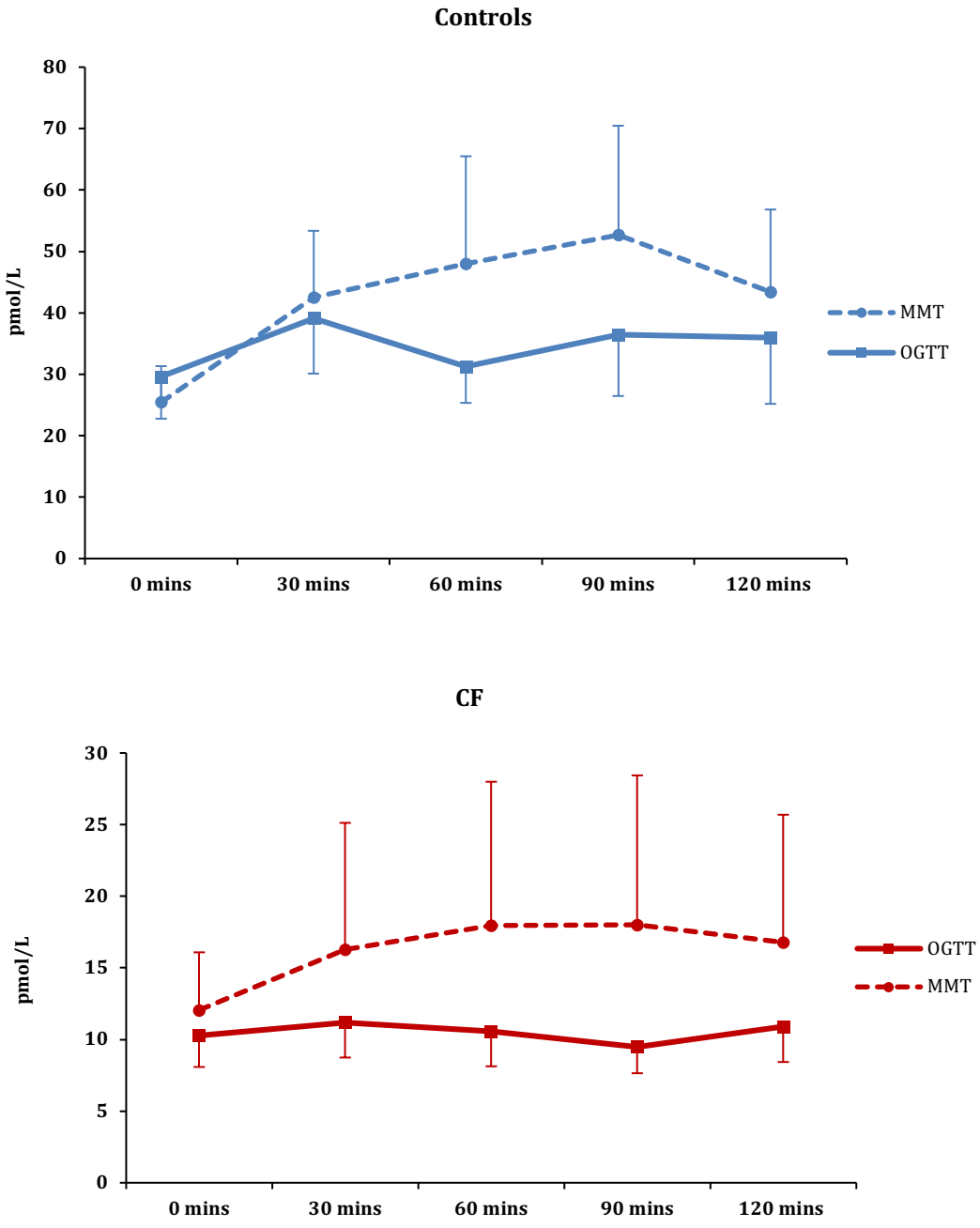
		0 mins	30 mins	60 mins	90 mins	120 mins
Controls	**OGTT**	29.6 ± 11.1	39.1 ± 14.5	31.3 ± 9.6	36.4 ± 16.1	35.9 ± 17.3
	MMT	25.5 ± 9.5	42.5 ± 17.5	48.0 ± 28.3	52.7 ± 28.7	43.4 ± 21.7
	p	0.28	0.56	0.1	0.14	0.35
CF	**OGTT**	10.3 ± 4.0	11.2 ± 4.5	10.6 ± 4.3	9.5 ± 3.4	10.9 ± 4.6
	MMT	12.1 ± 7.1	16.3 ± 16.3	18.0 ± 18.4	18.0 ± 19.2	16.8 ± 15.7
	p	0.36	0.26	0.15	0.11	0.2

Comparison of PYY (pmol/L) responses to a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using paired t-tests.

Table 5-14: PYY – Incremental and Total responses

		AUC_{30}	AUC_{120}
Controls	**OGTT**	1031 ± 326	4187 ± 1417
	MMT	1019 ± 374	5325 ± 2435
	p	0.59	0.19
CF	**OGTT**	322 ± 122	5325 ± 2435
	MMT	411 ± 267	2037 ± 1720
	p	0.23	0.13

Comparison of the initial (AUC_{30}) and total (AUC_{120}) responses in PYY (pmol/L) to a liquid and mixed meal over 120 minutes in control (n=10) and CF (n=13) subjects (mean ± SD). Statistical comparisons between tests were carried out using paired t-tests.



Comparison of PYY (pmol/L) responses to a liquid meal (OGTT) and mixed meal (MMT) over 120 minutes in control (n=10) (top panel) and CF (n=13) subjects (bottom panel). [mean ± CI].

Figure 5-11: PYY - Differences between OGTT and MMT

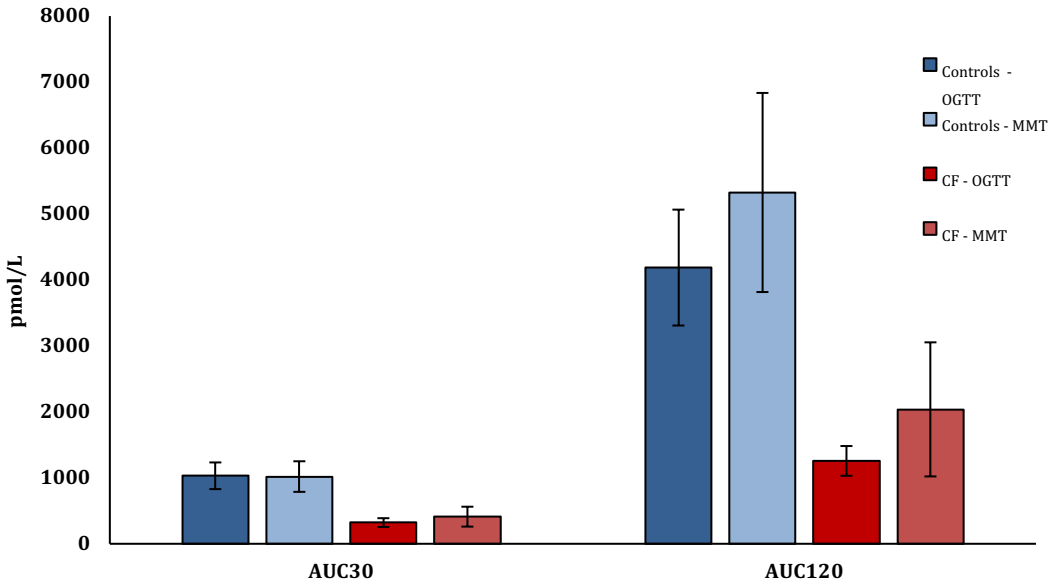


Figure 5-12: PYY - Incremental and total responses

5.4.4 Diurnal variation of GLP_1, GIP and PYY (MMT2 and MMT3)

As with gastric motility, glucose, insulin, c-peptide and glucagon, I compared changes in GLP_1, GIP and PYY through the day and evaluated the early response [30 minutes (AUC_{30})] and the total response [120-minutes (AUC_{120})].

5.4.4.1 GLP_1

See Table 5-15 , Table 5-16 on page 185 and Figure 5-13, Figure 5-14 on pages 186 and 187

A. Controls:

In the afternoon (MMT2), control subjects had a baseline level of 6.1 ± 1.4 pmol/L and a 120-minute level of 6.0 ± 1.1 pmol/L. 7 control subjects had a baseline below normal [Normal levels 7-36 pmol/L] and 8 subjects had 120-minute values lower than normal.

In the evening (MMT3), control subjects had a baseline level of 6.1 ± 2.0 pmol/L and a 120-minute level of 5.9 ± 1.8 pmol/L. Again, 7 control subjects had a baseline level below normal [Normal levels 7-36 pmol/L] and 7 subjects had 120-minute levels lower than normal.

In this group, there was no difference between times of the day with the corresponding time during the test. [Baseline: p=0.41, 30 minutes: p=0.17, 60 minutes: p=0.50, 90 minutes: p=0.49, 120-minutes: p=0.27).

When the incremental initial (AUC_{30}) and total (AUC_{120}) responses were evaluated, there were no differences within the group between the various tests [AUC_{30}: p=0.28; AUC_{120}: p=0.82].

B. Cystic Fibrosis

In the afternoon (MMT2), CF subjects had a baseline level of 5.2 ± 1.5 pmol/L and a 120-minute level of 6.6 ± 1.8 pmol/L. 7 CF subjects had a baseline level below normal [Normal

levels 7-36 pmol/L] and 8 subjects had low 120-minute levels. In 2 subjects, there was insufficient sample to carry out GLP_1 analysis at baseline.

In the evening (MMT3), CF subjects had a baseline level of 5.3 ± 1.3 pmol/L and a 120-minute level of 5.7 ± 1.7 pmol/L. 7 subjects had low baseline levels and 7 subjects had low 120-minute levels.

In CF subjects, there was no difference between times of the day with the corresponding time during the test. [Baseline: p=0.47, 30 minutes: p=0.62, 60 minutes: p=0.79, 90 minutes: p=0.97, 120-minutes: p=0.41).

When the incremental initial (AUC_{30}) and total (AUC_{120}) responses were evaluated, there was no difference within the control group between the various test [AUC_{30}: p=0.98; AUC_{120}: p=0.91]

C. Controls v Cystic Fibrosis

When the 2 groups were compared, there was no difference at the corresponding time during the test. [Afternoon: Baseline: p=0.17, 30 minutes: p=0.33, 60 minutes: p=0.83, 90 minutes: p=0.46, 120-minutes: p=0.34. Evening: Baseline: p=0.29, 30 minutes: p=0.28, 60 minutes: p=0.62, 90 minutes: p=0.91, 120-minutes: p=0.75).

Comparing the incremental responses between the groups, there was no difference in the AUC_{30} in the morning (p=0.98), afternoon (p=0.13) and evening (p=0.18). Similarly, there was no difference in the AUC_{120} between the 2 groups [Morning: p=0.55; Afternoon: p=044; Evening: p=0.68].

Table 5-15: GLP_1 levels through the day

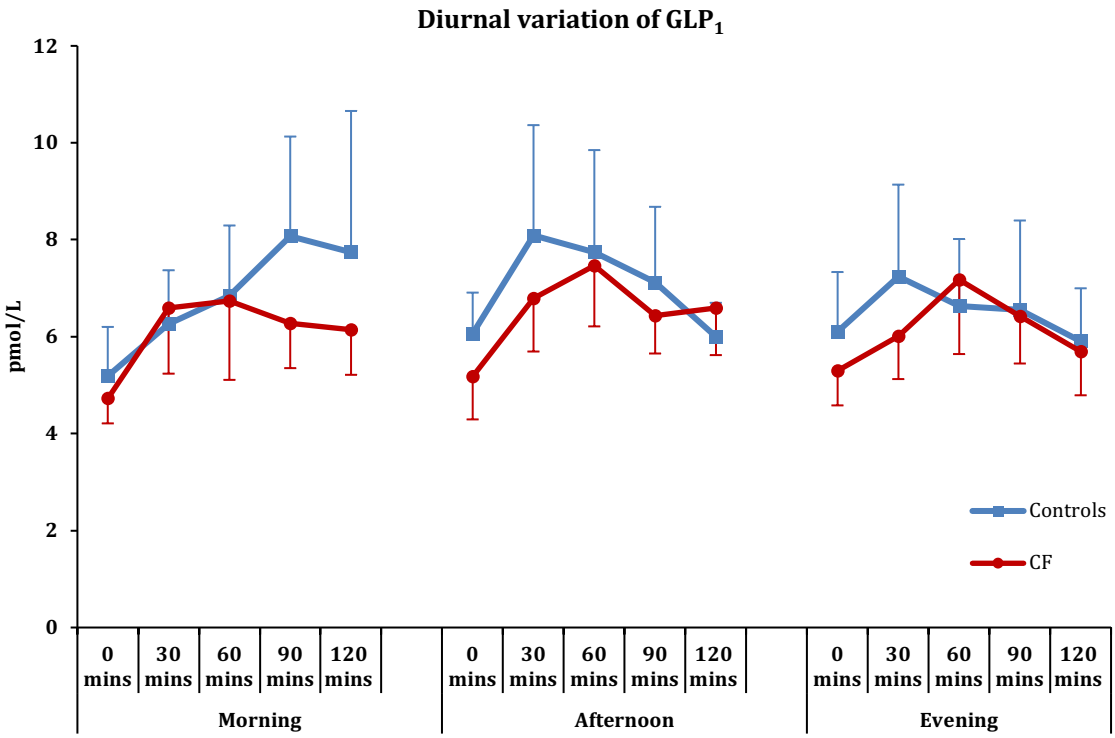
	Morning			Afternoon			Evening		
	Controls	**CF**	**p**	**Controls**	**CF**	**p**	**Controls**	**CF**	**p**
0 mins	5.2 ± 1.6	4.7 ± 0.9	0.38	6.1 ± 1.4	5.2 ± 1.5	0.17	6.1 ± 2.0	5.3 ± 1.3	0.29
30 mins	6.3 ± 1.8	6.6 ± 2.5	0.73	8.1 ± 3.7	6.8 ± 2.0	0.33	7.2 ± 3.1	6.0 ± 1.6	0.28
60 mins	6.8 ± 2.3	6.7 ± 3.0	0.93	7.7 ± 3.4	7.5 ± 2.3	0.83	6.6 ± 2.2	7.2 ± 2.8	0.62
90 mins	8.1 ± 3.3	6.3 ± 1.7	0.14	7.1 ± 2.5	6.4 ± 1.4	0.46	6.5 ± 3.0	6.4 ± 1.8	0.91
120 mins	7.7 ± 4.7	6.1 ± 1.7	0.33	6.0 ± 1.1	6.6 ± 1.8	0.34	5.9 ± 1.8	5.7 ± 1.7	0.75

Comparison between GLP_1 (pmol/L) responses over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-tests and a repeated measures ANOVA was performed to study differences within the group through the day. [There were no significant differences through the day in both groups].

Table 5-16: Incremental and total responses through the day

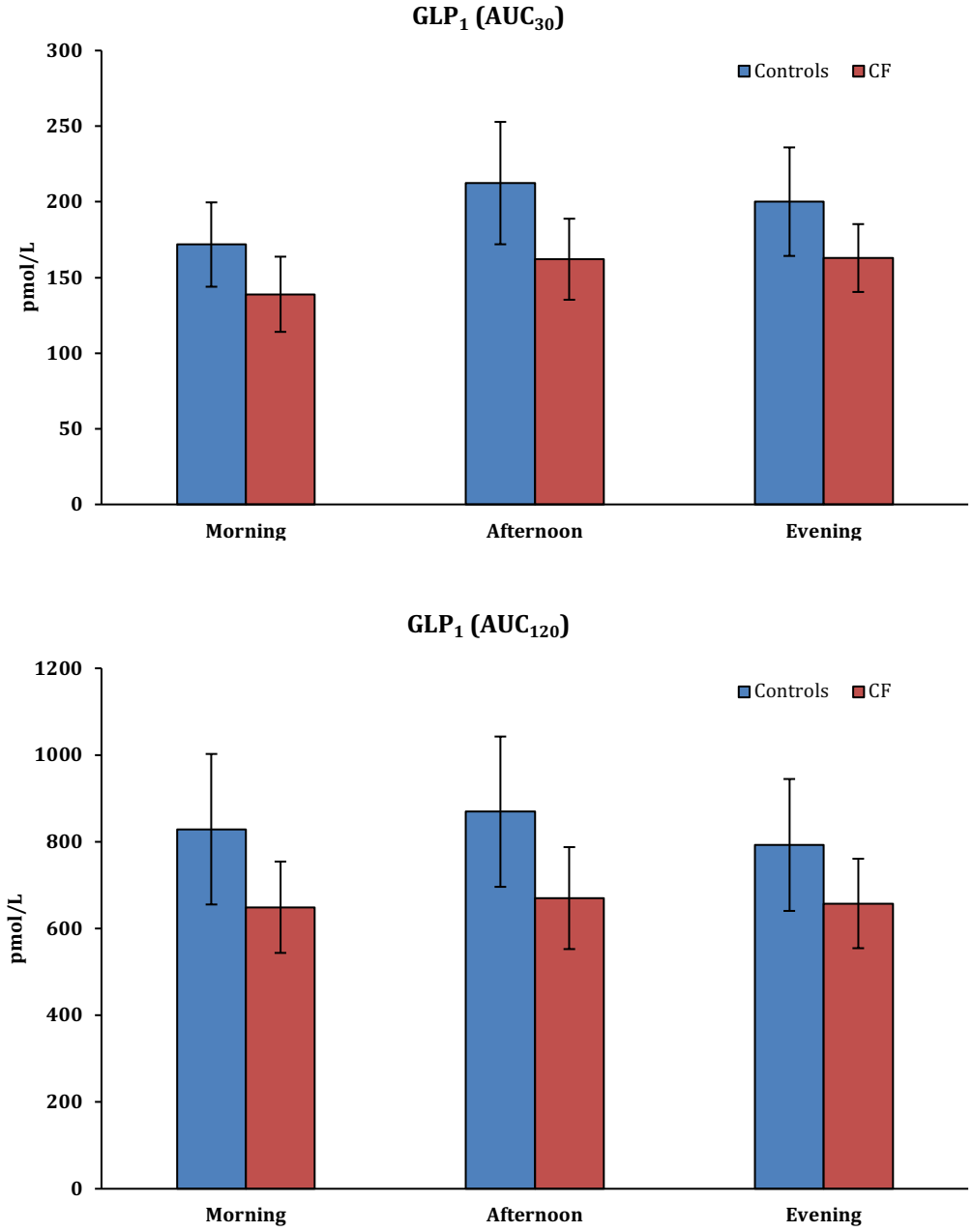
		Morning	Afternoon	Evening
AUC_{30}	**Controls**	172 ± 45	212 ± 65	200 ± 58
	CF	139 ± 17	162 ± 35	163 ± 36
	p	0.98	0.13	0.18
AUC_{120}	**Controls**	829 ± 280	869 ± 280	793 ± 245
	CF	649 ± 100	670 ± 91	658 ± 166
	p	0.55	0.44	0.68

Comparison between the initial (AUC_{30}) and total (AUC_{120}) GLP_1 (pmol/L) responses, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-tests and a repeated measures ANOVA was performed to study differences within the group through the day. [There were no significant differences through the day in both groups]



Comparison of GLP$_1$ (pmol/L) levels in response to a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening (mean ± CI). [For p-values see Table 5-15].

Figure 5-13: GLP$_1$ response through the day



Comparison of initial (AUC_{30}; top panel) and total (AUC_{120}; bottom panel) GLP_1 (pmol/L) response following a mixed meal (MMT) in the morning, afternoon and evening in control (n=10) and CF (n=13) subjects [mean ± CI].

Figure 5-14: GLP_1 – Incremental and total responses through the day

5.4.4.2 GIP

See Table 5-17, Table 5-18 on page 190 and Figure 5-15, Figure 5-16 on pages 191 and 192

A. Controls

In the afternoon (MMT2), 3 control subjects had concentrations < LOD at baseline. These data were censored for analysis and substituted with a constant value (LOD/√2).

At this time, subjects had a baseline GIP level of 74.2 ± 44.2 pmol/L and a 120-minute value of 99.9 ± 41.3 pmol/L.

In the evening (MMT3), only one subject had levels <LOD, at baseline. This data point was censored for analysis and substituted with a constant value (LOD/√2).

Subjects had a baseline level of 76.0 ± 50.5 pmol/L and a 120-minute value of 99.1 ± 40.6 pmol/L.

In this group, there was a diurnal variation observed at baseline (p=0.05) with the afternoon (p=0.01) and evening (p=0.01) values higher compared to the morning. At 30-minutes a diurnal variation was again observed (p=0.04) with the afternoon (p=0.01) and evening (p=0.001) values higher compared to the morning. There was no difference at 60 (p=0.38), 90 (p=0.98) and 120-minutes (p=0.88).

When the incremental initial (AUC_{30}) and total (AUC_{120}) responses were compared, there was a diurnal variation observed (p=0.02) in the AUC_{30} with the afternoon (p=0.002) and evening (p=0.0004) being higher than the morning. There was no difference with the AUC_{120} (p=0.85) between times of the day.

B. Cystic Fibrosis

In the afternoon (MMT2), 3 subjects had concentrations <LOD at baseline, one at 30 minutes and 90 minutes, one at 30 minutes, one at 90 and 120-minutes and one at 120-minutes. These data were censored for analysis and substituted with a constant value (LOD/√2).

At this time of the day, these subjects had a baseline level of 70.6 ± 38.8 pmol/L and a 120-minute value of 115.3 ± 85.4 pmol/L.

In the evening (MMT3), subjects had a baseline level of 74.2 ± 47.3 pmol/L and a 120-minute value of 113.7 ± 73.2 pmol/L. Four subjects had concentrations <LOD at baseline. Of these, one had concentrations <LOD at 30 minutes and 90 minutes and two at 120-minutes. These data were censored for analysis and substituted with a constant value (LOD/$\sqrt{2}$).

In CF subjects, there was no difference between times of the day with the corresponding time during the test [Baseline: p=0.16, 30 minutes: p=0.35, 60 minutes: p=0.63, 90 minutes: p=0.31, 120-minutes: p=0.53).

When the incremental initial (AUC_{30}) and total (AUC_{120}) responses were evaluated, there was no difference within the CF group between the various test [AUC_{30}: p=0.22; AUC_{120}: p=0.40].

C. Controls v Cystic Fibrosis

When the 2 groups were compared, there was no difference between the groups at the corresponding time during the test. (Afternoon: Baseline: p=0.84, 30 minutes: p=0.66, 60 minutes: p=0.59, 90 minutes: p=0.85, 120-minutes: p=0.39. Evening: Baseline: p=0.94, 30 minutes: p=0.17, 60 minutes: p=0.76, 90 minutes: p=0.47, 120-minutes: p=0.57).

Comparing the incremental responses between the groups, there was no difference in the AUC_{30} in the morning (p=0.54), afternoon (p=0.72) and evening (p=0.38). Similarly, there was no difference in the AUC_{120} between the 2 groups [Morning: p=0.98; Afternoon: p=0.72; Evening: p=0.38].

Table 5-17: GIP levels through the day

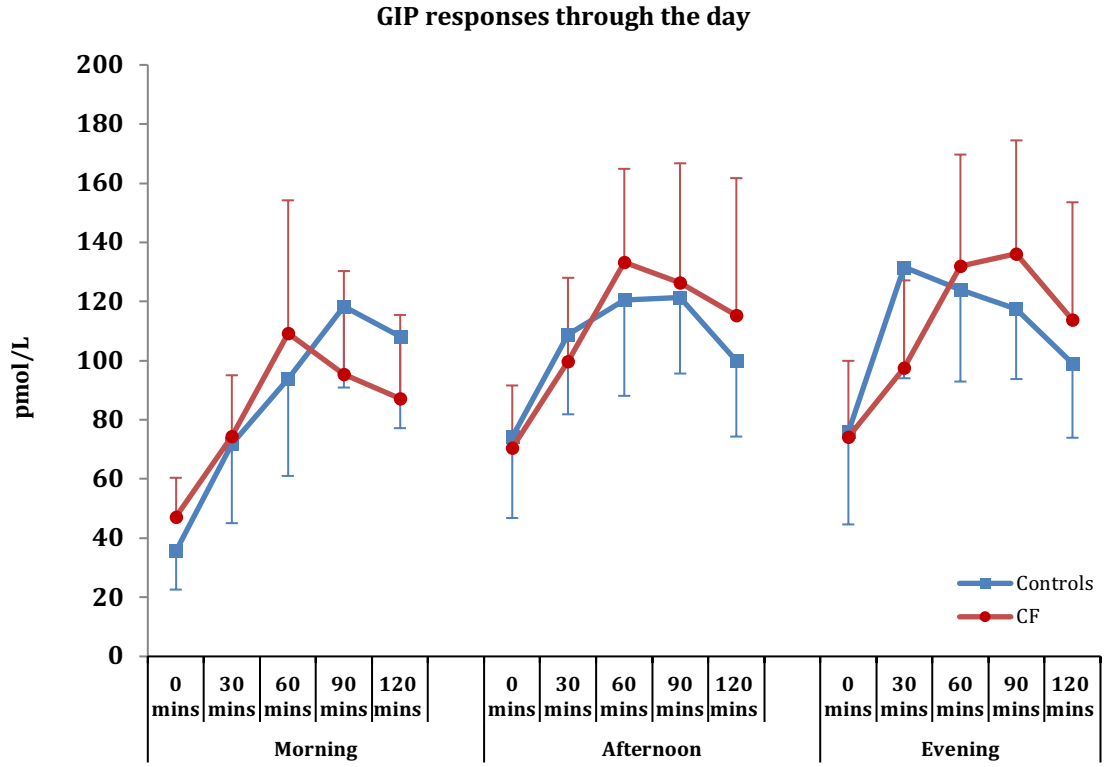
	Morning			Afternoon			Evening		
	Controls	**CF**	**p**	**Controls**	**CF**	**p**	**Controls**	**CF**	**p**
0 mins	35.6 ± 20.9	47.2 ± 24.4	0.24	74.2 ± 44.2 *	70.6 ± 38.8	0.84	76.0 ± 50.5 *	74.2 ± 47.3	0.94
30 mins	71.9 ± 43.2	74.3 ± 38.1	0.90	108.8 ± 43.4 ^	99.7 ± 52.0	0.66	131.6 ± 60.6	95.7 ± 54.5 £	0.17
60 mins	93.9 ± 53.0	109.2 ± 82.8	0.61	120.4 ± 52.1	133.3 ± 58.1	0.59	124.0 ± 50.1	132.0 ± 69.3	0.76
90 mins	118.4 ± 44.3	95.3 ± 64.4	0.34	121.3 ± 41.4	126.3 ± 74.4	0.85	117.5 ± 38.3	136.1 ± 70.5	0.47
120 mins	108.2 ± 50.1	87.1 ± 52.1	0.34	99.9 ± 41.3	115.3 ± 85.4	0.39	99.1 ± 40.6	113.7 ± 73.2	0.57

*Comparison between GIP (pmol/L) responses over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-tests and a repeated measures ANOVA was performed to study differences within the group through the day [Compared to morning: * p=0.01, ^ p=0.01, £ p=0.001]*

Table 5-18: GIP - Incremental and Total responses through the day

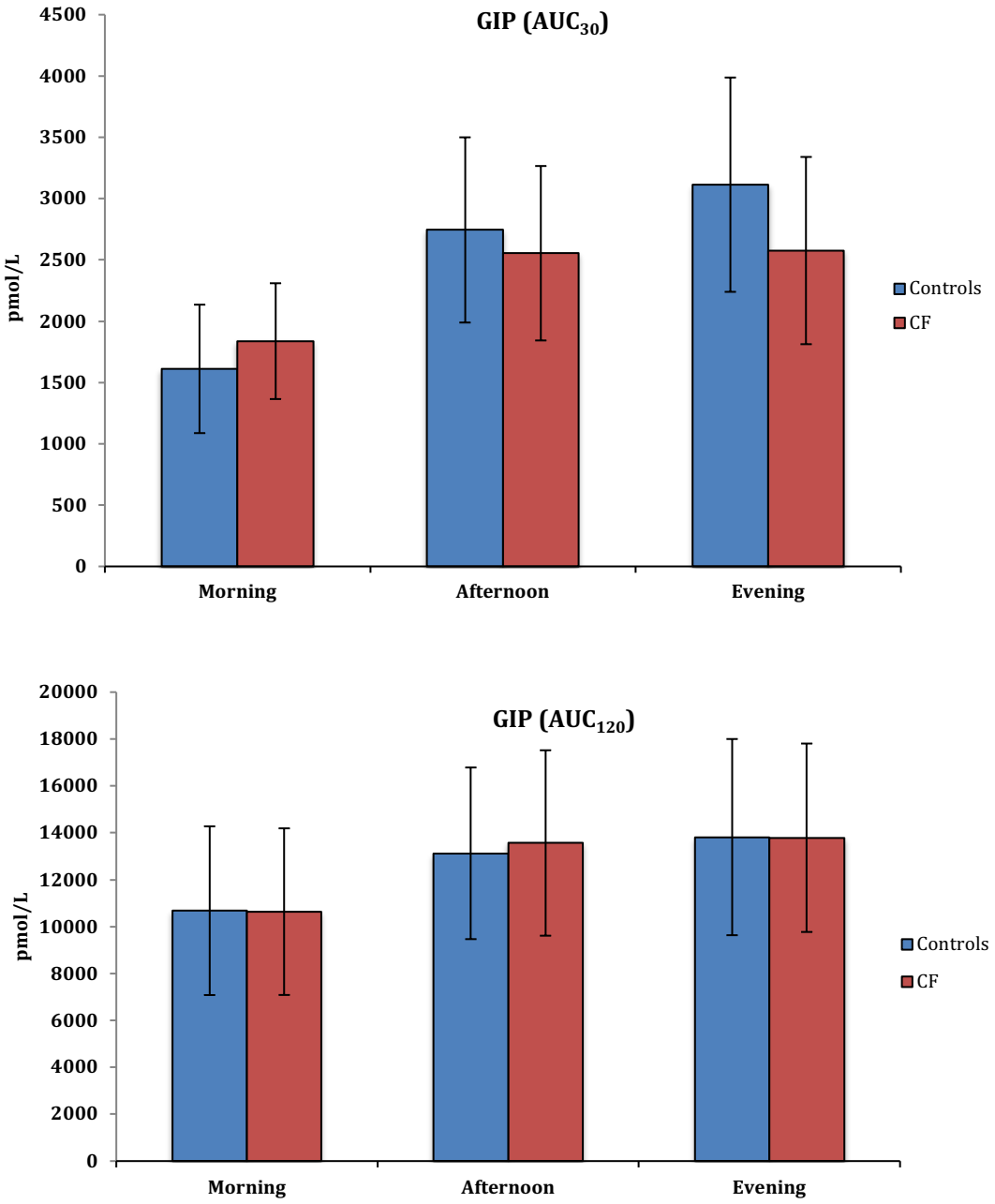
		Morning	Afternoon	Evening
AUC_{30}	**Controls**	1611 ± 845	2744 ± 1219 *	3113 ± 1410 ^
	CF	1837 ±869	2554 ± 1309	2576 ± 1405
	p	0.54	0.72	0.38
AUC_{120}	**Controls**	10680 ± 4699	13126 ± 4683	13817 ± 4843
	CF	10638 ± 6074	13566 ± 6750	13790 ± 6823
	p	0.98	0.86	0.99

*Comparison between the initial (AUC_{30}) and total (AUC_{120}) GIP (pmol/L) response, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-tests and a repeated measures ANOVA was performed to study differences within the group through the day. [Compared to morning: * p=0.002, ^ p=0.0004]*



Comparison of GIP (pmol/L) responses to a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening (mean ± CI). [For p-values see Table 5-17)

Figure 5-15: GIP responses through the day



Comparison of initial (AUC_{30}; top panel) and total (AUC_{120}; bottom panel) GIP (pmol/L) responses following a mixed meal (MMT) in the morning, afternoon and evening in control (n=10) subjects and CF (n=13) subjects [mean ± CI].

Figure 5-16: GIP – Incremental and Total responses through the day

5.4.4.3 PYY

See Table 5-19 and Table 5-20 on page 195 and Figure 5-17, Figure 5-18 on pages 196 and 197

A. Controls

In the afternoon (MMT2), one control subject had concentrations <LOD (10 pmol/L) at all-time points. These data points were censored for analysis and substituted with a constant value (LOD/√2).

At this time, subjects had a baseline level of 43.7 ± 23.2 pmol/L and a 120-minute value of 56.1 ± 24.0 pmol/L.

In the evening (MMT3), the same subject had levels <LOD, at baseline. This data point was censored for analysis and substituted with a constant value (LOD/√2).

Subjects had a baseline level of 43.3 ± 22.3 pmol/L and a 120-minute value of 61.8 ± 37.3 pmol/L.

In this group, although the afternoon and evening baseline levels were higher than the morning, this variation was not statistically significant (p=0.06).There was no differences at 30 (p=0.31) 60 (p=0.382), 90 (p=0.84) and 120-minutes (p=0.34).

When the incremental initial (AUC_{30}) and total (AUC_{120}) responses were compared, there was no diurnal variation observed (p=0.14) in the AUC_{30} or the AUC_{120} (p=0.40).

B. Cystic Fibrosis

In the afternoon (MMT2), 4 subjects had concentrations <LOD at baseline. Of these one had levels <LOD at all time points, two at 60 minutes, one at 60 and 120-minutes. One subject had detectable levels at baseline but levels <LOD at 30, 60 and 90 minutes.

These data were censored for analysis and substituted with a constant value (LOD/√2).

At this time of the day, these subjects had a baseline level of 18.6 ± 17.6 pmol/L and a 120-minute value of 21.2 ± 20 pmol/L.

In the evening (MMT3), subjects had a baseline PYY level of 17.2 ± 13.9 pmol/L and a 120-minute value of 26.1 ± 30.6 pmol/L. Five subjects had concentrations <LOD at baseline. Of these, two had concentrations <LOD at all time points and one at 30 and 120-minutes. One subject had levels <LOD at 30, 60, 90 and 120-minutes and one at 60, 90 and 120-minutes. These data were censored for analysis and substituted with a constant value (LOD/$\sqrt{2}$).

In CF subjects, there was no difference between times of the day with the corresponding time during the test. [Baseline: p=0.46, 30 minutes: p=0.47, 60 minutes: p=0.94, 90 minutes: p=0.76, 120-minutes: p=0.62).

When the incremental initial (AUC_{30}) and total (AUC_{120}) responses were evaluated, there was no difference within the CF group between the various test [AUC_{30}: p=0.39; AUC_{120}: p=0.50].

C. Controls v Cystic Fibrosis

When the 2 groups were compared, CF subjects had lower PYY levels at all time points during the test. (Afternoon: Baseline: p=0.01, 30 minutes: p=0.02, 60 minutes: p=0.006, 90 minutes: p=0.01, 120-minutes: p=0.002. Evening: Baseline: p=0.006, 30 minutes: p=0.02, 60 minutes: p=0.003, 90 minutes: p=0.007, 120-minutes: p=0.02).

Comparing the incremental responses between the groups, CF subjects had lower AUC_{30} in the morning (p=0.0002), afternoon (p=0.01) and evening (p=0.007). Similarly, they had lower AUC_{120} in the morning: p=0.002, afternoon: p=0.02 and evening: p=0.007.

Table 5-19: PYY (pmol/L) levels through the day

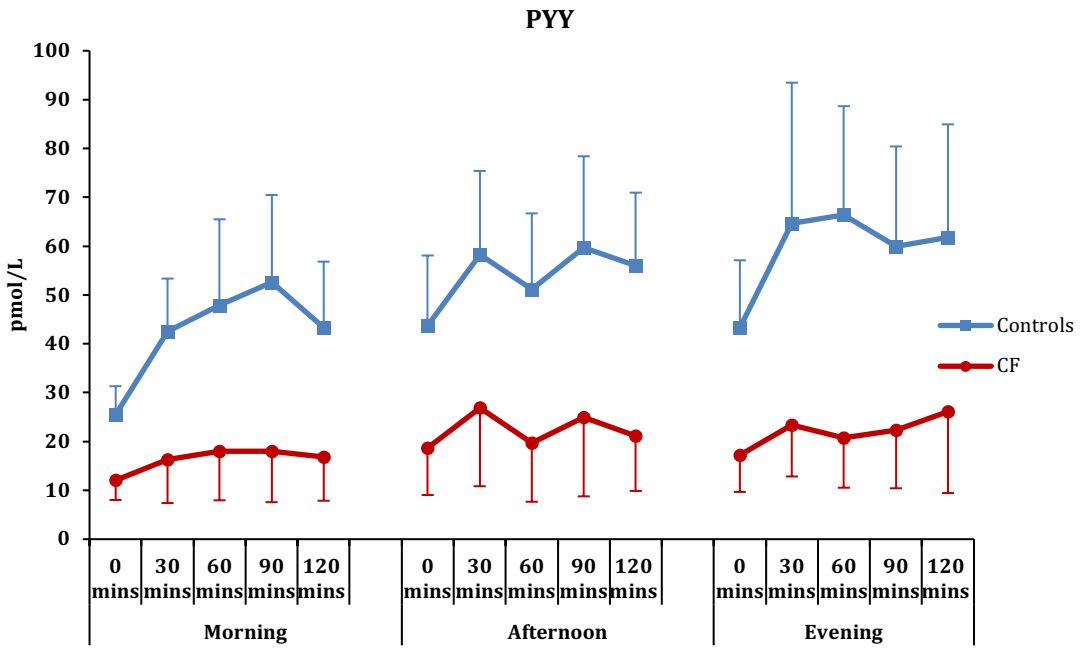
	Morning			Afternoon			Evening		
	Controls	**CF**	**p**	**Controls**	**CF**	**p**	**Controls**	**CF**	**p**
0 mins	25.5 ± 9.5	12.1 ± 7.1	0.001	43.7 ± 23.2	18.6 ± 17.6	0.01	43.3 ± 22.3	17.2 ± 13.9	0.006
30 mins	42.5 ± 17.5	16.3 ± 16.3	0.001	58.3 ± 27.6	26.9 ± 29.5	0.02	64.7 ± 46.4	23.4 ± 19.5	0.02
60 mins	48.0 ± 28.3	18.0 ± 18.4	0.01	51.1 ± 25.2	19.7 ± 22.1	0.006	66.4 ± 35.9	20.8 ± 18.9	0.003
90 mins	52.7 ± 28.7	18.0 ± 19.2	0.005	59.7 ± 30.1	24.9 ± 29.7	0.01	60.0 ± 32.9	22.3 ± 21.9	0.007
120 mins	43.4 ± 21.7	16.8 ± 15.7	0.006	56.1 ± 24.0	21.2 ± 20.0	0.002	61.8 ± 37.3	26.1 ± 30.6	0.02

Comparison between PYY (pmol/L) responses over 120 minutes, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-tests and a repeated measures ANOVA was performed to study differences within the group through the day. [There were no significant differences in the groups]

Table 5-20: Incremental and Total responses through the day

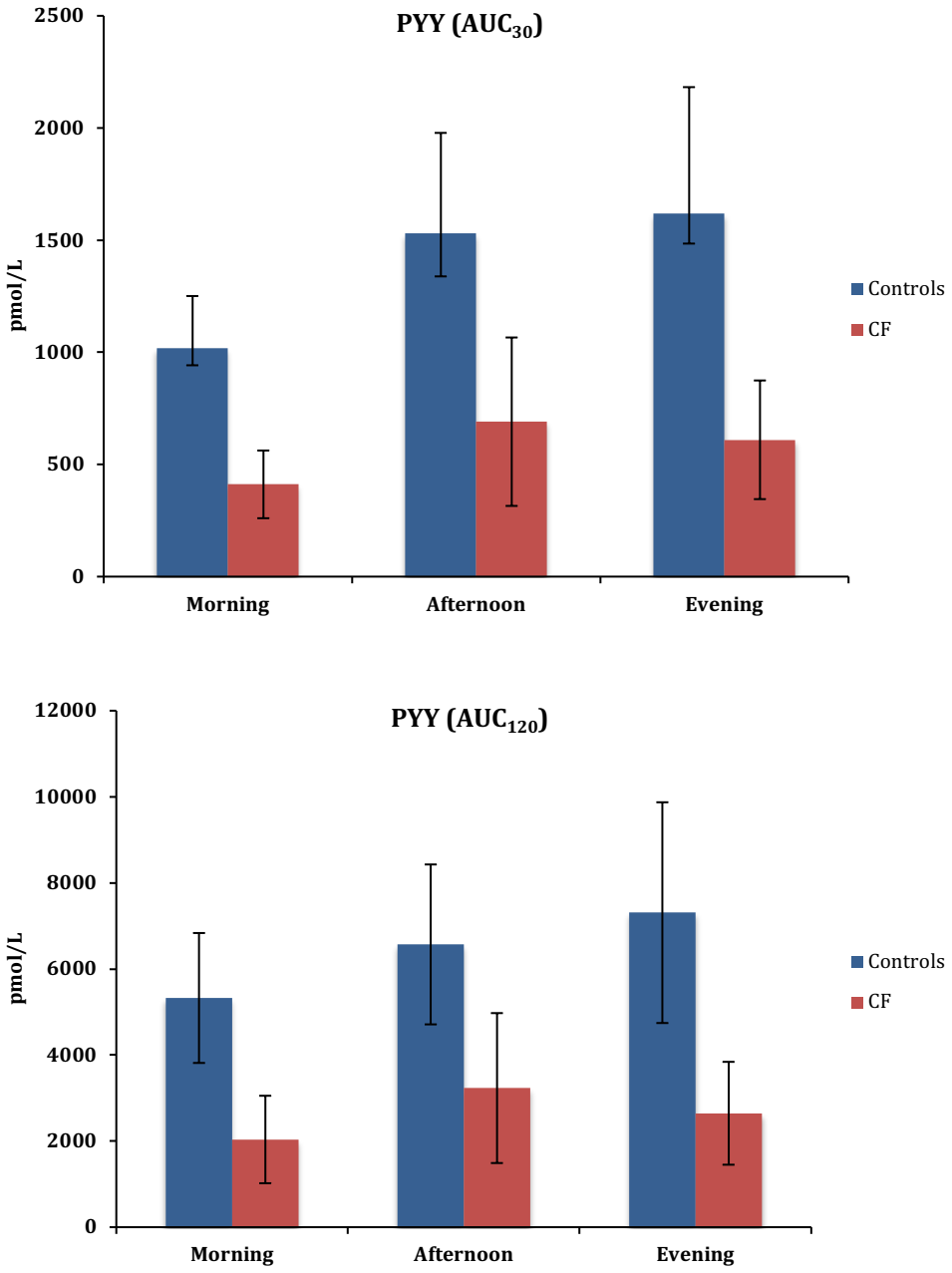
		Morning	Afternoon	Evening
AUC_{30}	**Controls**	1019 ± 374	1530 ± 723	1620 ± 908
	CF	411 ± 267	691 ± 691	610 ± 487
	p	0.002	0.01	0.007
AUC_{120}	**Controls**	5325 ± 2435	6570 ± 2997	7308 ± 4137
	CF	2037 ± 1720	3232 ± 3079	2647 ± 2199
	p	0.002	0.02	0.007

Comparison between the initial (AUC_{30}) and total (AUC_{120}) PYY (pmol/L) response, following the mixed meal in the morning, afternoon and evening (Mean ± SD). Statistical comparisons between control (n=10) and CF (n=13) subjects at each time point were carried out using independent t-test and a repeated measures ANOVA was performed to study differences within the group through the day. [There were no significant differences in the groups]



Comparison of GIP (pmol/L) levels in response to a mixed meal (MMT) over 120 minutes between control (n=10) and CF (n=13) subjects in the morning, afternoon and evening (mean ± CI). [For p-values see Table 5-19*)*

Figure 5-17: PYY release through the day



Comparison of initial (AUC_{30}; top panel) and total (AUC_{120}; bottom panel) PYY (pmol/L) responses following a mixed meal (MMT) in the morning, afternoon and evening in control (n=10) and CF (n=13) subjects [mean ± CI].

Figure 5-18: PYY – Incremental and total responses through the day

5.4.5 Experiment 3 summary

5.4.5.1 OGTT:

A. GLP_1

- There were low baseline and 120-minute values seen in both groups.
- There was no difference between the two groups at any time or with the initial (AUC_{30}) and total (AUC_{120}) responses.

B. GIP

- At 30-minutes, CF subjects had higher GIP levels than control subjects
- There was no difference between the two groups at any time or with the initial (AUC_{30}) and total (AUC_{120}) responses.

C. PYY

- CF subjects had lower levels at all time points.
- CF subjects had lower initial (AUC_{30}) and total (AUC_{120}) responses when compared.

5.4.5.2 MMT:

A. GLP_1

- All CF subjects had low baseline levels.
- Only 3 CF subjects had normal 120-minute levels
- There was no difference between the two groups at any time or with the initial (AUC_{30}) and total (AUC_{120}) responses.

B. GIP

- There was no difference between the two groups at any time or with the initial (AUC_{30}) and total (AUC_{120}) responses.

C. PYY

- CF subjects had lower levels at all time points,
- CF subjects had lower initial (AUC_{30}) and total (AUC_{120}) responses when compared to control subjects.

5.4.5.3 Difference between OGTT and MMT:

A. GLP_1

- There were no statistically significant differences between the two tests.

B. GIP

- In control subjects, there were higher GIP levels after the MMT at 90 and 120-minutes.
- In CF subjects, there were lower levels at 30-minutes following the MMT.
- There were no differences between the two tests with the initial (AUC_{30}) and total (AUC_{120}) responses.

C. PYY

- There were no differences between the two tests at the various times and with the initial (AUC_{30}) and total (AUC_{120}) responses.

5.4.5.4 Diurnal variation:

A. GLP_1

- There was no diurnal variation observed in both control and CF subjects.
- There was no difference between the controls and CF subjects at the different times of the day.

B. GIP

- In control subjects, there were higher GIP levels at baseline and 30 minutes in the afternoon and evening compared to the morning.
- In the control group, the initial (AUC_{30}) response in the afternoon and evening was higher than the morning.
- In CF subjects, there was no diurnal variation and there was no difference when compared to control subjects at various time points during the day.

C. PYY

- In control subjects, there was no statistically significant diurnal variation.
- In CF subjects, there was no diurnal variation observed, but there were lower PYY levels at all times of the day, compared with controls.

5.5 Discussion

In experiment 2, I have shown that in CF subjects without frank diabetes, there is deficient glucose handling throughout the OGTT and early morning MMT. I have also shown that fasting and total insulin secretion is similar in CF and healthy subjects, although healthy subjects achieve an earlier peak level. The experiment reiterated a β cell defect, as CF subjects had lower 30 and 60-minute c-peptide levels and took a longer time to reach a peak level. By evaluating α cell activity, I have shown that glucagon does not contribute to the increased glucose levels in CF patients with NGT, possibly explaining why abnormal fasting plasma glucose is a late occurrence in CF and why the OGTT is a poor test in CF.

By studying diurnal variation, I have shown that glucose handling in CF patients is variable throughout the day, with the 120-minute value in the evening deserving further attention since it may be a better indicator of glucose intolerance later in the day. Interestingly, I found that in the CF group glucose handling improved in the afternoon, with these subjects insulinopaenic at different times of the day, with the lowest insulin level in the afternoon and the longest time taken to reach a peak insulin level in the evening, suggesting β cells working harder at this time of the day.

It is also possible to attribute the loss of glucose tolerance to a diminished pancreatic response to a glycaemic stimulus. Dietary stimuli other than glucose affect insulin secretion via the incretin system.

To explain the changes I observed in experiment 2, I set out to evaluate the impact of the incretins GLP_1 and GIP and also PYY on glucose handling throughout the day.

In this chapter (experiment 3), I have shown that in fasting CF subjects without frank diabetes, there was no difference in GLP_1 secretion when compared to healthy control subjects following a liquid or a mixed meal. Although CF subjects had low baseline levels, this was similar to the control group and has been demonstrated before [280, 281]. Hillman et al [281] have previously shown that total GLP_1 did not differ between healthy controls and CF subjects with and without diabetes. However, in their study active GLP_1 was lower in CF

individuals, indicating that the inactive form of GLP_1 was more pronounced in the CF group. It is thought that individuals with CF, irrespective of glycaemic status, hypersecrete total GLP_1 to achieve similar levels of the active hormone seen in matched controls suggesting that the CF condition is associated with higher levels of incretin degradation even in stable non-diabetic individuals [280].

When the OGTT and MMT were compared, control subjects tended to have higher GLP_1 levels at 90-minutes after the MMT and a higher initial response to the MMT. In contrast, CF subjects tended to have higher levels at 120-minutes after the MMT, suggesting a later peak in the CF group. Although the total amount secreted was no different between the groups, it is possible that those with CF have this delayed response due to decreased gastric motility (as I demonstrated in experiment 1) and the delayed presentation of food substrate to the duodenum where GLP_1 is secreted.

Traditionally, is it thought that GLP_1 secretion rises early, starting 10-15 minutes after ingestion of food, peaking during the second hour, then slowly declining to baseline over the next two hours [164, 282]. However, this mono-phasic response is disputed, with some studies suggesting a "biphasic" pattern, where an early peak takes place within a few minutes after nutrient ingestion and lasts for approximately 30-60 min, followed by a nadir and then a second rise in GLP_1 which continues for up to 3 hours [283-285].

In my experiment, a monophasic pattern was observed, with levels back to baseline at 120-minutes. One reason for this response could be explained by the meal size and meal composition that I used in this study, since the levels of GLP_1 secretion are known to change with the intake of different nutrients [286].

Only two studies have previously looked at diurnal rhythms of plasma GLP_1 levels, and these were in non-CF individuals. Munoz et al [287] did not demonstrate a diurnal variation in healthy subjects but demonstrated a disruption in GLP_1 secretion in overweight and obese subjects. Brubaker et al [288] evaluated the secretory patterns of GLP_1 following short-term sleep deprivation with nocturnal light exposure. They demonstrated a diurnal variation in GLP_1 responses to identical meals (850 kcal), served 12-hours apart in normal dark and light

periods and suggested the existence of a daily pattern of activity GLP_1 secretion. I did not demonstrate a diurnal variation in GLP_1 secretion in normal subjects: however, these two studies are not comparable to my experiment, since meal sizes and constituents were different, my subjects were not over-weight or obese and were well rested prior to the experiment. GLP_1 secretory patterns throughout the day have not been evaluated in CF subjects before and my experiment addressed this: I was unable to find any diurnal variation. Thus, it is unlikely that GLP_1 contributes to the changes in glucose handing through the day seen in my study population.

With regards to GIP, I have shown that although there are no differences in the total GIP secreted following the OGTT, CF subjects had higher levels at 30-minutes compared to controls. Hypersecretion of GIP in non-diabetic children with CF was shown in 1981 by Ross and colleagues [205]. Despite apparently normal glucose handling, in that study, CF children demonstrated significant insulinopenia compared to the normal control subjects supporting the hypothesis that insulin exerts feedback control on GIP secretion. Subsequent to my study, Jones et al [280] showed using an isoglycaemic clamp the incretin effect is lost in those with CFRD and although incretin hormones are produced appropriately there is a failure of β cells to respond. My study evaluated non-diabetic CF individuals and the hypersecretion of GIP demonstrated can be explained by the effect of the gut that would not occur with isoglycaemic clamping.

As regards the MMT, my study showed no difference between the control and CF groups, suggesting an intact and appropriate GIP response. This differs from the study by Kuo et al [206] where CF subjects were found to have abnormally low postprandial stimulation of GIP, but in that work the level was normalised with pancreatic supplementation. In my study, subjects had their usual pancreatic supplements (Creon®) with the mixed meal correcting for the lack of pancreatic enzyme. Although my study differs in methodology from the study of Kuo, my results concur that GIP secretion is normal in those with CF without diabetes.

More recently, Sheikh and colleagues [256] showed that in a group of CF pancreatic insufficient subjects there was less GIP produced over a 4-hour period when compared to pancreatic sufficient CF subjects. However, they found no difference in GIP secretion

between healthy control subjects and those CF subjects with pancreatic insufficiency, similar to my results.

In my study, towards the latter part of the test, control subjects had higher GIP levels after the MMT compared to the OGTT. A number of studies have compared postprandial metabolic responses between OGTTs and MMTs in healthy subjects.

GIP may be higher after the MMT due to a number of reasons. The incretin effect itself is a major contributor [164] to gastric emptying that is more rapid after the OGTT than after a solid meal, and the proteins and fats present in a mixed meal delay gastrointestinal glucose absorption, leading to an attenuated postprandial glucose gradient [289, 290]. While my study responses are similar to those in other studies, once again comparisons between studies of metabolic responses to meal tests are limited by the differences in contents and sizes of meals [290-293].

No study has previously looked at the differences in response to a liquid and a mixed meal in the CF population. The initial (30-minute) lower GIP response in CF subjects after the MMT compared to the OGTT was unexpected and has not been described before. One explanation is that the gut-sensing or GIP-release patterns to a mixed meal and other nutritional constituents are defective in CF. Also, the glycaemic index of the mixed meal is less than that of the OGTT, resulting in lower GIP stimulation. Furthermore, individuals with CF are advised to consume at least 3000 calories/day, most of which will have a high glycaemic index, and this may condition the GIP response.

As regards the diurnal variation in secretion, the only study [294] previously evaluating GIP responses through the day looked at 12 healthy male subjects who consumed a standard breakfast at 0800 hrs and 1700 hrs on the same day. The authors found that the early release of GIP was more pronounced in the morning: in my study control subjects had higher baseline and 30-minute levels in the afternoon and evening - i.e. - the early response. These two studies, however, are not directly comparable: Lindgren fasted subjects for 11 hours between tests, which is not a normal meal ingestion pattern, whereas in my study meals were provided at more physiological intervals (5 hours).

It is also possible that a period of prolonged fasting between the two tests alters the cephalic phase of digestion [282], impacting on insulin release and its interaction with GIP. This is yet to be studied.

For the first time my study has looked at the diurnal variation of GIP in CF. I have shown that there is no diurnal variation in GIP secretion in CF and there were no differences in comparison to control subjects. I did not find higher responses in the afternoon and evening in this group similar to the control group suggesting an intact and appropriate GIP response. This was similar to my findings comparing the 2 groups during MMT1 (the morning MMT).

Despite my findings, there are a number of possible reasons why diurnal variation might be expected – variation in autonomic nervous activity, changes in gastric motility (as shown in experiment 1) or changes in growth hormone and cortisol levels through the day, all known to be present in CF subjects. There is also the possibility of a diurnal variation in K and L cell activity and an alteration in DPP-4 activity due to neural effect (which does not occur in healthy subjects).

Finally, I studied pancreatic polypeptide (PYY) a hormone that is known to be decreased secondary to pancreatic exocrine damage [52, 150, 151], but has received little attention in CF where its response to stimuli has not been previously studied.

PYY release is stimulated via the vagus, GIP, gastric distension, gastrin and vasoactive intestinal peptide. It is localised almost entirely to the pancreas where it is produced by discrete, small, granular endocrine cells, scattered throughout the exocrine parenchyma. PYY levels rise rapidly after food, especially protein or fat and remain raised for several hours. PYY inhibits gastric emptying of solid food and delays the postprandial rise in plasma glucose and insulin.

The pancreas is the only significant source of PYY and intravenous infusions of amino acids, glucose or lipids do not alter PYY levels, suggesting an entero-insular axis, an area I wished to evaluate in this experiment.

In response to the OGTT and MMT, CF subjects had lower levels at all time points when compared to control subjects. This would be in keeping with literature [52, 150, 151] where low circulating levels of PYY have been reported. In my study, I have shown for the first time a lack of PYY response to an appropriate stimulus – sugar, protein and fat. This might be related to existing pancreatic insufficiency in my study group.

As PYY release is mediated via the vagus, one future consideration would be to study the effect of vagal stimulation and the possibility of autonomic dysfunction secondary to dysglycaemia in the CF population.

In control subjects, although the afternoon and evening baseline levels were higher than the morning, this variation did not achieve significance. A diurnal variation in PYY has been described, where concentrations are lowest in the morning and peak during the evening [295]. This might be secondary to the diurnal variation in vagal tone, with parasympathetic vagal activity increasing through the day [296]. I did not see a significant change through the day in my study group. This could be due to the inability to analyse some samples (< LOD) in my study population, the younger age range of my study group or related to the small sample size in my study.

I did not see a diurnal variation in the CF subjects although there were lower PYY levels at all times of the day, compared with controls. This diurnal variation has not been studied before. One explanation for this lack of diurnal variation might be an underlying autonomic neuropathy [297] in those with CF, who are known to be sympathetically overactive. Whether there is a lack of diurnal variation in autonomic activity in this patient group remains to be studied.

I acknowledge my study has limitations.

Firstly, the small sample size that may have reduced its ability to detect a statistical difference through the day.

Secondly, GLP_1 is quickly deactivated by the serine DPP-4 as an important regulatory mechanism. I did not use DPP-4 inhibitors while collecting blood samples as I wanted to

evaluate the total GLP_1 response. However, active GLP_1 (using DPP-4 inhibitor) needs to be looked at in CF individuals with NGT using insulin clamp studies through the day and this was beyond the scope of my study but should be considered in future studies.

Thirdly, due to limitations of the analytical procedures a number of samples analysed from my study (GIP and PYY) were below the LOD and couldn't be precisely measured. I needed to censor and substitute these values with a constant value ($LOD/\sqrt{2}$) value – a factor that might have influenced my results and the ability to show significant changes.

6 Summary discussion, Limitations, Future considerations and Conclusions

6.1 Discussion

With increasing survival, CFRD is now the most common comorbidity in individuals with CF. The additional diagnosis of CFRD to an already complex clinical condition has a negative impact on pulmonary function and survival in CF.

The pathophysiology of diabetes in CF has not been fully understood and there is a long-held view that CFRD stems from physical damage to the pancreas. While it shares features of type 1 and type 2 diabetes, CFRD is a unique clinical entity and is unlike other types of diabetes for several pertinent reasons as I have described in my literature review at the beginning of this thesis.

Firstly, gastric emptying and motility are likely to be altered in the condition, changing the way in which nutrients are presented to the gut compared to normal individuals. Secondly, relative progressive insulinopaenia occurring over time may result in diurnal changes in glucose and other nutrient handling that has not previously been explained. Thirdly, abnormalities in the small intestine due to the CF condition may alter the secretion of incretins and the associated pancreatic enzymes compared to non-CF individuals.

With this background, this study was set up to look at factors that could possibly contribute to the development of CFRD and I studied individuals who were not known to be diabetic or had previously developed diabetes. Being aware that these individuals had not developed diabetes and their chances of doing so were high as their condition and age advances, allowed me to evaluate the early changes that occur in insulin regulation and whether CF causes a second problem which underlies diabetes, separate from pancreatic structural damage.

The main purpose of this study was to look at possible additional factors that might be related to the development of CFRD. The aims of this thesis were to determine some of the hormonal and gastric influences on glucose handling. The results of the studies presented in this thesis centre around three main themes, which address my hypothesis at the beginning of this study that are discussed below.

6.1.1 Gastric emptying in CF

Gastric motility within CF has received little attention and previous investigations comparing the gastric emptying rate (GER) in CF have shown inconsistent results [206, 237-241]. An altered GER in patients with CF can have important clinical consequences.

A number of factors affect GER in CF - pancreatic enzyme supplementation, glycaemia and the high fat/high energy diets prescribed for these individuals. Gastric emptying is itself influenced by acute changes in blood glucose concentrations, with hyperglycaemia delaying gastric emptying, which in turn slows the absorption of ingested carbohydrate and reduces the propensity for further hyperglycaemia [236].

Circadian changes in glucose handling are well described in the normal population [209, 212, 213, 217] and gastric emptying, which is in turn slowed by hyperglycaemia [242] may influence these changes. However there have been no studies of circadian changes in gastric emptying in CF subjects, a group with poor glucose handling and there was a need to explore gastric motility in CF and the effects this might have on glucose handling and CFRD.

The study presented in experiment 1 of this thesis was designed to investigate GER in healthy control subjects and individuals with CF. Several different methods have been used to evaluate gastric emptying in adults. The gold standard test to assess gastric emptying is scintigraphy which is not practicable for daily use and not practicable for the nature of my study as scintigraphy requires subjects fasted for at least 12 hours, which would not have been possible to do in a study assessing diurnal changes. Moreover, scintigraphy requires expensive equipment not readily available and has an associated radiation burden. I chose ultrasound measurements, a validated and comparable method [298] with advantages over

other techniques in that it is non-invasive, does not entail radiation and makes use of standard ward based ultrasound equipment, which is easily accessed.

I assessed GER using an ultrasound method that is safe, easy to use, requires a standard ultrasound transducer and gives real time results. This method has previously been standardized [243] and validated [244-246] with good intra- and inter-observer agreement [243]. I was able to reliably measure GER in 96% of my subjects.

My study is the first to use ultrasound to assess gastric emptying in the adult CF population.

Using this method, I have shown that healthy control subjects have normal gastric emptying for both liquid and mixed meals after an overnight fast, in keeping with existing literature [248].

For the first time, I have shown that although adult CF subjects without known CFRD have a normal antral size, following an overnight fast, they have delayed gastric emptying for both liquid and mixed meals.

In my study cohort, although the mean gastric emptying rate in response to a liquid meal was normal, 42% of CF subjects had decreased gastric emptying at 60-minutes. These subjects also had reduced emptying of a mixed meal.

I have also shown that more CF subjects have decreased gastric emptying in response to a mixed meal compared to a liquid meal, demonstrating that the mixed meal test is the more physiological test in CF individuals.

My study is the first to explore gastric emptying in adult CF patients throughout the day. I have shown that not only do CF individuals without known CFRD have delayed gastric emptying in the morning, but this delay continues throughout the day.

A number of studies have demonstrated that pre-existing hyperglycaemia slows gastric emptying in healthy humans [232], Type 1 [250-252] and Type 2 diabetics [231]: I chose CF individuals without known CFRD to minimise this and also prevent the possibility of occult gastroparesis due to diabetic-related autonomic neuropathy. Similarly, by using the same

carefully measured constituents in each experiment I sought to control for differences in food composition and volume. Nevertheless, these CF subjects still had a delay in gastric emptying and the subsequent experiments in my thesis explored dynamic glucose handling and the influence of incretins and other hormones that might influence gastric emptying.

In experiment 2, I had shown CF subjects had higher glucose responses at 30 to 90 minutes after the OGTT and 30 to 120-minutes after the MMT. Coinciding with this hyperglycaemia the GER was delayed at 90 and 120-minutes after the OGTT and 60 to 120-minutes after the MMT and it is likely that this lag is due to the preceding hyperglycaemia delaying GER. Among the other factors that may determine postprandial glucose excursions is glucose influx from the gut [299] which appears to be determined to a certain extent by the rate of gastric emptying: since as much as 40–50% of variation in postprandial glucose excursions may be explained by differences in the rate of gastric emptying [233]. In Type 1 diabetes, gastroparesis is a late diabetic complication resulting from irreversible intestinal nerve damage [299] and is different from the physiological inhibitory effects of acute hyperglycemia on gastric motility [300, 301]. The latter has been proposed as a defence mechanism to minimize postprandial hyperglycemia by reducing the rate of efflux of glucose into the circulation from the gut [232]. Whether this mechanism exists in CF remains to be determined and it is possible that this mechanism is responsible for the delayed initial emptying seen in the evening in this study.

It is also possible that an increased GIP early on following the OGTT, that I found in experiment 3, contributed to some delayed emptying. However, I was unable to demonstrate significant changes during the MMT or during the day, making a significant contribution of GIP to a delayed emptying unlikely.

Finally, in a real world and practical setting, gastric ultrasound may also be useful in the clinical setting, by providing a simple cheap non-invasive way of assessing gastric emptying.

6.1.2 Glucose handling in CF and its diurnal variation

Clinical decline in CF precedes the detection of diabetes by current OGTT criteria and its early treatment confers a clinical advantage. CFRD is characterised by post-prandial hyperglycaemia rather than fasting hyperglycaemia and is frequently evident in CF patients with a normal OGTT.

In experiment 2, I showed that in CF subjects without frank diabetes, there is deficient glucose handling throughout the OGTT and early morning MMT and was able to demonstrate significant differences in subjects examined in the afternoon compared to the morning and evening. Whether this might be clinically significant remains to be determined. Importantly, the 120-minute result on a test of glucose handling is of no significance when done in the morning in CF subjects and the 120-minute value on an evening test deserves more attention.

This emphasises the need for tests of serial glucose handling with tests that provide 'glucose profiles'. The standard mixed meal test considered a more physiological test, both at the time of diagnosis and during follow-up has been proposed for clinical practice. Carrying out routine evening MMTs would not be practicable on a larger scale in clinical practice. Hence, there is a role for ambulatory Continuous Glucose Monitoring (CGM) systems that have been validated for use in those with CF [88-90].

In experiment 2, I sought to evaluate the impact of pancreatic β cell function on glucose handling. I showed that fasting and total insulin secreted are similar in CF and healthy subjects with an insulin lag in CF subjects. This has been shown before [101, 260, 261] and it is likely to be this insulin lag that contributed to the initial glucose excursions I saw in this study. Moreover, in addition to lower c-peptide levels, CF subjects took a longer time to reach a peak level reiterating a β cell defect and suggests that altered glucose handling is related to decreased insulin and c-peptide levels, a reflection of decreased β cell activity. This reduced β cell activity appears to go together with decreased α cell activity and the counter-regulation by glucagon to decreased insulin levels. In my study, it appears that glucagon does not contribute to the increased glucose levels in CF patients with NGT. This is in keeping

with the studies of Lanng [36], Lippe [138] and Moran [52] who demonstrated that CF patients are not able to appropriately increase glucagon secretion in response to arginine or hypoglycaemia using clamp studies, consistent with a reduced α cell mass.

The main focus of my study was to evaluate pancreatic function through the day. Surprisingly, I found CF subjects had better glucose handing in the afternoon and at the same time had the lowest insulin levels. As discussed in experiment 2, it is possible that insulin liberated post-prandially in the morning conserves a certain activity at the moment of the next meal, and still intervenes in maintaining glucose homeostasis. In addition, when I studied insulin sensitivity, this was highest in the afternoon and appears to play a significant part in improving glucose handling in the afternoon. Importantly the time taken to reach a peak insulin level in the evening was the longest, suggesting the β cells take longer to produce insulin at this time of the day – a concept I referred to as 'CF pancreatic fatigue'.

6.1.3 The contribution of the entero-insular axis to altered glucose handling

Gastric emptying and glucose handling is regulated by a complex interaction of neuronal and hormonal input. Many of these hormones produced and secreted by the gastrointestinal tract have simultaneous effects on glucose handling via the release of insulin. Dietary constituents other than glucose affect insulin secretion. The addition of protein, amino acids or fat to carbohydrate is known to enhance insulin secretion, via the incretin system [265] The main incretins - GIP and GLP_1 are stimulated by fat [266]. I also sought to measure PYY, which is secreted solely by the pancreas but is under autonomic vagal control and has received little attention in CF.

I have shown that in fasting CF subjects without frank diabetes, there was no difference in GLP_1 secretion when compared to healthy control subjects following a liquid or a mixed meal or through the day. However, CF subjects in contrast to controls tended to have higher levels later during the MMT. It is possible that those with CF have this delayed response due to decreased gastric motility and the delayed presentation of food substrate to the duodenum where GLP_1 is secreted. It is unlikely that GLP_1 contributed to the changes in glucose handing

seen through the day seen in my study population. I did not measure active GLP_1, by using DPP-4 inhibitors and this is a limitation of the study that I have addressed in the subsequent section.

When measuring GIP, CF subjects had early hypersecretion of GIP following the OGTT. Hypersecretion of GIP in non-diabetic children with CF was shown in 1981 by Ross and colleagues [205] and my study is the first to show this in adults. I did not find any significant differences after the MMT or on evaluation of GIP through the day suggesting an intact and appropriate GIP response to food substances.

When I set out at the beginning of this study, I had hoped to show differences in the incretin responses and a significant contribution of the entero-insular axis to glucose handing and the diurnal variation in glucose handling. This did not occur and there are several possible explanations for it. The CF patients I studied were comparatively well: CFRD tends to manifest in the sicker population and it might be that those I studied had yet to develop significant limitations of the entero-insular axis. Another factor that might have contributed is the calorific size of the meal I administered. Although I used a standard mixed meal, this may not have been sufficient to stimulate the incretin system in a population with a high metabolic rate who are used to consuming meals with high calorific values.

In my PYY experiment I showed, for the first time a lack of response in CF subjects to oral stimuli with no diurnal variation. This is likely to be secondary to the known existing pancreatic exocrine insufficiency in my CF study group. PYY inhibits gastric emptying of solid food and delays the postprandial rise in plasma glucose and insulin. The lack of PYY in my CF study group would not have contributed to the delayed gastric emptying I showed in experiment 1. It is also unlikely to contribute to the delayed insulin response that has been well described in the CF population.

6.2 Limitations

This study does have its limitations. I have discussed some of these in previous chapters. Firstly, a limitation of the experiments in this thesis is the small sample size. However, although the number of subjects was relatively small, it was still adequate to demonstrate significant differences among groups in several independently measured variables.

Secondly, there were more male subjects in the CF study group. However, females with CF develop CFRD earlier than males (on average 5–7 years) [302] and tend to be sicker than their male counterparts [86], limiting the period of clinical stability required for recruitment. Why this occurs is not clear, but female hormones, lack of anabolic male hormones, and oral contraceptives are all possible explanations [29].

Thirdly, the screening OGTT (or the liquid meal) carried out was a 'single' test. Given the up to 50% variability in OGTT results in CF patients [23], some may have shown a different response to a second OGTT on another day.

The small sample size might have limited the ability to detect statistical differences in insulin and c-peptide at some time points or later in the day. It could very well be that secretion of insulin and c-peptide change, albeit at a later time, but continuous monitoring of insulin secretion to measure its pulsatility is not practicable.

One criticism of my study could be the non-measurement of active GLP_1. GLP_1 is quickly deactivated by the serine protease dipeptidyl peptidase IV (DPP-4) as an important regulatory mechanism. I did not use DPP-4 inhibitors as I wanted to evaluate the total GLP_1 response. However, active GLP_1 (using DPP-4 inhibitor) needs to be looked at in CF individuals with NGT using insulin clamp studies through the day and this was beyond the scope of my study. On reflection, at the end of this work, it would have been prudent to collect an additional blood sample with DPP-4 inhibitor, as the results might have strengthened my work and thesis.

As discussed in experiment 1, with the assessment of gastric motility, I was not blinded to subjects' responses due to the nature of the study. I was trained and had a competency

assessment by an experienced radiologist, but I did not evaluate reproducibility and repeatability. However, four complete studies for each subject were carried out on two different days, three of which were performed on the second day and the analysis indicated an acceptable level of repeatability. With regards to reproducibility, I had initially set out to compare the GER_{30} in the morning on separate days. However, since the volumes of substance used to make up the OGTT and MMT were different, stomach distension was dissimilar such that comparison was not possible.

A number of samples analysed from my study (GIP and PYY) were below the LOD and therefore could not be precisely measured. I censored and substituted these values with a constant ($LOD/\sqrt{2}$), in keeping with accepted statistical practice, but nevertheless this might have influenced the ability to show significant changes.

Finally, some tests of diurnal variation (Repeated ANOVA) were significant, but the post-hoc tests (Bonferroni tests) were not. This might have been due to small sample size and low power. The Bonferroni correction might have been too conservative a test and a larger sample size and Bonferroni correction might have confirmed results.

Some of the limitations of this study need to be addressed in future studies discussed in the next section.

6.3 Future considerations

The studies presented in this thesis provide insights into glucose handling in CF patients without known diabetes.

Future work in this area should consider the following:

Following completion of data collection in my study, Hillman et al [281] showed that active GLP_1 was significantly decreased in patients with CF and CFRD compared to healthy controls. GLP_1 analogues are now used as anti-diabetic drugs in patients with T2DM, but it is not known whether these drugs are clinically effective in CF patients. Further work needs to be done looking at active GLP_1, in particular in response to a mixed meal.

I have also shown the predominant defect in CFRD is poor β cell function. In my study, it appeared the incretin system functions normally in those with CF. However, further work needs to be done in this area to confirm this. Using matching isoglycaemic and hyperglycaemic clamps would enable measurement of the dependency of insulin secretion on incretin hormones. By comparing levels following OGTT, MMT and then an intravenous glucose infusion, the incretin effect could be evaluated. Measuring active GLP_1, at the same time and throughout the day would improve our understanding of the pathogenesis of CFRD.

My study did not evaluate the effect of individual components of the mixed-meal. This is important to consider for future research, as the responses to various concentrations of fat, carbohydrate or protein might vary through the day and the meals I administered had fixed amounts of nutrients. It is possible that the raised sugars seen commonly at the end of the day might be related to abnormal handing of nutrients later in the day. As discussed earlier, a limitation of my study is the calorific size of the meal I administered which might not be high enough to stimulate the CF pancreas and altering meal constituents at different times of the day might address this.

Elder et al [303] showed that patients with CF have reduced insulin secretion during an OGTT regardless of exocrine pancreatic status. This would suggest abnormal insulin secretion in all CF patients and may predispose them to developing glucose intolerance,

particularly when challenged by inflammation, infection, or nutritional deficiencies. I chose pancreatic insufficient subjects for my study. Future work should evaluate the same aims of my study, but in pancreatic sufficient individuals with CF. At the time of writing this thesis, Sheikh et al [256] published data demonstrating reduced β cell secretory capacity in pancreatic-insufficient, but not pancreatic-sufficient CF patients with normal glucose tolerance. The study by Sheikh did not evaluate changes through the day and this needs further attention in the pancreatic sufficient group.

Following the availability of CFTR modulators such as Ivacaftor (Kalydeco®) which corrects the gating defect of the CFTR channel associated with the G551D genetic mutation, there have been reports of resolution [304] or the delay in the onset [305] of CFRD. A direct role for CFTR in islet function has been demonstrated in the ferret and pig models of CF [306, 307] and there is a possible regulatory mechanism of CFTR on insulin production [308]. Treatments such as these might affect CFTR function, but the impact of CFTR on β cells in humans is not known. Studying pancreatic sufficient individuals with CF, might give further insight into the effect of glucose handing and incretin responses.

Insulin secretion is regulated through a complex of different signals stimulated by nutrients, incretins, and important autonomic stimuli. The cephalic phase insulin response, which lasts for about 10 minutes, is initiated by meal ingestion and has been demonstrated before [309]. I did not measure this in my study. Whether this has an impact on glucose handling in CF is not known and has not been evaluated before and deserves further attention.

6.4 Conclusions

The experimental studies described within this thesis have extended knowledge on the gastric emptying rate, glucose handling and gut hormone responses in CF subjects without known CFRD. The main conclusions that can be drawn from this work are:

This is the first study to use ultrasound to assess gastric motility in the adult CF population and demonstrates delayed gastric emptying throughout the day in CF subjects, compared to matched controls, for both liquid and mixed meals. Moreover, this study shows that this novel inexpensive bedside technique provides a simple method of assessing GER in CF and will have a role in the clinical setting.

This is the first study to show that although adult CF subjects without known CFRD have a normal antral size, following an overnight fast they have delayed gastric emptying for both liquid and mixed meals.

CF subjects have decreased gastric emptying in response to a mixed meal compared to a liquid meal, demonstrating that the mixed meal test is more physiological in CF individuals.

In CF subjects without frank diabetes, there is deficient glucose handling throughout the OGTT and early morning MMT.

There are significant differences in glucose handling in the afternoon compared to the morning and evening, with the 120-minute value at an evening test deserving more attention.

Fasting and total insulin secreted are similar in CF and healthy subjects with an insulin lag in CF subjects and glucagon does not appear to contribute to elevated blood sugars.

Insulin sensitivity is highest in the afternoon and appears to play a significant part in improving glucose handling in the afternoon.

The time taken to reach a peak insulin level in the evening is the longest, suggesting that β cells work harder at this time of the day.

In fasting CF subjects without frank diabetes, there is no difference in GLP_1 secretion when compared to healthy control subjects following a liquid or a mixed meal.

In fasting CF subjects without frank diabetes, there is no difference in GIP secretion when compared to healthy control subjects following a mixed meal, but hypersecretion exists early in the response to the OGTT.

CF subjects have lower PYY levels in response to the OGTT and MMT with no diurnal variation, likely to be secondary to the existing pancreatic insufficiency.

7 Appendices

Appendix 1: Demographic details

Appendix 2: Gastric motility data

Appendix 3: REC approval letter

Appendix 4: Consent form

Appendix 5: Participant information sheet (CF)

Appendix 6: Participant information sheet (Controls)

Appendix 7: DRG® Insulin ELISA (EIA-2935) test

Appendix 8: DRG® C-Peptide ELISA (EIA-1293) test

Appendix 1: Demographic data

	Age (years)	FEV_1 (%)	FVC (%)	Weight (kg)	Height (m)	BMI (kg/m^2)	Genotype	Genotype	Comments
Control 1	29	NA	NA	60	1.6	23.4		NA	
Control 2	32	NA	NA	64	1.84	18.9å		NA	
Control 3	23	NA	NA	72	1.76	23.2		NA	
Control 4	26	NA	NA	62	1.63	23.3		NA	
Control 5	31	NA	NA	77	1.8	23.8		NA	
Control 6	28	NA	NA	59	1.64	21.9		NA	
Control 7	27	NA	NA	90	1.7	31.1		NA	
Control 8	29	NA	NA	71	1.68	25.2		NA	
Control 9	41	NA	NA	67.6	1.7	23.4		NA	
Control 10	31	NA	NA	86.36	1.75	28.2		NA	
***Subject 1**	21	68	105	69	1.71	23.6	F508del	F508del	
***Subject 2**	26	97	112	75.6	1.73	25.3	F508del	G551D	

	Age (years)	FEV_1 (%)	FVC (%)	Weight (kg)	Height (m)	BMI (kg/m^2)	Genotype	Genotype	Comments
*Subject 3	29	112	114	77	1.7	26.6	R117H |	3272-26A->G	Pancreatic sufficient
*Subject 4	27	96	137	68	1.74	22.5	F508del	P2055	Diabetic
*Subject 5	27	75	105	60	1.65	22	F508del	G551D	Diabetic
*Subject 6	21	90	100	54	1.57	21.9	F508del	G551D	Diabetic
*Subject 7	36	96	112	68	1.69	23.8	F508del	Positive sweat test	
*Subject 8	22	96	94	72	1.9	19.9	621+1G->T	Positive sweat test	
*Subject 9	22	75	104	62	1.67	22.2	F508del	F508del	
*Subject 10	24	72	96	57	1.6	22.3	F508del	F508del	
*Subject 11	21	80	91	69.35	1.73	23.2	F508del	F508del	
*Subject 12	25	73	111	65	1.81	19.8	F508del	F508del	IGT
*Subject 13	26	65	77	72.6	1.73	24.3	F508del	F508del	
*Subject 14	18	88	103	86.5	1.76	27.9	F508del	F508del	
*Subject 15	39	47	79	91	1.75	29.7	F508del	2789+5G->A	Diabetic

	Age (years)	FEV_1 (%)	FVC (%)	Weight (kg)	Height (m)	BMI (kg/m^2)	Genotype	Genotype	Comments
*Subject 16	38	44	59	72.1	1.7	24.9	G551D	R553X	
*Subject 17	25	99	103	76	1.8	23.5	F508del	F508del	Liver disease
*Subject 18	25	66.9	79.83	79.9	1.81	24.4	F508del	F508del	
*Subject 19	25	93.44	98.3	65.4	1.78	20.6	F508del	R560T	
*Subject 20	27	97	103.97	64.2	1.62	24.5	F508del	F508del	

*Included in final analysis

Appendix 2: Individual data for gastric motility

	OGTT					MMT 1					MMT 2					MMT 3			
	30 mins	60 mins	90 mins	120 mins		30 mins	60 mins	90 mins	120 mins		30 mins	60 mins	90 mins	120 mins		30 mins	60 mins	90 mins	120 mins
Control 1	36	60	63	76		51	59	77	81		26	56	71	74		36	44	68	72
Control 2	35	47	76	76		47	60	74	82		52	65	67	79		43	56	68	72
Control 3	32	47	72	74		34	48	69	79		42	50	68	82		46	52	69	82
Control 4	35	45	66	70		38	44	72	76		23	40	69	74		34	50	70	72
Control 5	34	52	67	76		35	48	74	80		41	51	69	80		30	56	73	79
Control 6	43	61	75	77		39	61	74	80		34	68	76	82		30	45	70	77
Control 7	38	48	69	71		36	43	70	77		25	47	70	73		28	49	66	74
Control 8	38	61	68	73		33	55	70	79		32	47	72	76		37	50	74	79
Control 9	42	47	68	77		33	55	71	81		37	57	70	77		32	53	70	76
Control 10	34	46	66	76		44	57	71	76		43	62	69	74		36	45	70	77
Subject 1	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-
Subject 2	13	34	64	71		31	34	53	60		35	41	52	55		27	32	54	69
Subject 7	41	68	68	75		36	44	66	80		35	46	60	75		22	45	57	74
Subject 8	39	53	70	71		39	48	60	72		37	58	68	78		23	36	57	75
Subject 9	23	40	41	52		22	32	39	41		33	43	56	70		19	35	57	68
Subject 10	41	42	49	62		48	57	59	72		36	49	62	69		28	37	60	69

	OGTT					MMT 1					MMT 2					MMT 3			
Subject 11	20	29	43	45		32	41	51	54		26	34	54	57		16	41	61	70
Subject 13	27	48	53	57		27	45	64	69		17	37	58	68		19	39	54	65
Subject 14	42	47	54	64		18	26	41	56		36	42	53	60		25	55	67	76
Subject 16	31	48	50	56		46	57	60	68		30	44	62	71		34	57	62	69
Subject 18	18	39	65	71		24	44	62	65		29	37	58	66		25	40	59	72
Subject 19	33	52	62	74		38	46	57	67		31	47	56	68		43	52	63	72
Subject 20	39	53	60	70		42	48	54	61		26	39	51	64		28	45	61	70

Appendix 3

NHS

National Research Ethics Service

NRES Committee North West - Greater Manchester East
3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7820

27 September 2011

Private & Confidential
Dr D S Nazareth, Clinical Research Fellow
Adult CF Unit
Liverpool Heart and Chest Hospital NHS Trust
Thomas Drive
Liverpool
L14 3PE

Dear Dr Nazareth

Study title: **Factors responsible for the development of diabetes in adults with Cystic Fibrosis**

REC reference: **11/NW/0552**

Thank you for your letter of 7 September 2011 responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Alternate Vice Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Additional condition

On page 5 of the information sheet (CF) version 1.4, 'Creon/Pancreozyme' should read 'Creon/Pancreozymin'.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Please notify the REC in writing once all conditions have been met (except for site approvals from host organisations) **and provide copies of any revised documentation with updated version numbers.** Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Advertisement	1.1	01 September 2011
Covering Letter		25 July 2011
GP/Consultant Information Sheets	Controls - 1	21 July 2011
GP/Consultant Information Sheets	Patients - 1	21 July 2011
Investigator CV		
Other: CV Martin Walshaw		
Participant Consent Form: Patient	1.3	05 September 2011
Participant Consent Form: Controls	1.3	05 September 2011
Participant Information Sheet: Patient	1.4	01 September 2011
Participant Information Sheet: Controls	1.4	01 September 2011
Protocol	3.3	02 September 2011
REC application	3.2	26 July 2011
Response to Request for Further Information		
Summary/Synopsis	3.1	18 July 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/NW/0552	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Elaine Hutchings

pp **Mr Francis Chan**
Chair

Email: elaine.hutchings@northwest.nhs.uk

Enclosure: "After ethical review – guidance for researchers"

Copy to: Dr Margarita Perez-Casal,
Research, Audit and Effectiveness Manager
Liverpool Heart and Chest Hospital

Dr Martin Walshaw, Consultant Chest Physician
Liverpool Heart and Chest Hospital

Health Research Authority
National Research Ethics Service

NRES Committee North West - Greater Manchester East
3rd Floor, Barlow House
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Manchester
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Tel: 0161 625 7830
Fax: 0161 625 7299

18 June 2012

Dr Dilip S Nazareth
Clinical Research Fellow
Liverpool Heart and Chest Hospital NHS Trust
Adult CF Unit
Thomas Drive
Liverpool
L14 3PE

Dear Dr Nazareth

Study title:	**Factors responsible for the development of diabetes in adults with Cystic Fibrosis**
REC reference:	**11/NW/0552**
Amendment number:	**Substantial amendment 1**
Amendment date:	**20 May 2012**

The above amendment was reviewed June 2012 by the Sub-Committee in correspondence.

Ethical opinion

The sub- committee reviewed the amendment and have no ethical concerns.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Participant Consent Form	1.1	19 May 2012
Participant Information Sheet	1.5	19 May 2012
Protocol	3.5	18 May 2012
Notice of Substantial Amendment (non-CTIMPs)	Substantial amendment 1	20 May 2012

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/NW/0552:	**Please quote this number on all correspondence**

Yours sincerely

Mr Francis Chan
Chair

E-mail: nicola.burgess@northwest.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Dr Margarita Perez-Casal*

NRES Committee North West - Greater Manchester East

Attendance at Sub-Committee of the REC meeting on 18 June 2012

Name	*Profession*	*Capacity*
Mr Francis Chan	Consultant Orthopaedic Surgeon	Expert
Dr Michael Hollingsworth	Retired Senior Lecturer in Pharmacology	Lay
Professor Janet Marsden	Professor of Ophthalmology and Emergency Care	Expert

28 September 2011 Version 1.4

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

Consent Form

Study Number

Participant Identification Number for this Study:

Title of Project: Factors responsible for the development of diabetes in adults with Cystic Fibrosis

Name of Researcher: Dr. Dilip Nazareth

Please Initial Box

1. I confirm that I have read the information sheet dated 28-September-2011 (version 1.5) of the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities, the research team, or from the NHS Trust, were it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I understand that blood samples collected will be frozen and analysis may take place at a later date and may occur in an external laboratory. ☐

6. I agree to gift the samples collected for use in future research projects which have been reviewed by a Research Ethics Committee. ☐

7. I agree to participate in this study. ☐

Name of Participant _____ Date _____

Researcher _____

When complete, 1 copy for participant: 1 copy for researcher site file: 1 (original) to be kept in medical notes

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

Participant Information Sheet (CF) Summary

Factors responsible for the development of diabetes in adults with Cystic Fibrosis

Cystic Fibrosis (CF) affects over 8,500 people and affects multiple parts of the body. One organ that is affected is the pancreas that is responsible for the release of insulin and those with CF are prone to develop diabetes. The type of diabetes in CF is different from the other types of diabetes and is 'unique'. The exact nature of why diabetes develops is not known but is thought that both the release and action of insulin may be responsible and it has been found that insulin enhances muscle mass and improves lung function.

The purpose of this study is to look at possible factors that might be related to the development of diabetes and to develop a new test for diabetes that may be suited for use in someone with CF as the current tests are derived from information available from people with diabetes who do not have cystic fibrosis.

You have been invited to take part in this study as our records indicate that you have cystic fibrosis but currently **do not** have a diagnosis of cystic fibrosis related diabetes. We want to assess some factors (stomach movements, hormones and insulin release) that will allow us to determine, more precisely, the risk of individuals like you developing diabetes. We intend to recruit 30 participants with CF to take part in this study.

Text

The participant information sheet attached gives details of the study and how it would be carried out.

Your participation is entirely voluntary and you would be able to withdraw from the study at any time.

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

Participant Information Sheet (CF)

Factors responsible for the development of diabetes in adults with Cystic Fibrosis

We would like to invite you to take part in our research study. However, before you decide, we need you to understand the reason for the research and what it involves. You may talk to others about the study if you so wish. Part 1 tells you the purpose of the study and what to expect if you take part. Part 2 gives you more detailed information about the conduct of the study. Please ask if there is anything you do not understand. Take time to decide whether or not you wish to take part in this research study.

If you are willing to participate please telephone **Mrs. Patricia Stanley** (Pulmonary Function Administrator) on 01516001780 to indicate that you are interested in taking part in the study and your contact information will then be passed on to the Investigator of the study who will then contact you. A member of the Research Team will go through the information sheet with you and answer any questions you may raise.

PART 1

Background

Cystic Fibrosis (CF) is the UK's most common life-threatening inherited disease and according to the CF Trust (UK) the disease affects over 8,500 people. The genetic defect is responsible for abnormal salt and water movements across cells causing thickened secretions. One organ that is affected is the pancreas that is responsible for the release of insulin. Diabetes mellitus is a well-recognised complication of cystic fibrosis, is unique and distinct, sharing features of other types of diabetes. The current recommendation is that a test called the oral glucose tolerance test be carried out to diagnose diabetes in CF. However, the test and its normal and abnormal values are drawn from a diabetic population who do not have CF. The exact nature of why diabetes develops in individuals with CF is not known, but is thought that both a defect in the release of insulin and a sensing of insulin by tissues may be responsible.

It is known that falls in weight and lung function occur prior to the development of diabetes and that diabetes in CF is associated with early death. It is also known that insulin enhances muscle mass and improves lung function, thereby improving quality of life.

What is the purpose of the study?

The purpose of this study is to look at possible factors that might be related to the development of diabetes and to develop a new test that may be suited for use in someone with CF.

The main purposes of this research is to study the following:

1. Does the pancreas tire as the day progresses and does this contribute to the altered insulin release that is seen in people with CF.

2. Should a glucose tolerance test be done at a later time in the day, as against first thing in the morning as is currently done?

3. To determine if stomach movements affect the release of insulin?
4. To study the changes in hormones that affect insulin in response to a liquid and solid meal and patterns of change through the day.

Why have I been invited?

You have been invited to take part in this study as our records indicate that you have cystic fibrosis but currently **do not** have a diagnosis of cystic fibrosis related diabetes. We want to assess some factors (stomach movements, hormones and insulin release) that will allow us to determine, more precisely, the risk of individuals like you developing diabetes. We intend to recruit 30 individuals with CF take part in the project.

Do I have to take part?

Your participation is completely voluntary, and you can withdraw your consent at any time without penalty. This will not affect your medical care in any way.
If you choose to participate in this study you must sign the Informed Consent Form before your participation in the study is initiated. Read this information carefully before you decide if you want to participate. You have the right to have time for reflection before you give your informed consent.
In addition to this information document you will receive a copy of the signed Informed Consent Form. If you need further information you can contact the study doctor.

What will happen to me if I take part?

- Reasonable expenses including travel costs incurred to participate in this study would be reimbursed for the duration you are in the study. Taking part in this study will involve 3 visits to the hospital. If you agree to take part in this study you will be asked to visit the clinic (visit 1) to discuss the study with the investigator, sign the consent form, talk about your medical history (including smoking, exercise, alcohol habits), your Cystic Fibrosis history (including date of diagnosis, previous and current CF medications), undergo a routine physical examination (including body weight , height blood pressure, heart rate and oxygen levels). You will need to undergo lung function testing (simple spirometry). At this visit you will be required to undergo one blood test to ensure your blood levels are not too low and you are not anaemic. This visit will last approximately 45 minutes.

If following completion of the screening test you are still eligible to take part and wish to continue in the study, your participation may last up to a week.

We will be performing a number of tests which will require two further visits (visits 2 and 3) to hospital. These visits should occur within a week of each other, following your consent to take part in the project. Visit 2 will last approximately 3 hours and visit 3 will last approximately 12.5 hours.

With your permission, information will be gathered by the research team from your medical notes (medical history, medications, and investigation results) and your GP will be informed that you are taking part in this study.

The only people who will have access to your medical notes as part of this study are a few members of the research team who are qualified doctors or nurses working in the NHS, and the regulatory bodies overseeing the research.

This study has 2 distinct parts involving meals, blood tests and ultrasound scanning of the stomach to evaluate movements. These tests are described below:

- Oral and mixed meals: For both parts of the study you would need to come to the hospital after having fasted overnight (12 hours). For the first part of the study you will be given a glucose drink (75 grams of glucose in water) called Procal ® and blood tests and ultrasound

scanning (see below) will be performed over 2 hours. This then completes this part of the study.
Within a week you will be required to come back to have a similar test done and having fasted overnight (12 hours). This time instead of being asked to drink a liquid you will be given what is called a 'mixed meal' to complete within 10 minutes. This meal contains a certain amount of solid and liquid foods (37% carbohydrate, 18% protein, 44% fat) and blood samples will be collected over 2 hours. This test will be carried out twice more at 1300 and 1800 hrs to coincide with usual meal times. The usual meal would consist of a ham and cheese sandwich with butter, orange juice and digestive biscuits. You will need to fast between each meal.

- Blood tests: As part of this study 20 blood samples (each of 9 mls, overall 180 mls of blood) will be collected for analysis. For each part of the study (2 hour duration), one sample at the beginning followed by 4 samples at 30, 60, 90 and 120 minutes will be collected from a cannula (see below). These blood samples will be stored in the Biochemistry Laboratory at Liverpool Heart and Chest Hospital. Most of these tests will be performed at the above laboratory at Liverpool Heart and Chest Hospital, but with your permission, we may have to send some of these samples, to an outside laboratory for analysis.

- Ultrasound test: Ultrasound is a safe procedure. You will be asked to lie on your back or slightly towards your left-hand side during this scan, which may be uncomfortable. The scan will be carried out for 2-3 minutes following a blood sample collection to study the size of the stomach. This test will be done in both parts of the study.

- Inserting a cannula (Cannulation) (described below) in the arm or forearm would avoid the need for repetitive blood collection using needles. Usually 1 cannula is required for each part of the study (visit 2 and 3) but occasionally the cannula does not work any more and you might need another one inserted. To ensure that the cannula remains patent 3-5mLs of Normal Saline would be used as a flush after insertion and after each blood collection point. At the end of each visit the cannula would be removed.

- A summary of investigations and time points is represented in the table below:

	Time Points	**Baseline**	**30 mins**	**60 mins**	**90 mins**	**120 mins**
Visit 1	**1 blood sample**					
Visit 2	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x
Visit 3	**Time Points**	**Baseline**	**30 mins**	**60 mins**	**90 mins**	**120 mins**
0800 – 1000 hrs	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x
1300 – 1500 hrs	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x

1800 – 2000 hrs	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x

- All your usual medication and supplements (including Creon/Pancreozymin) will continue as usual and you will be asked to take any routine medication at least one hour before the start of tests at the second and third visits. You will not be required to stop or withhold any medication.

What will I have to do?

If, after reading this leaflet, you wish to take part, one of our research team will speak with you to make sure that you understand what is involved. You will then be asked to sign a consent form.
We will arrange a mutually convenient time in order for us to carry out the study.

What are the possible disadvantages or risks of taking part?

- Inconvenience of having to come into hospital thrice
- Inconvenience of having investigations
- Inconvenience of being contacted by the research team

The possible disadvantages and risks of the additional investigations are:

- Blood tests: There may be discomfort, bleeding and bruising as a result of having additional blood tests. Blood tests will only be taken by a member of staff qualified to do so using appropriate hospital equipment.

- Cannulation: A cannula is inserted using a needle similar to those used in taking standard blood tests. This may cause pain, bleeding or bruising. This is usually slightly more uncomfortable than a blood test but once the cannula is in place should not cause you any real discomfort. This will be placed by a member of staff trained to do so. This cannula will be removed once the test is complete unless you become unwell during the test and require this cannula for medical reasons. An additional risk of cannulation is infection. A previous study of hospital patients reported the risk of serious infection of 2/10,000. The risk should be less as part of this study as the cannula will only be in place for a short time.

What are the side effects of any treatment received when taking part?

You will only be asked to drink a glucose drink called Procal ® that is routinely used in tests to diagnose diabetes. No side effects have been reported. The components of the meal are a part of a usual Western diet.

What are the possible benefits of taking part?

We cannot promise that the study will have a direct benefit to you, but the information we gather may help improve the management of people with cystic fibrosis who develop diabetes and identify those who are at an increased risk of developing diabetes in order to optimise lung function to the maximum possible.
As a result of the investigations we may discover that you have diabetes or are prone to developing diabetes, which may require further evaluation or treatment. Any abnormal results will be discussed with you and arrangements made for further management. Your GP may need to be informed or you may require referral to another clinic.

What happens when the research study stops?

If there is an important finding that has an implication in the way you should be managed, we may invite you to attend for further discussions/tests, if we think you are at increased risk of having diabetes.

Once the initial investigations are collected, you will as part of your routine Cystic Fibrosis care, be followed up on a regular basis in clinic (usually 2 monthly). If during these routine clinic visits it might be suspected that you may be diabetic then these tests described above may help to clarify this. If needed, at that point, with your permission, these results will be entered into your clinical record.

What if there is a problem?

Any complaint about the way you have been treated during the study or any possible harm you may suffer will be addressed. Detailed information regarding this option is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practices and all information about you will be handled in confidence. The details are included in Part 2. If the information in Part 1 has interested you and you are considering participating, please read the additional information in Part 2 before making a decision.

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

PART 2

What if relevant new information becomes available?

On receiving new information, your doctor may consider it to be in your best interests to withdraw from further investigations in the study. For instance, if your blood tests suggest there is a reason why it may be unsafe to put you through the study. If anything untoward does arise your research doctor will explain the reasons and inform your GP.

Once the study is completed and you wish to know the results, we will be happy to discuss these with you at a routine clinic visit. If during these routine clinic visits it might be suspected that you may be diabetic then these tests described above may help to clarify this. If needed, at that point, with your permission, these results will be entered into your clinical record.

What will happen if I don't want to carry on with the study?

If you wish to withdraw from the study, you can contact the Research Team to notify them whether you want to withdraw from the study entirely, or just from part of it.

- We will remove you from our follow-up database if you do not want to be contacted again.
- Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on you.
- We would be unable to destroy the results of investigations already performed as part of this study which may assist standard medical care.
- We would be unable to destroy any information from your general medical notes.

If you lose capacity or are unable to consent at any part of the study then we will not carry out any further part of the study. We would analyse samples that have been collected while you consented and had capacity to make that decision.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions and can be contacted via The Regional Adult CF Centre, Liverpool Heart and Chest Hospital on 0151 228 1616. If you remain unhappy and wish to complain formally, you can do this via the NHS Complaints Procedure. Details can be obtained from the Research Team or through the Patient Advice and Liaison Services on 0151 228 1616.

This study is covered by The NHS Indemnity Scheme. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

With your consent, we would normally inform your general practitioner that you are participating in the study. Reports of these investigations will be placed in your medical notes. All patient information collected in the study will be stored on password protected computers or in locked rooms at Liverpool Heart and Chest Hospital. When the results of the study are reported, individuals who have taken part will not be identified in any way.

All data will remain confidential and no personal details will be made available to any third parties or transferred outside of the hospital. Details about you will be stored on computer during the research project but your data will only be reviewed by members of the research team. Blood will be stored in a secure storage facility at the Liverpool Heart and Chest Hospital NHS Trust. The data will only be used for research purposes in this project. No other use of the data will be undertaken without seeking your prior consent.

Involvement of the General Practitioner/Family doctor (GP)

With your consent, your GP will be notified of your participation in the study. If any tests show results which may affect the way you should be treated medically, your GP will be made aware of these changes.

What will happen to any samples I give?

Blood samples will be frozen and stored on-site in the biochemistry laboratory at Liverpool Heart and Chest Hospital NHS Trust. All blood samples will be handled as any other blood samples are at the Hospital, will be kept securely and only accessed by research and laboratory staff. All samples taken as part of this study will be taken and labelled in accordance with standard trust policy. A code will link your data and samples through a name list. This list that links your name to the code will be kept by the chief investigator. This is to ensure that if the results are needed in the future for your routine clinical care then they can be referred back to and that we can be sure the results apply to the correct participant. Some blood tests may need to be sent to different laboratories and if this is needed then your name and date of birth will be included on the sample.

Only the investigator for the study will have access to the results and if the tests show information of clinical significance, a member of the research team will inform you of the results in person in clinic and explain any action needed.

We will not routinely inform you of the results of tests. If you agree to take part in the study, we will ask you to gift any samples we take so that we may use them for any future research. Serum samples obtained from blood tests will be stored for up to 10 years in a tissue bank approved under the Human Tissue Act 2004. At the end of this time the samples will be destroyed.
When all the participants have been recruited we may analyse stored blood samples taken during the study for additional biomarkers; these again would be biomarkers related to diabetes or cystic fibrosis. The storage of blood and tissue samples are guided by strict regulations and would be stored in accordance with this.

Will any genetic tests be done?

No

What will happen to the results of the research study?

You will not be personally identified in any report or publication. The results of the study will be disseminated via electronic and paper medical journals as well as presentations in various major medical societies throughout the world.

Who is organising and funding the research?

The study is being funded by in-house funds from Liverpool Heart and Chest Hospital NHS Trust. The research group performing this research study has no conflict of interests. Your research nurse/doctor will not receive any additional money for including you in this study.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by the National Research Ethics Service (NRES) Committee North West – Greater Manchester East.

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

Further information and contact details

Further information regarding this project can be obtained from The Research Team, who can be contacted by calling the Regional Adult CF Unit on 0151 228 1616.

Dr Dilip Nazareth, Clinical Research Fellow, Principal Investigator

Dr Kamlesh Mohan, Consultant Chest Physician

Dr Martin Walshaw, Consultant Chest Physician and Clinical Director

Further information about research in general can be found at the National Institute for Health Research, at the following web address: www.nihr.ac.uk/research

If you develop any concerns about the study, you can contact any member of the research team above.

Liverpool Heart and Chest Hospital NHS

NHS Foundation Trust

Participant (Control) Information Sheet Summary

Factors responsible for the development of diabetes in adults with Cystic Fibrosis

Cystic Fibrosis (CF) affects over 8,500 people and affects multiple parts of the body. One organ that is affected is the pancreas that is responsible for the release of insulin and those with CF are prone to develop diabetes. The type of diabetes in CF is different from the other types of diabetes and is 'unique'. The exact nature of why diabetes develops is not known but is thought that both the release and action of insulin may be responsible and it has been found that insulin enhances muscle mass and improves lung function.

The purpose of this study is to look at possible factors that might be related to the development of diabetes and to develop a new test for diabetes that may be suited for use in someone with CF as the current tests are derived from information available from people with diabetes who do not have cystic fibrosis. In order to do so, we need to compare 2 groups of people – those with CF and those without.

You have been invited to take part in this study as you currently **do not** have a diagnosis of cystic fibrosis or diabetes. We want to assess some factors (stomach movements, hormones and insulin release) that will allow us to determine, more precisely, the risk of individuals with CF developing diabetes. We intend to recruit 6 volunteers to take part in this study.
The Participant information sheet attached gives details of the study and how it would be carried out.
Your participation is entirely voluntary and you would be able to withdraw from the study at any time.

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

Participant Information Sheet

Factors responsible for the development of diabetes in adults with Cystic Fibrosis

We would like to invite you to take part in our research study. However, before you decide, we need you to understand the reason for the research and what it involves. You may talk to others about the study if you so wish. Part 1 tells you the purpose of the study and what to expect if you take part. Part 2 gives you more detailed information about the conduct of the study. Please ask if there is anything you do not understand. Take time to decide whether or not you wish to take part in this research study.

If you are willing to participate please telephone **Mrs. Patricia Stanley** (Pulmonary Function Administrator) on 01516001780 to indicate that you are interested in taking part in the study and this information will then be passed on to the Investigator of the study who will then contact you. A member of the Research Team will go through the information sheet with you and answer any questions you may raise

PART 1

Background

Cystic Fibrosis (CF) is the UK's most common life-threatening inherited disease and according to the CF Trust (UK) the disease affects over 8,500 people. The genetic defect is responsible for abnormal salt and water movements across cells causing thickened secretions. One organ that is affected is the pancreas that is responsible for the release of insulin. Diabetes mellitus is a well-recognised complication of cystic fibrosis, is unique and distinct, sharing features of other types of diabetes. The current recommendation is that a test called the oral glucose tolerance test be carried out to diagnose diabetes in CF. However, the test and its normal and abnormal values are drawn from a diabetic population who do not have CF. The exact nature of why diabetes develops in individuals with CF is not known, but is thought that both a defect in the release of insulin and a sensing of insulin by tissues may be responsible.

It is known that falls in weight and lung function occur prior to the development of diabetes and that diabetes in CF is associated with early death. It is also known that insulin enhances muscle mass and improves lung function, thereby improving quality of life.

What is the purpose of the study?

The main purposes of this research is to study the following:

1. Does the pancreas tire as the day progresses and does this contribute to the altered insulin release that is seen in people with CF.

2. Should a glucose tolerance test be done at a later time in the day, as against first thing in the morning as is currently done?

3. To determine if stomach movements affect the release of insulin?
4. To study the changes in hormones that affect insulin in response to a liquid and solid meal and patterns of change through the day

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

Why have I been invited?

You have been invited to take part in this study as you **do not** have cystic fibrosis or diabetes. We want to assess some factors (stomach movements, hormones and insulin release) that will allow us to determine, more precisely, the risk of individuals with CF developing diabetes. We intend to recruit 6 healthy Participants (controls) for this study, in order to compare the differences between individuals like yourself and those with CF.

Do I have to take part?

Your participation is completely voluntary, and you can withdraw your consent at any time without penalty.

If you choose to participate in this study you must sign the Informed Consent Form before your participation in the study is initiated. Read this information carefully before you decide if you want to participate. You have the right to have time for reflection before you give your informed consent. In addition to this information document you will receive a copy of the signed Informed Consent Form. If you need further information you can contact the study doctor.

What will happen to me if I take part?

- Reasonable expenses including travel costs incurred to participate in this study would be reimbursed for the duration you are in the study. Taking part in this study will involve 3 visits to the hospital. If you agree to take part in this study you will be asked to visit the clinic (visit 1) to discuss the study with the investigator, sign the consent form, talk about your medical history (including smoking, exercise, alcohol habits), your Cystic Fibrosis history (including date of diagnosis, previous and current CF medications), undergo a routine physical examination (including body weight , height blood pressure, heart rate and oxygen levels). You will need to undergo lung function testing (simple spirometry). At this visit you will be required to undergo one blood test to ensure your blood levels are not too low and you are not anaemic. This visit will last approximately 45 minutes.

If following completion of the screening test, you are still eligible to take part and wish to continue in the study, your participation may last up to a week.

We will be performing a number of tests which will require two further visits (visits 2 and 3) to hospital. These visits should occur within a week of each other, following your consent to take part in the project. Visit 2 will last approximately 3 hours and visit 3 will last approximately 12.5 hours.

With your permission, information will be gathered by the research team from your medical notes (medical history, medications, and investigation results) and your GP will be informed that you are taking part in this study.

The only people who will have access to your medical notes as part of this study are a few members of the research team who are qualified doctors or nurses working in the NHS, and the regulatory bodies overseeing the research.

This study has 2 distinct parts involving meals, blood tests and ultrasound scanning of the stomach to evaluate movements. These tests are described below:

- Oral and mixed meals: For both parts of the study you would need to come to the hospital after having fasted overnight (12 hours). For the first part of the study you will be given a glucose drink (75 grams of glucose in water) called Procal ® and blood tests and ultrasound

scanning (see below) will be performed over 2 hours. This then completes this part of the study.

Within a week you will be required to come back to have a similar test done and having fasted overnight (12 hours). This time instead of being asked to drink a liquid you will be given what is called a 'mixed meal' to complete within 10 minutes. This meal contains a certain amount of solid and liquid foods (37% carbohydrate, 18% protein, 44% fat) and blood samples will be collected over 2 hours. This test will be carried out twice more at 1300 and 1800 hrs to coincide with usual meal times. The usual meal would consist of a ham and cheese sandwich with butter, orange juice and digestive biscuits. You will need to fast between each meal.

- Blood tests: As part of this study 20 blood samples (each of 9 mls, overall 180 mls of blood) will be collected for analysis. For each part of the study (2 hour duration), one sample at the beginning followed by 4 samples at 30, 60, 90 and 120 minutes will be collected from a cannula (see below). These blood samples will be stored in the Biochemistry Laboratory at Liverpool Heart and Chest Hospital. Most of these tests will be performed at the above laboratory at Liverpool Heart and Chest Hospital, but with your permission, we may have to send some of these samples, to an outside laboratory for analysis.
- Ultrasound test: Ultrasound is a safe procedure. You will be asked to lie on your back or slightly towards your left-hand side during this scan, which may be uncomfortable. The scan will be carried out for 2-3 minutes following a blood sample collection to study the size of the stomach. This test will be done in both parts of the study.
- Inserting a cannula (Cannulation) (described below) in the arm or forearm would avoid the need for repetitive blood collection using needles. Usually 1 cannula is required for each study but occasionally the cannula does not work any more and you might need another one inserted. To ensure that the cannula remains patent 3-5mLs of Normal Saline would be used as a flush after insertion and after each blood collection point. At the end of each visit the cannula would be removed.

- A summary of investigations and time points is represented in the table below:

	Time Points	**Baseline**	**30 mins**	**60 mins**	**90 mins**	**120 mins**
Visit 1	**1 blood sample**					
Visit 2	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x
Visit 3	**Time Points**	**Baseline**	**30 mins**	**60 mins**	**90 mins**	**120 mins**
0800 – 1000 hrs	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x
1300 – 1500 hrs	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x
1800 – 2000 hrs	**Blood collection**	x	x	x	x	x
	Ultrasound study	x	x	x	x	x

- All your usual medications (if any) will continue as usual and you will be asked to take any routine medication at least one hour before the start of tests at the second and third visits. You will not be required to stop or withhold any medication.

What will I have to do?

If, after reading this leaflet, you wish to take part, one of our research team will speak with you to make sure that you understand what is involved. You will then be asked to sign a consent form.
We will arrange a mutually convenient time in order for us to carry out the study.

What are the possible disadvantages or risks of taking part?

- Inconvenience of having to come into hospital twice
- Inconvenience of having investigations
- Inconvenience of being contacted by the research team

The possible disadvantages and risks of the additional investigations are:

- Blood tests: There may be discomfort, bleeding and bruising as a result of having additional blood tests. Blood tests will only be taken by a member of staff qualified to do so using appropriate hospital equipment.

- Cannulation: a cannula is inserted using a needle similar to those used in taking standard blood tests. This may cause pain, bleeding or bruising. This is usually slightly more uncomfortable than a blood test but once the cannula is in place should not cause you any real discomfort. This will be placed by a member of staff trained to do so. This cannula will be removed once the test is complete unless you become unwell during the test and require this cannula for medical reasons. An additional risk of cannulation is infection. A previous study of hospital patients reported the risk of serious infection of 2/10,000. The risk should be less as part of this study as the cannula will only be in place for a short time.

What are the side effects of any treatment received when taking part?

You will only be asked to drink a glucose drink called Procal ® that is routinely used in tests to diagnose diabetes. No side effects have been reported. The components of the meal are a part of a usual Western diet.

What are the possible benefits of taking part?

The information we gather may help improve the management of people with cystic fibrosis who develop diabetes and identify those who are at an increased risk of developing diabetes in order to optimise lung function to the maximum possible.
As a result of the investigations we may discover that you have diabetes or are prone to developing diabetes, which may require further evaluation or treatment. Any abnormal results will be discussed with you and arrangements made for further management. Your GP may need to be informed or you may require referral to another clinic.

What happens when the research study stops?

If there is an important finding that has an implication in the way you should be managed, we may invite you to attend for further discussions/tests, if we think you are at increased risk of having diabetes.

What if there is a problem?

Any complaint about the way you have been treated during the study or any possible harm you may suffer will be addressed. Detailed information regarding this option is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practices and all information about you will be handled in confidence. The details are included in Part 2. If the information in Part 1 has interested you and you are considering participating, please read the additional information in Part 2 before making a decision.

PART 2

What if relevant new information becomes available?

On receiving new information, your doctor may consider it to be in your best interests to withdraw from further investigations in the study. For instance, if your blood tests suggest there is a reason why it may be unsafe to put you through the study. If anything untoward does arise your research doctor will explain the reasons and inform your GP.

Once the study is completed and you wish to know the results, we will be happy to discuss these with you once all the results have been analysed. You can contact a member of the research team to discuss these results if you wish.

What will happen if I don't want to carry on with the study?

If you wish to withdraw from the study, you can contact the Research Team to notify them whether you want to withdraw from the study entirely, or just from part of it.

- We will remove you from our follow-up database if you do not want to be contacted again.
- Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on you.
- We would be unable to destroy the results of investigations already performed as part of this study which may assist standard medical care.
- We would be unable to destroy any information from your general medical notes.

If you lose capacity or are unable to consent at any part of the study then we will not carry out any further part of the study. We would analyse samples that have been collected while you consented and had capacity to make that decision.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions and can be contacted via The Regional Adult CF Centre, Liverpool Heart and Chest Hospital on 0151 228 1616. This study is covered by The NHS Indemnity Scheme and the normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

With your consent, we would normally inform your general practitioner that you are participating in the study. Reports of these investigations will be placed in your medical notes. All patient information collected in the study will be stored on password protected computers or in locked rooms at Liverpool Heart and Chest Hospital. When the results of the study are reported, individuals who have taken part will not be identified in any way.
All data will remain confidential and no personal details will be made available to any third parties or transferred outside of the hospital. Details about you will be stored on computer during the research project but your data will only be reviewed by members of the research team. Blood will be stored in a secure storage facility at the Liverpool Heart and Chest Hospital NHS Trust. The data will only be used for research purposes in this project. No other use of the data will be undertaken without seeking your prior consent.

Involvement of the General Practitioner/Family doctor (GP)
Your GP will be informed only if any study investigations reveal information of which the GP should be made aware of.

What will happen to any samples I give?
Blood samples will be frozen and stored on-site in the biochemistry laboratory at Liverpool Heart and Chest Hospital NHS Trust. All blood samples will be handled as any other blood samples are at the Hospital, will be kept securely and only accessed by research and laboratory staff. All samples taken as part of this study will be taken and labelled in accordance with standard Trust policy. A code will link your data and samples through a name list. This list that links your name to the code will be kept by the chief investigator. This is to ensure that if the results are needed in the future for your routine clinical care then they can be referred back to and that we can be sure the results apply to the correct participant. Some blood tests may need to be sent to different laboratories and if this is needed then your name and date of birth will be included on the sample.

Only the investigator for the study will have access to the results and if the tests show information of clinical significance, a member of the research team will inform you of the results in person in clinic and explain any action needed.

We will not routinely inform you of the results of tests. If you agree to take part in the study, we will ask you to gift any samples we take so that we may use them for future research. Serum samples obtained from blood tests will be stored for up to 10 years in a tissue bank approved under the Human Tissue Act 2004. At the end of this time the samples will be destroyed.
When all the participants have been recruited we may analyse stored blood samples taken during the study for additional biomarkers; these again would be biomarkers related to diabetes or cystic fibrosis. The storage of blood and tissue samples are guided by strict regulations and would be stored in accordance with this.

Will any genetic tests be done?
No

What will happen to the results of the research study?
You will not be personally identified in any report or publication. The results of the study will be disseminated via electronic and paper medical journals as well as presentations in various major medical societies throughout the world.

Who is organising and funding the research?

Liverpool Heart and Chest Hospital NHS
NHS Foundation Trust

The study is being funded by in-house funds from Liverpool Heart and Chest Hospital NHS Trust. The research group performing this research study has no conflict of interests. Your research nurse/doctor will not receive any additional money for including you in this study.

Who has reviewed the study?

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Further information and contact details

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Dr Dilip Nazareth, Clinical Research Fellow, Principal Investigator

Dr Kamlesh Mohan, Consultant Chest Physician

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Further information about research in general can be found at the National Institute for Health Research, at the following web address: www.nihr.ac.uk/research

If you develop any concerns about the study, you can contact any member of the research team above.

Appendix 7

DRG® Insulin ELISA (EIA-2935)

Revised 13 Dec. 2013 rm (Vers. 8.1)

IVD

1 INTRODUCTION

1.1 Intended Use

The **DRG Insulin ELISA** is an enzyme immunoassay for the quantitative *in vitro diagnostic* measurement of Insulin in serum and plasma

1.2 Summary and Explanation

Insulin is the principal hormone responsible for the control of glucose metabolism. It is synthesized in the β-cells of the islets of Langerhans as the precursor, proinsulin, which is processed to form C-peptide and insulin. Both are secreted in equimolar amounts into the portal circulation. The mature insulin molecule comprises two polypeptide chains, the A chain and B chain (21 and 30 amino acids respectively). The two chains are linked together by two inter-chain disulphide bridges. There is also an intra-chain disulphide bridge in the A chain.
Secretion of insulin is mainly controlled by plasma glucose concentration, and the hormone has a number of important metabolic actions. Its principal function is to control the uptake and utilisation of glucose in peripheral tissues via the glucose transporter. This and other hypoglycaemic activities, such as the inhibition of hepatic gluconeogenesis and glycogenolysis are counteracted by the hyperglycaemic hormones including glucagon, epinephrine (adrenaline), growth hormone and cortisol.
Insulin concentrations are severely reduced in insulin-dependent diabetes mellitus (IDDM) and some other conditions such as hypopituitarism. Insulin levels are raised in non-insulin-dependent diabetes mellitus (NIDDM), obesity, insulinoma and some endocrine dysfunctions such as Cushing's syndrome and acromegaly.

2 PRINCIPLE OF THE TEST

The DRG Insulin ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle.
The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the Insulin molecule.
An aliquot of patient sample containing endogenous Insulin is incubated in the coated well with enzyme conjugate, which is an anti-Insulin antibody conjugated with Biotin. After incubation the unbound conjugate is washed off.
During the second incubation step Streptavidin Peroxidase Enzyme Complex binds to the biotin-anti-Insulin antibody.
The amount of bound HRP complex is proportional to the concentration of Insulin in the sample.
Having added the substrate solution, the intensity of colour developed is proportional to the concentration of Insulin in the patient sample.

3 WARNINGS AND PRECAUTIONS

1. This kit is for in vitro diagnostic use only. For professional use only.
2. All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
3. Before starting the assay, read the instructions completely and carefully. Use the valid version of instructions for use provided with the kit. Be sure that everything is understood.
4. The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided.
5. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
6. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution coloured. Do not pour reagents back into vials as reagent contamination may occur.
7. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
8. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
9. Allow the reagents to reach room temperature (21 °C - 26 °C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
10. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
11. Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
12. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
13. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
14. Do not use reagents beyond expiry date as shown on the kit labels.
15. All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiter plate readers.
16. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
17. Avoid contact with *Stop Solution* containing 0.5 M H_2SO_4. It may cause skin irritation and burns.
18. Some reagents contain Proclin 300, BND and/or MIT as preservatives. In case of contact with eyes or skin, flush immediately with water.
19. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
20. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
21. For information on hazardous substances included in the kit please refer to Material Safety Data Sheets. Material Safety Data Sheets for this product are available upon request directly from DRG.

4 REAGENTS

4.1 Reagents provided

1. ***Microtiterwells***, 12 x 8 (break apart) strips, 96 wells;
   Wells coated with anti-Insulin antibody (monoclonal).
2. ***Zero Standard***, 1 vial, 3 mL, ready to use
   0 µIU/mL
   Contains non-mercury preservative.
3. ***Standard (Standard 1-5)***, 5 vials, 1 mL, ready to use;
   Concentrations: 6.25 - 12.5 – 25 - 50 and 100 µIU/mL,
   Conversion: µIU/mL x 0.0433 = ng/mL,
   ng/mL x 23.09 = µIU/mL
   The standards are calibrated against international WHO approved Reference material NIBSC 66/304.;
   Contain non-mercury preservative.
4. ***Enzyme Conjugate***, 1 vial, 5 mL, ready to use,
   mouse monoclonal anti-Insulin conjugated to biotin;
   Contains non-mercury preservative.
5. ***Enzyme Complex***, 1 vial, 7 mL, ready to use,
   Streptavidin-HRP Complex
   Contains non-mercury preservative.
6. ***Substrate Solution***, 1 vial, 14 mL, ready to use,
   Tetramethylbenzidine (TMB).
7. ***Stop Solution***, 1 vial, 14 mL, ready to use,
   contains 0.5 M H_2SO_4,
   Avoid contact with the stop solution. It may cause skin irritations and burns.
8. ***Wash Solution***, 1 vial, 30 mL (40X concentrated),
   see „Preparation of Reagents“.

Note: Additional Zero *Standard* for sample dilution is available upon request.

4.2 Materials required but not provided

- A microtiter plate calibrated reader (450 ± 10 nm) (e.g. the DRG Instruments Microtiter Plate Reader).
- Calibrated variable precision micropipettes.
- Absorbent paper.
- Distilled or deionised water
- Timer
- Graph paper or software for data reduction

4.3 Storage Conditions

When stored at 2 °C to 8 °C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.
Opened reagents must be stored at 2 °C to 8 °C. Microtiter wells must be stored at 2 °C to 8 °C. Once the foil bag has been opened, care should be taken to close it tightly again.
Opened kits retain activity for 8 weeks if stored as described above.

4.4 Reagent Preparation

Bring all reagents and required number of strips to room temperature prior to use.

Wash Solution

Add deionized water to the 40X concentrated Wash Solution.
Dilute 30 mL of concentrated *Wash Solution* with 1170 mL deionized water to a final volume of 1200 mL.
The diluted Wash Solution is stable for 2 weeks at room temperature.

4.5 Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheet.

4.6 Damaged Test Kits

In case of any severe damage to the test kit or components, DRG has to be informed in writing, at the latest, one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5 SPECIMEN COLLECTION AND PREPARATION

Serum or plasma (only heparin- or citrate plasma) can be used in this assay.
Do not use haemolytic, icteric or lipaemic specimens.
Please note: Samples containing sodium azide should not be used in the assay.

5.1 Specimen Collection

Serum:

Collect blood by venipuncture (e.g. Sarstedt Monovette for serum), allow to clot, and separate serum by centrifugation at room temperature. Do not centrifuge before complete clotting has occurred. Patients receiving anticoagulant therapy may require increased clotting time.

Plasma:

Whole blood should be collected into centrifuge tubes containing anti-coagulant (e.g. Sarstedt Monovette with the appropriate plasma preparation) and centrifuged immediately after collection.

5.2 Specimen Storage and Preparation

Specimens should be capped and may be stored for up to 5 days at 2 °C to 8 °C prior to assaying.
Specimens held for a longer time (at least one year) should be frozen only once at -20 °C prior to assay. Thawed samples should be inverted several times prior to testing.

5.3 Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with *Zero Standard* and re-assayed as described in Assay Procedure.
For the calculation of the concentrations this dilution factor has to be taken into account.

Example:
a) dilution 1:10: 10 µL sample + 90 µL *Zero Standard* (mix thoroughly)
b) dilution 1:100: 10 µL dilution a) 1:10 + 90 µL *Zero Standard* (mix thoroughly).

6 ASSAY PROCEDURE

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

6.2 Test Procedure

Each run must include a standard curve.

1. Secure the desired number of Microtiter wells in the frame holder.
2. Dispense **25 µL** of each ***Standard, control*** and **samples** with new disposable tips into appropriate wells.
3. Dispense **25 µL** ***Enzyme Conjugate*** into each well.
   Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
4. Incubate for **30 minutes** at room temperature.
5. Briskly shake out the contents of the wells.
   Rinse the wells **3 times** with diluted *Wash Solution* (400 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6. Add **50 µL** of ***Enzyme Complex*** to each well.
7. Incubate for **30 minutes** at room temperature.
8. Briskly shake out the contents of the wells.
   Rinse the wells **3 times** with diluted *Wash Solution* (400 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.
9. Add **50 µL** of ***Substrate Solution*** to each well.
10. Incubate for **15 minutes** at room temperature.
11. Stop the enzymatic reaction by adding **50 µL** of ***Stop Solution*** to each well.
12. Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader.
    It is recommended that the wells be read **within 10 minutes** after adding the *Stop Solution*.

6.3 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the Instructions for Use have been calculated automatically using a 4-Parameter curve fit. (4 Parameter Rodbard or 4 Parameter Marquardt are the preferred methods.)
   Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as > 100 µIU/mL. For the calculation of the concentrations this dilution factor has to be taken into account.

6.3.1 Example of Typical Standard Curve

The following data is for demonstration only and **cannot** be used in place of data generations at the time of assay.

Standard	Optical Units (450 nm)
Standard 0 (0 µIU/mL)	0.03
Standard 1 (6.25 µIU/mL)	0.07
Standard 2 (12.5 µIU/mL)	0.14
Standard 3 (25 µIU/mL)	0.35
Standard 4 (50 µIU/mL)	0.88
Standard 5 (100 µIU/mL)	2.05

7 EXPECTED NORMAL VALUES

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

In a study conducted with apparently normal healthy adults, using the DRG Insulin ELISA the following values are observed:

2 µIU/mL to 25 µIU/mL

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

8 QUALITY CONTROL

Good laboratory practice requires that controls be run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance.
It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.
The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.
It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.
Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid.
In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.
After checking the above mentioned items without finding any error contact your distributor or DRG directly.

9 PERFORMANCE CHARACTERISTICS

9.1 Assay Dynamic Range

The range of the assay is between 1.76 – 100 µIU/mL.

9.2 Specificity of Antibodies (Cross Reactivity)

The cross reactivities were determinated by addition of different analytes to serum containing 4 ng/mL (≅ 100 µIU/mL) Insulin and measuring the apparent Insulin concentration.

Added analyte to a high value serum (4 ng/mL)		Observed Insulin value (ng/mL)	Cross reaction (%)
Porcine Insulin	8 ng/mL	17	> 100
Bovine Insulin	8 ng/mL	17.8	> 100
Dog Insulin	16 ng/mL	17.2	82
Rabbit Insulin	16 ng/mL	14.1	63
Rat Insulin	16 ng/mL	4.0	0
Human Proinsulin	32 ng/mL	4.1	0
Porcine Proinsulin	16 ng/mL	4.0	0
Bovine Proinsulin	16 ng/mL	4.1	0

9.3 Sensitivity

The analytical sensitivity of the DRG ELISA was calculated by adding 2 standard deviations to the mean of 20 replicate analyses of the Zero Standard and was found to be 1.76 µIU/mL.

9.4 Reproducibility

9.4.1 Intra-Assay

The within assay variability is shown below:

Sample	n	Mean (µIU/mL)	CV (%)
1	20	17.5	2.6
2	20	66.4	1.8

9.4.2 Inter-Assay

The between assay variability is shown below:

Sample	n	Mean (µIU/mL)	CV (%)
1	12	17.4	2.9
2	12	66.9	6.0

9.5 Recovery

Samples have been spiked by adding Insulin solutions with known concentrations in a 1:1 ratio.
The expected values were calculated by addition of half of the values determined for the undiluted samples and half of the values of the known solutions. The % Recovery has been calculated by multiplication of the ratio of the measurements and the expected values with 100.

Sample	Added Concentration 1:1 (v/v) (µIU/mL)	Measured Conc. (µIU/mL)	Expected Conc. (µIU/mL)	Recovery (%)
1		21.2		
	100	66.4	60.6	109.6
	50	38.8	35.6	108.9
	25	23.4	23.1	101.1
	12.5	17.37	16.9	102.9
2		69.0		
	100	84.6	84.5	100.1
	50	58.4	59.5	98.1
	25	43.2	47.0	91.8
	12.5	37.5	40.8	91.9

9.6 Linearity

Sample	Dilution	Measured Conc. (µIU/mL)	Expected Conc. (µIU/mL)	Recovery (%)
1	None	21.2	21.2	
	1:2	9.4	10.6	88.5
	1:4	5.2	5.3	98.5
	1:8	2.8	2.7	105.9
	1:16	1.5	1.3	110.3
2	None	69.0	69.0	
	1:2	30.5	34.5	88.4
	1:4	17.6	17.3	102.0
	1:8	8.7	8.6	101.2
	1:16	4.8	4.3	110.4

10 LIMITATIONS OF USE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

Haemoglobin (up to 4 mg/mL), bilirubin (up to 0.5 mg/mL) and triglyceride (up to 30 mg/mL) have no influence on the assay results.

10.2 Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of Insulin in a sample.

10.3 High-Dose-Hook Effect

No hook effect was observed in this test up to 1600 µIU/mL of Insulin.

11 LEGAL ASPECTS

11.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.
The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact DRG.

11.2 Therapeutic Consequences

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 11.1. Any laboratory result is only a part of the total clinical picture of a patient.
Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived.
The test result itself should never be the sole determinant for deriving any therapeutic consequences.

11.3 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.
Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2. are also invalid.
Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

12 REFERENCES

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Rev. 12/12/13cc

Appendix 8

DRG® C-Peptide ELISA (EIA-1293)

CE

Revised 5 Sept. 2012 rm (Vers. 11.1)

IVD

1 INTRODUCTION

1.1 Intended Use

The **DRG C-Peptide ELISA** is an enzyme immunoassay for the quantitative *in vitro diagnostic* measurement of C-Peptide in serum, plasma and urine

1.2 Summary and Explanation

Insulin is synthesized in the pancreatic beta cells as a 6000 MW component of an 86 amino acid polypeptide called proinsulin (1, 2, 3). Proinsulin is subsequently cleaved enzymatically, releasing insulin into the circulation along with a residual 3000 MW fragment called connection ("C") peptide, so-named because it connects A and B chains of insulin within the proinsulin molecule (1, 2, 3, 4). Human C-Peptide, a 31 amino acid residue peptide, has a molecular mass of approximately 3000 daltons. C-Peptide has no metabolic function. However, since C-Peptide and insulin are secreted in equimolar amounts, the immunoassay of C-Peptide permits the quantitation of insulin secretion (4, 5, 6). This is the reason for the clinical interest of serum and urinary determinations of C-Peptide. Moreover, C-Peptide measurement has several advantages over immunoassays of insulin.

The half-life of C-Peptide in the circulation is between two and five times longer than that of insulin (7). Therefore, C-Peptide levels are a more stable indicator of insulin secretion than the more rapidly changing levels of insulin. A very clear practical advantage of C-Peptide measurement arising from its relative metabolic inertness as compared to insulin is that C-Peptide levels in peripheral venous blood are about 5-6 times greater than insulin levels (3). Also, relative to an insulin assay, the C-Peptide assay's advantage is its ability to distinguish endogenous from injected insulin.

Thus, low C-Peptide levels are to be expected when insulin is diminished (as in insulin-dependent diabetes) or suppressed (as a normal response to exogenous insulin), whereas elevated C-Peptide levels may result from the increased β-cell activity observed in insulinomas (3, 6, 9).

C-Peptide has also been measured as an additional means for evaluating glucose tolerance and glibenclamide glucose tests (2, 3, 9, 10).

C-Peptide levels are in many ways a better measurement of endogenous insulin secretion than peripheral insulin levels. C-Peptide may be measured in either blood or urine (9). With improved sensitive C-Peptide immunoassays, it is now possible to measure C-Peptide values at extremely low levels. The clinical indications for C-Peptide measurement include diagnosis of insulinoma and differentation from factitious hypoglycemia, follow-up of pancreatectomy, and evaluation of viability of islet cell transplants (11, 12, 13). Recently, these indications have been dramatically expanded to permit evaluation of insulin dependence in maturity onset diabetes mellitus.

1.3 Clinical Indications for the DRG C-Peptide ELISA

- Assessment of residual β-cell function in diabetics under insulin therapy
- Detection and monitoring of the remission phase of type I diabetes
- Adjunct in the differential diagnosis between type I (insulin dependent) and type II (non-insulin-dependent) diabetes
- Diagnosis of insulin-induced factitious hypoglycemia.
- Contribution to the diagnosis of insulinoma (insulin suppression test)
- Prognostic index of fetal outcome in pregnant diabetic women
- Evaluation of insulin secretion in liver disease
- Monitoring of pancreasectomy

2 PRINCIPLE OF THE TEST

The DRG C-Peptide ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding.
The microtiter wells are coated with anti-mouse antibody, which binds a monoclonal antibody directed towards a unique antigenic site on the C-Peptide molecule. Endogenous C-Peptide of a patient sample competes with a C-Peptide-horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off.
The amount of bound peroxidase conjugate is inversely proportional to the concentration of C-Peptide in the sample. After addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of C-Peptide in the patient sample.

3 WARNINGS AND PRECAUTIONS

1. This kit is for in vitro diagnostic use only. For professional use only.
2. All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
3. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
4. The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided.
5. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
6. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
7. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
8. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
9. Allow the reagents to reach room temperature (21 °C – 26 °C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
10. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
11. Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
12. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
13. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
14. Do not use reagents beyond expiry date as shown on the kit labels.
15. All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
16. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
17. Avoid contact with *Stop Solution* containing 0.5 M H_2SO_4. It may cause skin irritation and burns.
18. Some reagents contain Proclin 300, BND and/or MIT as preservatives. In case of contact with eyes or skin, flush immediately with water.

19. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
20. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
21. For information on hazardous substances included in the kit please refer to Material Safety Data Sheets. Material Safety Data Sheets for this product are available upon request directly from DRG.

4 REAGENTS

4.1 Reagents provided

1. ***Microtiterwells***, 12 x 8 (break apart) strips, 96 wells;
   Wells coated with anti-mouse-antibody
2. ***Standard (Standard 0-5)***, 6 vials, lyophilized, 0.75 mL
   Concentrations: 0 – 16 ng/mL (see exact value on the vial label or on the QC-Datasheet).
   The standards are calibrated against WHO approved Reference material IRR C-Peptide, code 84/510.
   see „Preparation of Reagents“
   Contain non-mercury preservative.
3. ***Sample Diluent***, 1 vial, 3 mL, ready to use,
   Contains non-mercury preservative.
4. **Antiserum**, 1 vial, 7 mL, ready to use
   monoclonal mouse anti C-Peptide antibody
   Contains non-mercury preservative.
5. **Enzyme Conjugate**, 1 vial, 14 mL, ready to use
   biotinylated C-Peptide
   Contains non-mercury preservative.
6. **Enzyme Complex**, 1 vial, 14ml, ready to use
   contains horseradish Peroxidase
   Contains non-mercury preservative.
7. **Substrate Solution**, 1 vial, 14 mL, ready to use
   TMB
8. **Stop Solution**, 1 vial, 14 mL, ready to use
   contains 0.5 M H_2SO_4
   Avoid contact with the stop solution. It may cause skin irritations and burns.
9. **Wash Solution**, 1 vial, 30 mL (40X concentrated)
   see „Preparation of Reagents“

Note: Additional *Sample Diluent* for sample dilution is available upon request.

4.2 Materials required but not provided

- A microtiter plate calibrated reader (450 ± 10 nm) (e.g. the DRG Instruments Microtiter Plate Reader).
- Calibrated variable precision micropipettes.

- Absorbent paper.
- Distilled or deionized water
- Timer
- Semi logarithmic graph paper or software for data reduction

4.3 Storage Conditions

When stored at 2 °C to 8 °C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.
Opened reagents must be stored at 2 °C to 8 °C. Microtiter wells must be stored at 2 °C to 8 °C. Once the foil bag has been opened, care should be taken to close it tightly again.
Reagent Preparation
Bring all reagents and required number of strips to room temperature prior to use.

4.4 Reagent Preparation

Standards
Reconstitute the lyophilized contents of the standard vial with 0.75 mL Aqua dest.
Note: *The reconstituted standards are stable for 3 days at 2 °C to 8 °C.*
For longer storage the reconstituted standards should be aliquoted and stored at -20 °C.

Wash Solution
Add deionized water to the 40X concentrated Wash Solution.
Dilute 30 mL of concentrated *Wash Solution* with 1170 mL deionized water to a final volume of 1200 mL.
The diluted Wash Solution is stable for 2 weeks at room temperature.

4.5 Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheet.

4.6 Damaged Test Kits

In case of any severe damage to the test kit or components, DRG has to be informed in writing, at the latest, one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5 SPECIMEN COLLECTION AND PREPARATION

Serum, plasma (EDTA-, heparin- or citrate plasma) or urine can be used in this assay.
Do not use haemolytic, icteric or lipaemic specimens.
Please note: Samples containing sodium azide should not be used in the assay.

5.1 Specimen Collection

Serum:
Collect blood by venipuncture (e.g. Sarstedt Monovette for serum), allow to clot, and separate serum by centrifugation at room temperature. Do not centrifuge before complete clotting has occurred. Patients receiving anticoagulant therapy may require increased clotting time.

Plasma:
Whole blood should be collected into centrifuge tubes containing anti-coagulant (e.g. Sarstedt Monovette with the appropriate plasma preparation) and centrifuged immediately after collection.

Urine:
The total volume of urine excreted during a 24 hour period should be collected and mixed in a single container.
Note: Specimens should be stored at 2 °C - 8 °C during collection period and total volume collected should be recorded.

5.2 Specimen Storage and Preparation

Serum / Plasma:
Specimens should be capped and may be stored for up to 24 hours at 2 °C to 8 °C prior to assaying.
Specimens held for a longer time should be frozen only once at -20 °C prior to assay. Thawed samples should be inverted several times prior to testing.

Urine:
Aliquot a well-mixed sample to be used in the assay. Centrifuge sample to clear. Urine samples may be stored for up to 36 hours at 2 °C - 8 °C prior to assaying.
Specimens held for a longer time should be frozen only once at -20 °C prior to assay.

5.3 Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with *Sample Diluent* and reassayed as described in Assay Procedure.
For the calculation of the concentrations this dilution factor has to be taken into account.
Example:
a) dilution 1:10: 10 µL Serum + 90 µL *Sample Diluent* (mix thoroughly)
b) dilution 1:100: 10 µL dilution a) 1:10 + 90 µL *Sample Diluent* (mix thoroughly).

Urine Samples
Prior to use dilute urine samples **1:20** with *Sample Diluent*.

If the Sample Diluent included in the kit is insufficient, you can order additional *Sample Diluent* (40 mL vial) with REF.: EIA-1293DIL

6 ASSAY PROCEDURE

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

6.2 Test Procedure

Each run must include a standard curve.

1. Secure the desired number of Microtiter wells in the frame holder.
2. Dispense **100 µL** of each Standard, controls and samples with new disposable tips into appropriate wells.
3. Dispense **50 µL** Antiserum into each well
4. Dispense **100 µL** Enzyme Conjugate into each well.
   Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
5. Incubate for **60 minutes** at room temperature with shaking (400 - 500 rpm).
6. Briskly shake out the contents of the wells.
   Rinse the wells 3 times with diluted Wash Solution (400 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
7. Add **100 µL** of Enzyme Complex to each well.
8. Incubate for **30 minutes** at room temperature with shaking (400 - 500 rpm).
9. Briskly shake out the contents of the wells.
   Rinse the wells 3 times with diluted Wash Solution (400 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.
10. Add **100 µL** of Substrate Solution to each well.
11. Incubate for **20 minutes** at room temperature.
12. Stop the enzymatic reaction by adding **100 µL** of Stop Solution to each well.
13. Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader.
    It is recommended that the wells be read **within 10 minutes** after adding the *Stop Solution*.

6.3 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using semi-logarithmic graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as > 16 ng/mL. For the calculation of the concentrations this dilution factor has to be taken into account.

6.3.1 Example of Typical Standard Curve

The following data is for demonstration only and **cannot** be used in place of data generations at the time of assay.

Standard	Optical Units (450 nm)
Standard 0 (0 ng/mL)	1.82
Standard 1 (0.2 ng/mL)	1.64
Standard 2 (0.7 ng/mL)	1.46
Standard 3 (2.0 ng/mL)	1.02
Standard 4 (6.0 ng/mL)	0.47
Standard 5 (16 ng/mL)	0.21

7 EXPECTED NORMAL VALUES

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

In a study conducted with apparently normal healthy adults, using the DRG C-Peptide ELISA the following values are observed:

	n	Mean ± 2SD
Serum (Post 12-hour Fasting)	60	0.5 – 3.2 ng/mL
Urine		1 - 200 µg/day

he results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

8 QUALITY CONTROL

Good laboratory practice requires that controls be run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance.
It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.
The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.
It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.
Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid.
In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.
After checking the above mentioned items without finding any error contact your distributor or DRG directly.

9 PERFORMANCE CHARACTERISTICS

9.1 Assay Dynamic Range

The range of the assay is between 0.06 – 16 ng/mL.

9.2 Specificity of Antibodies (Cross Reactivity)

The cross-reactivity of intact or split-Proinsulin is clinically not significant.

9.3 Sensitivity

The analytical sensitivity of the DRG ELISA was calculated by subtracting 2 standard deviations from the mean of 20 replicate analyses of the Zero Standard (S0) and was found to be be 0.064 ng/mL.

9.4 Reproducibility

9.4.1 Intra Assay

The within assay variability is shown below:

Sample	n	Mean (ng/mL)	CV (%)
1	20	0.48	6.54
2	20	2.30	6.70
3	20	3.86	5.13

9.4.2 Inter Assay

The between assay variability is shown below:

Sample	n	Mean (ng/mL)	CV (%)
1	12	0.42	9.33
2	12	2.05	9.92
3	12	4.23	8.38

9.5 Recovery

Samples have been spiked by adding C-Peptide solutions with known concentrations in a 1:1 ratio.
The % Recovery has been calculated by multiplication of the ratio of the measurements and the expected values with 100.

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Serum Sample	Endogenus C-Peptide ng/mL	Added C-Peptide ng/mL	Measured Conc. ng/mL	Expected Conc ng/mL	Recovery (%)
1	5.36	0.00	5.36		
		8.00	10.31	10.68	96.6
		3.00	5.57	5.68	98.0
		1.00	3.63	3.68	98.7
		0.35	3.08	3.03	101.8
2	9.70	0.00	9.70		
		8.00	12.49	12.85	97.2
		3.00	8.23	7.85	104.8
		1.00	5.15	5.85	87.9
		0.35	4.54	5.20	87.2
3	12.12	0.00	12.12		
		8.00	15.52	14.06	110.4
		3.00	9.72	9.06	107.3
		1.00	7.30	7.06	103.4
		0.35	5.65	6.41	88.1

Urine Sample	Endogenous C-Peptide (ng/mL)	Added Conc. 1:1 (v/v) (ng/mL)	Measured Conc. (ng/mL)	Expected Conc. (ng/mL)	Recovery (%)
1	2.1				
		8.0	10.9	10.1	107.9
		3.0	5.57	5.1	109.2
		1.0	2.6	2.62	99.2
2	1.01				
		8.0	9.2	9.01	102.1
		3.0	4.03	4.01	100.5
		1.0	2.2	2.01	109.5
3	2.5				
		8.0	10.1	10.5	96.2
		3.0	5.3	5.5	96.4
		1.0	3.8	3.5	108.6

9.6 Linearity

Sample	Dilution	Measured Conc. ng/mL	Expected Conc. ng/mL	Recovery (%)
1	undil	6.10	6.10	
Serum	1 : 2	3.25	3.05	106.7
	1 : 4	1.61	1.52	105.3
	1 : 8	0.84	0.76	110.6
	1:16	0.41	0.38	107.6
2	undil	9.90	9.90	
Serum	1 : 2	5.59	4.95	112.8
	1 : 4	2.48	2.48	100.3
	1 : 8	1.29	1.24	104.0
	1:16	0.69	0.62	111.8
3	undil	13.25	13.25	
Serum	1 : 2	6.97	6.62	105.1
	1 : 4	3.22	3.31	97.1
	1 : 8	1.70	1.66	102.8
	1:16	0.85	0.83	103.1

Urine Sample	Dilution	Measured Conc. ng/mL	Expected Conc. ng/mL	Recovery (%)
1	undil	8.7	8.7	
	1 : 2	4.29	4.35	98.6
	1 : 4	2.01	2.18	92.4
	1 : 8	1.09	1.09	100.2
2	undil	9.2	9.2	
	1 : 2	4.7	4.6	102.2
	1 : 4	2.25	2.3	97.8
	1 : 8	1.12	1.15	97.5
3	undil	13.9	13.9	
	1 : 2	6.6	6.95	95.0
	1 : 4	3.3	3.48	95.0
	1 : 8	1.8	1.74	103.6

10 LIMITATIONS OF USE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

Haemoglobin (up to 4 mg/mL), Bilirubin (up to 0.5 mg/mL) and Triglyceride (up to 30 mg/mL) have no influence on the assay results.

10.2 Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of C-Peptide in a sample.

10.3 High-Dose-Hook Effect

No hook effect was observed in this test.

11 LEGAL ASPECTS

11.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.
The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact DRG.

11.2 Therapeutic Consequences

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 11.1. Any laboratory result is only a part of the total clinical picture of a patient.
Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived.
The test result itself should never be the sole determinant for deriving any therapeutic consequences.

11.3 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.
Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2. are also invalid.
Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

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