INVESTIGATING THE POTENTIAL OF A METABOLOMIC BIOMARKER FOR PANCREATIC EXOCRINE INSUFFICIENCY

By

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Abstract

Pancreatic exocrine insufficiency (PEI) is a reduction in pancreatic enzymes to a level below that required to maintain normal digestion. It is a consequence of many diseases, leading to malnutrition, impaired of quality of life and poor clinical outcomes. Diagnosis of PEI is challenging. This thesis investigates the potential for a novel diagnostic measure of PEI using metabolomics as a discovery tool.

The use of metabolomics, the study of the small products of metabolism, in developing novel biomarkers is a rapidly expanding field. This body of work is based on the hypothesis that: *A fatty test meal will create a dynamic, measurable response in the food metabolome and that response will be different in those with PEI compared to healthy controls.* To test this hypothesis, a trial called DETECTION was designed to obtain fed and fasted plasma samples from patients with PEI alongside healthy controls and use untargeted metabolomics to explore differences in their metabolomic response to a test meal. To accurately determine PEI status, the ^{13}Carbon mixed triglyceride breath test (^{13}C MTGT) has been used alongside other markers of PEI. Much of this work has been directed towards developing and refining the ^{13}C MTGT for use alongside blood metabolite analysis.

In summary, I have created an evidence-based protocol and novel test meal for the ^{13}C MTGT that is compatible with post-prandial metabolite analysis. With the results of the DETECTION trial PEI screen, I have evaluated the prevalence of PEI in various patient groups and explored concordance between existing diagnostic tests for PEI. Finally, I have compared the post-prandial metabolome of those with and without PEI and found significant differences that have the potential to be used as a biomarker for PEI, this is novel, ground-breaking and has the potential to greatly improve diagnosis and treatment of PEI.

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Finally, a special thank you to my Husband and my parents for all their help. These four years have taken us through so much and I wouldn't change a second.

Dedication

I dedicate this work to my sister, Annie, who despite a very troubled mind is still the most unconditionally kind person I know.

Table of Contents

List of Figures

List of Tables

List of abbreviations

PEI	Pancreatic exocrine insufficiency
PERT	Pancreatic enzyme replacement therapy
CP	Chronic pancreatitis
CF	Cystic fibrosis
NET	Neuroendocrine tumour
SSA	Somatostatin analogue
^{13}CMTG	13C Mixed Triglyceride ([13C]2-octanoyl,1,3-distearin)
^{13}CMTGT	13C Mixed Triglyceride Test
cPDR	Cumulative percent dose recovered
FE-1	Faecal elastase-1
SSA	Somatostatin analologue
PDAC	Pancreatic ductal adenocarcinoma
CRP	C-reactive protein
IAPP	Islet amyloid polypeptide
DP	Distal pancreatectomy
PD	Pancreaticoduodenectomy (Whipple's)
PPPD	Pylorus preserving pancreaticoduodenectomy
TP	Total pancreatectomy
CCK	Cholecystokinin
PRO	Patient reported outcome
CFA	coefficient of fat absorption
USP	United States Pharmacopeia
IU	International units
QOL	Quality of life
HMBD	Human Metabolite Database
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
LC-MS	Liquid Chromatography Mass Spectrometry
GC-MS	Gas Chromatography Mass Spectrometry
RT	Retention time
m/z	Mass to charge ratio
QA	Quatlity assurance
UoB	University Of Birmingham
ECMC	Experimental Cancer Medicine Centre
NSA	Non substantial amendment
IRAS	Integrated Research Application System
HPB	Hepatopancreatobiliary
UHB	University Hospitals Birmingham
PPIE	Patient and Public Involvement and Engagement
CRAG	Clinical Research Ambassadors Group
PIL	Patient information leaflet
SOP	Standard operating procedures
BMI	Body mass index
SR	Systematic review
MA	Meta-analysis
ACF	Ante-cubital fossa
HC	Healthy control
UHPLC-MS	Ultrahigh performance liquid chromatography mass spectrometry
HILIC	Hydrophilic interaction liquid chromatography
QC	Quality control
PB	Process Blank
FWHM	Full width at half maximum
RF	Radiofrequency
XCMS	Xml cryptographic message syntax (bioinformatics software for analysing MS data)
BEAMSpy	Birmingham mEtabolite Annotation for Mass SPectrometrY
KNN	K-nearest neighbour
PC	Principle component

Publications arising from this work as 1st or 2nd Author. (For full list, including presentations, see Appendix 9)

Powell-Brett S, Halle-Smith J, Hall LA, Hodson J, Phillips ME, Roberts K. Comprehensive, long-term evaluation of pancreatic exocrine insufficiency after pancreatoduodenectomy: Accepted Pancreatology 01/11/2023. Included in results Section 4.5

Hall LA, Powell-Brett S, Halle-Smith J, Ward L, Wiggins T, Markar SR, Roberts KJ. Pancreatic exocrine insufficiency after non-pancreatic upper gastrointestinal surgery: meta-analysis. Br J Surg. 2023 Dec 8:znad369. Doi: 10.1093/bjs/znad369. Epub ahead of print. PMID: 38064682. Results not included in thesis.

Powell-Brett S, Hall LA, Roberts KJ. A standardised nutritional drink as a test meal for the ^{13}C mixed triglyceride breath test for pancreatic exocrine insufficiency: A randomised, two-arm crossover comparative study. J Hum Nutr Diet. 2023 Sep 18. Doi: 10.1111/jhn.13237. PMID: 37723653. Included in results section 3.3

Hall LA, Powell-Brett S, Thompson O, Smith D, Bradley E, Smith S, Vickrage S, Kemp-Blake J, Roberts KJ, Shah T. Casting a Wider NET: Pancreatic Exocrine Insufficiency Induced by Somatostatin Analogues among Patients with Neuroendocrine Tumours? Cancers (Basel). 2023 Mar 23;15(7):1933. doi: 10.3390/cancers15071933. PMID: 37046594; PMCID: PMC10093494. Included in results section 4.4

Powell-Brett S, Hall L, Edwards M, Roberts K. A systematic review and meta-analysis of the accuracy and methodology of the 13C mixed triglyceride breath test for the evaluation of pancreatic function. Pancreatology. 2023 Apr;23(3):283-293. doi: 10.1016/j.pan.2023.02.004. Epub 2023 Feb 16. PMID: 36805050. Included in results section 3.2

Powell-Brett S, de Liguori Carino N, Roberts K. Understanding pancreatic exocrine insufficiency and replacement therapy in pancreatic cancer. Eur J Surg Oncol. 2021 Mar;47(3 Pt A):539-544. doi: 10.1016/j.ejso.2020.03.006. Epub 2020 Mar 8. PMID: 32178962. Parts included in background section 1.3, 1.4 and 1.5.

Powell-Brett S, Chinuck R, Roberts K. Management of pancreatic exocrine insufficiency. Textbook of pancreatic cancer: Principles and practice of surgical oncology. Springer Nature. 2021 Feb: 43: 665-680. Doi: 10.1007/978-3-030-537. Parts included in background section 1.5

Chapter 1 Introduction and Background

Summary of Chapter 1

This thesis is the collation of 3 years' work on pancreatic exocrine insufficiency (PEI). The original concept was to develop a novel biomarker to diagnose PEI using metabolomics (the study of the small products of metabolism). The concept is based on the theory that the metabolomic response to a challenge meal would be very different in people with exocrine dysfunction and people without. Before a trial could be started, a recognised diagnostic test for PEI had to be established locally (the ^{13}C MTGT), and a study to challenge the exocrine pancreas and acquire post-prandial plasma samples for metabolomic analysis had to be designed.

This introduction explains the physiology of the exocrine pancreas and the aetiology, incidence, consequences, and treatment of PEI. To explain why a novel diagnostic test is required, the different PEI diagnostic tools and their limitations are discussed. Finally, the concept of metabolomics, high-throughput analytical platforms and the hypothesis behind using metabolomics to explore exocrine dysfunction are discussed.

1.1 Background

'Normal' pancreatic exocrine function is essential to life and yet, despite this, PEI is not perceived as organ failure. Inadequate production or impaired function of pancreatic enzymes results in pancreatic exocrine insufficiency (PEI) which leads to profound malabsorption and malnutrition. Untreated PEI results in impaired quality of life and increased mortality among patients with cystic fibrosis, chronic pancreatitis or pancreatic cancer (both resected and unresected).[1] Treatment of PEI with Pancreatic exocrine replacement therapy (PERT) in the form of capsules taken with meals is highly effective and has been shown to correct maldigestion, malabsorption, improve symptoms and overall quality of life.[2] In addition, there is emerging evidence of survival benefit among patients with pancreatic cancer taking PERT.[3, 4]

Despite its clear advantages, PERT is often not routine. The 2018 prospective national audit of pancreatic cancer, RICOCHET,[5, 6] found that 60-80% of patients with pancreatic cancer were not receiving PERT, this is consistent with data from other European countries, America and Australia.[7-9] Reasons for undertreatment are not well defined, but in large part relate to a lack of a diagnostic test that is both accurate and easy to perform. There are various diagnostic tests, but all have significant limitations, which will be discussed later in this chapter. A reliable diagnostic test is essential as the symptoms of PEI are subtle and often confused with the underlying disease states.

This work aimed to develop a novel method for PEI diagnosis using a metabolomic approach. The ^{13}C labelled mixed triglyceride breath test (^{13}C MTGT), alongside standard laboratory testing of PEI (FE-1, faecal elastase-1), will be used as the gold standard.

1.2 The exocrine pancreas and pancreatic exocrine insufficiency

Exocrine function of the pancreas is an essential component of macronutrient digestion. Pancreatic exocrine insufficiency (PEI) can be defined as 'A reduction in pancreatic enzyme activity in the intestinal lumen to a level that is below the threshold required to maintain normal digestion'.[10] It is difficult to put a precise value on the diagnosis of PEI but in 1973 DiMagno et al correlated duodenal enzyme outputs to steatorrhea and found that steatorrhea and creathorrhea was not observed until enzyme outputs (lipase and trypsin) had fallen to below 10% of normal.[11] PEI is an extremely broad term that does not always reflect a primary pancreatic parenchyma problem, but any interruption in the chain of events from enzyme secretion to their end hydrolytic function.

The physiological role of the pancreas consists of exocrine and endocrine functions; 2-3 litres of pancreatic juice is secreted per day, which is an isotonic alkaline, containing digestive enzymes (mainly amylase (7%), proteases (80%), lipase (4%), and nucleases (1%) [12, 13]. The exocrine component is made up of acinar, centroacinar, and ductal cells and makes up 90% of pancreatic volume, the endocrine component (Islets of Langerhan) accounts for below 2%, and the residual volume comprises extracellular matrix, lymphatics, nerves, and blood vessels [14]. The acini produce and secrete digestive enzymes by exocytosis and the epithelial cells lining the small/proximal ducts secrete large amounts of bicarbonate rich fluid [12].

The result of stimulation of the exocrine pancreas is postprandial injection of inactive enzyme precursors into the duodenum. A complex network of neural, humoral, and paracrine factors mediates exocrine pancreatic secretions. Pancreatic enzyme secretion has 3 phases: cephalic, gastric, and intestinal. The mixture of bicarbonate and enzyme precursors must then be delivered via the pancreatic and common bile duct into the duodenum, where they are activated.

This section presents an overview of the three phases of pancreatic enzyme secretion and factors influencing the level and duration of enzymatic secretion, summarised in **Figure 1**.

1.2.1 The Cephalic and Gastric phase of pancreatic enzyme secretion

These are both largely mediated by neuro-vagal stimulation. The cephalic phase is initiated by various sensory inputs, such as sight, smell, and taste, and has been shown by sham feeding to account for approximately 20-25% of the total pancreatic exocrine secretion. Intra-pancreatic postganglionic neurons receive central parasympathetic input from the neurons of the dorsal motor nucleus of the vagus (DMV) and ultimately secrete acetylcholine, which then acts on muscarinic receptors on acinar cells to elicit enzyme secretion. The gastric phase is initiated by the entry of food into the stomach, animal studies have shown that antral distension leads to pancreatic secretion via vagal stimulation. Vagotomy has been shown to block both the cephalic and gastric phase, reinforcing the evidence that vagal efferents are the primary mechanism by which the cephalic and gastric phase lead to exocrine secretion.[15]

1.2.2 The intestinal phase of pancreatic enzyme secretion

The intestinal phase begins when chyme (gastric acid, free fatty acids, essential amino acids, and carbohydrates) reaches the duodenum. This phase is regulated by hormonal and neural mechanisms. The key regulatory hormones are secretin and cholecystokinin (CCK) and the main neural pathway is the enteropancreatic reflex. Secretin is released into the blood from S cells in response to the low pH of gastric chyme, the role of secretin is to stimulate the release of bicarbonate from pancreatic ductal cells. CCK is considered the most important mediator of postprandial pancreatic enzyme secretion. Interestingly, CCK works both directly on pancreatic acinar cells (which have a functional CCK receptor) that activate enzyme secretion

in response to CCK, and through the cholinergic nervous system (Like the cephalic and gastric phases). Atropine has been shown to almost entirely halt the pancreatic exocrine output stimulated by CCK [10] (implying that CCK stimulates the pancreas mainly via interaction with cholinergic nerves). Later evidence suggests there is a lack of significant amounts of CCK receptor proteins on acinar cells and a lack of response of acinar cells to CCK receptor agonists; it is therefore likely that the cholinergic route is the most significant mechanism of action. [11, 12]

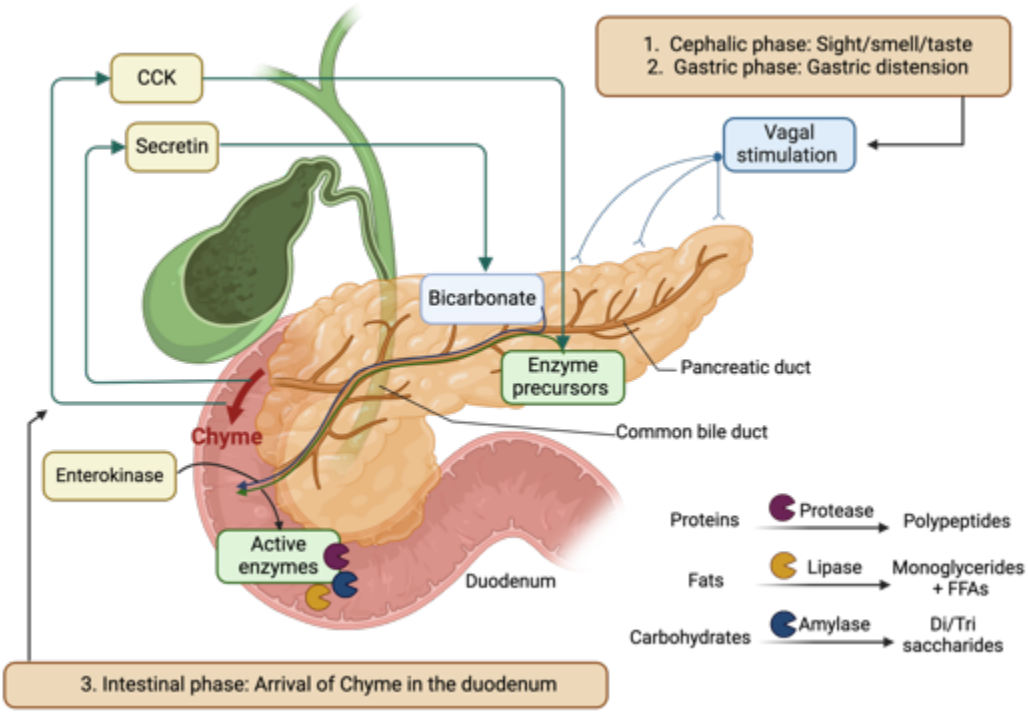


Figure 1 Pancreatic enzyme secretion: Created in Biorender ®

1.2.3 Timing of enzyme secretion

Pancreatic enzymatic secretion has an interdigestive level (which behaves in a low-level cyclic fashion) and a postprandial level (which is a higher, constant rate). The delivery of enzymes into the duodenum has been shown to reach a peak in the first hour post-prandial, which then returns slowly to its inter-digestive level by approximately 3-4 hours postprandially. [16, 17] The level and duration of stimulation is affected by several factors, including calorie content, nutrient composition, and physical properties. [18]

1.2.4 Impact of calorie content on exocrine secretion

Brunner et al looked at the correlation between post-prandial amylase secretion and meal energy density found that maximal enzyme response was achieved by meals above 20 kcal/kg daily intake divided into 3 meals, thus for an average 75kg person, meals containing greater than 500kcal should induce maximal enzymatic response. [14] Ishii et al found that post-prandial changes in blood pancreatic peptide at 4 and 6 hours was significantly lower following a 200kcal or 400kcal meal in comparison to an 800kcal meal. [15]

1.2.5 Impact of nutrient components on exocrine secretion

Boivin et al compared postprandial enzymatic output in five different diets differing in proportions of carbohydrate, fat, and protein, and concluded that diets with a higher carbohydrate content are associated with lower enzymatic output in comparison to diets with a higher fat content. Chronic modifications of diet altered both interdigestive and postprandial enzymatic secretion whereas acute changes only influenced postprandial enzymatic secretion [15]. These influences were independent of caloric content, as each diet's caloric content was adjusted to body weight and daily activity.

1.2.6 Impact of form in which a meal is ingested.

The pancreatic secretory response to a solid meal appears to be more sustained than to a homogenised meal with the same nutritional and caloric properties. A solid meal results in slower gastric emptying and increased gastric acid secretion. Studies using homogenised meals show a similar level of enzyme secretion, but with an earlier postprandial peak and a faster return to the interdigestive range. [16]

To summarise, pancreatic enzymatic output will be at its maximum in the first hour post-prandially, a meal of more than 500kCal with a higher fat content and lower carbohydrate content will stimulate higher enzyme outputs, and a homogenised meal creates an earlier peak in output.

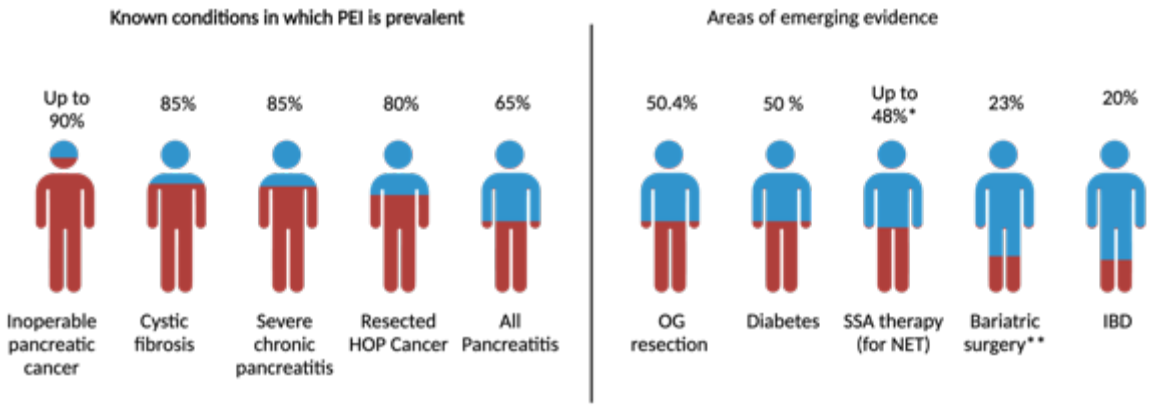
1.3 Aetiology of pancreatic exocrine insufficiency

PEI can arise from pathology that affects any part of the sequence from enzymatic production to their end function of hydrolysis and therefore should be thought of as pancreatic malabsorption, not just the failure of exocrine function. PEI can be primary (from a problem with the pancreas itself) or secondary (anything out with the pancreas that disrupts the process of pancreatic enzyme action). Most often PEI results from diseases that affect the pancreatic parenchyma such as cystic fibrosis, chronic pancreatitis, pancreatic cancer, and pancreatic resection [10, 18]. Other causes include diabetes mellitus, inflammatory bowel disease, coeliac disease, somatostatin analogue therapy and upper gastrointestinal surgery. (See **Table 1)** In some conditions the evidence base is poor and ascertaining incidence of PEI is difficult. (See **Figure 2).**

Table 1 Primary and secondary causes of Pancreatic Exocrine Insufficiency

Primary PEI (intrinsic/pancreatic)	Secondary PEI (extrinsic/intestinal)
Pancreatic fibrosis/chronic pancreatitis	Intestinal motility
Replacement of pancreatic tissue with tumour	Low intestinal pH (ulcer disease)
Removal of pancreatic tissue (surgery)	Anatomic alteration (surgery)
Diabetes mellitus (pancreatic exocrine atrophy)	Stimulation/denervation (surgery, drugs e.g. somatostatin analogues, diabetes)
Pancreatic duct obstruction	

Figure 2 Incidence of PEI in different conditions (figure created in Biorender ®)



*HOP = Head of pancreas, OG = oesophagogastric, SSA = Somatostatin Analogue, NET = Neuroendocrine tumour, IBD = inflammatory bowel disease, * poor quality literature, ** Does not include gastric bands*

1.3.1 Chronic Pancreatitis

Chronic Pancreatitis (CP) tends to encompass an initial pancreatic insult followed by an ongoing inflammatory process that leads to irreversible and progressive loss in pancreatic parenchyma and pancreatic fibrosis affecting both endocrine and exocrine functions. PEI occurs in around 65% of all patients with chronic pancreatitis. PEI is progressive and correlates

to severity; prevalence of PEI in mild CP is around 30%, increasing to 85 % with severe CP. [19, 20] The timing of onset is difficult to quantify but in a large natural history study, PEI occurred at a median time of 13.1 years from diagnosis of alcoholic pancreatitis and 26.3 years from diagnosis of early onset idiopathic chronic pancreatitis.[21] Stevens et al reviewed the literature around the pathogenesis of chronic pancreatitis and summarised the following as plausible aetiologies; oxidative stress, loss of ductal function or obstruction, toxic metabolic derangements and necrosis-fibrosis. [5, 22]

1.3.2 Cystic Fibrosis

The autosomal recessive disorder Cystic Fibrosis (CF) is caused by a genetic mutation affecting the gene encoding for the CF transmembrane conducting regulator (CFTR). CFTR is highly expressed in ductal epithelial cells where it regulates the transport of fluid and ions into the lumen. CFTR protein dysfunction causes a reduction in chloride/bicarbonate transport which results in a reduced volume of luminal fluid and a decrease in luminal pH causing the precipitation of proteins in the ductal lumen, obstruction of the pancreatic ducts and subsequent pancreatic parenchyma destruction or autodigestion (See **Figure 3**). [23, 24] Around 85% of CF patients have PEI requiring pancreatic enzyme replacement therapy. [25]

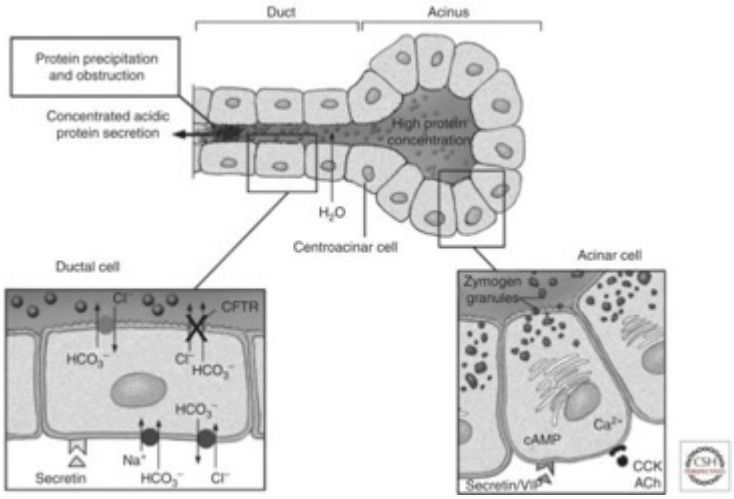


Figure 3 Mechanism of PEI in cystic fibrosis

1.3.3 Pancreatic cancer

Pancreatic cancer is the 6th commonest cause of cancer death in the UK and the 4th commonest cause of cancer death in the US. Less than 3% of people diagnosed with pancreatic cancer in England will survive for 5 years and only 1% will survive 10 years. With an irresectable cancer survival is extremely poor.[26] For those with resectable disease (accounting for approximately 20% of patients), surgery alone still only confers a 10% 5-year survival.[27] Evolving regimens of neo-adjuvant and adjuvant chemotherapy have resulted in markedly improved duration and chance of survival. [28-32] Despite these significant survival advantages population studies have shown that only around half of pancreatic cancer patients received adjuvant therapy after resection.[33] High rates of perioperative morbidity, suboptimal use of neoadjuvant or adjuvant therapy and malnutrition all contribute to poor outcomes in this patient cohort. Malnutrition and PEI in pancreatic cancer needs to be better recognised and treated to improve access to surgery, neoadjuvant and adjuvant treatments and maintain quality of life.

There are multiple contributing factors to malnutrition and PEI in pancreatic cancer, to fully appreciate this it is essential to understand the physiology of pancreatic digestive enzyme release. Pancreatic cancer, both of itself and post pancreaticoduodenectomy can cause PEI and weight loss through a variety of mechanisms. Over 80% of those with pancreatic cancer have lost weight by the time of diagnosis and over a third of these patients have lost more than 10% of their body weight.[34] Irresectable and resectable pancreatic cancer have different contributing factors to PEI and malnutrition (summarised **Figure 4)**, hence it is worth looking at them separately.

1.3.3.1 PEI and malnutrition in irresectable pancreatic cancer

The incidence of PEI is reported to be between 66% and 92% of patients with inoperable pancreatic cancer.[35] The most obvious cause of PEI in pancreatic cancer is direct damage to the acini and obstruction of the pancreatic ducts by the cancer itself, especially as the majority of tumours occur in the pancreatic head resulting in the obstruction of the main pancreatic duct, preventing any passage of digestive enzymes from the pancreas to the duodenum. Bicarbonate is secreted solely by the pancreas and neutralises gastric acid. Thus, obstruction leads not only to a reduction in enzyme secretion but a reduction in the ability to normalise the luminal pH of small bowel. This reduces the function of exogenous or endogenous pancreatic enzymes. Factors other than PEI that compound malnutrition in this group are:

- Warburg effect; wasting from increased energy expenditure (tumour metabolism) [36, 37],
- Chronic, subclinical inflammation; raised C-reactive protein (CRP) is present in many solid tumours, including pancreatic cancer, contributing to increased energy expenditure and loss of appetite (CRP can be correlated to the degree of cachexia and prognosis[38]). [39]
- Presence of the tumour derived islet amyloid polypeptide (IAPP) also contributes to weight loss.[40]
- The psychological effect of a cancer diagnosis, especially one with such a poor prognosis as irresectable pancreatic cancer can lead to loss of appetite.

1.3.3.2 PEI and malnutrition in resectable pancreatic cancer

Pancreatic resection with curative intent is attempted in about 20% of patients with pancreatic cancer.[41] There are several variations on resection depending on the position and extent of the tumour. Broadly, these are Distal pancreatectomy (DP), Pancreaticoduodenectomy (PD,

otherwise known as Whipple's), pylorus preserving PD (PPPD) and total pancreatectomy (TP). It is difficult to pick apart the prevalence of PEI post pancreatic resection as many studies compare different types of resections rather than pre- and post-surgery and the methods of PEI diagnosis are varied. In patients undergoing pancreatic resection there is physical loss of pancreatic tissue combined with physiological and anatomical alterations that inhibit enzymatic function. These include denervation from lymph node dissection, asynchrony between pancreatic secretion and gastric emptying and the effects of duodenal resection.[42, 43] Duodenal resection reduces the CCK mediated intestinal phase of secretion and the reconstructive element results in pancreatic enzymes being released into a more distal part of small intestine that is more acidic (causing inactivation of digestive enzymes) and less rich in enterokinase (reducing enzyme activation). (For a summary of this, refer to **Figure 4)** Of note, different types of reconstruction (Pancreaticogastrostomy (PG) vs pancreaticojejunostomy (PJ)) affect the incidence of PEI following pancreaticoduodenectomy. One study evaluating PEI after pancreaticoduodenectomy in 99 patients found PG to be an independent risk factor for PEI and a retrospective review of PEI (based on PERT usage) after pancreaticoduodenectomy found significantly higher rates of PEI in the PG group when compared to PJ (75% compared to 46% had PEI at 1 year after surgery, respectively, $p<0.001$).[44] [45]

In addition to PEI post resection, pre-operatively there will be a degree of PEI and malnutrition secondary to the malignancy itself (as described above for inoperable pancreatic cancer) thus patients awaiting resection may already have significant malnutrition.

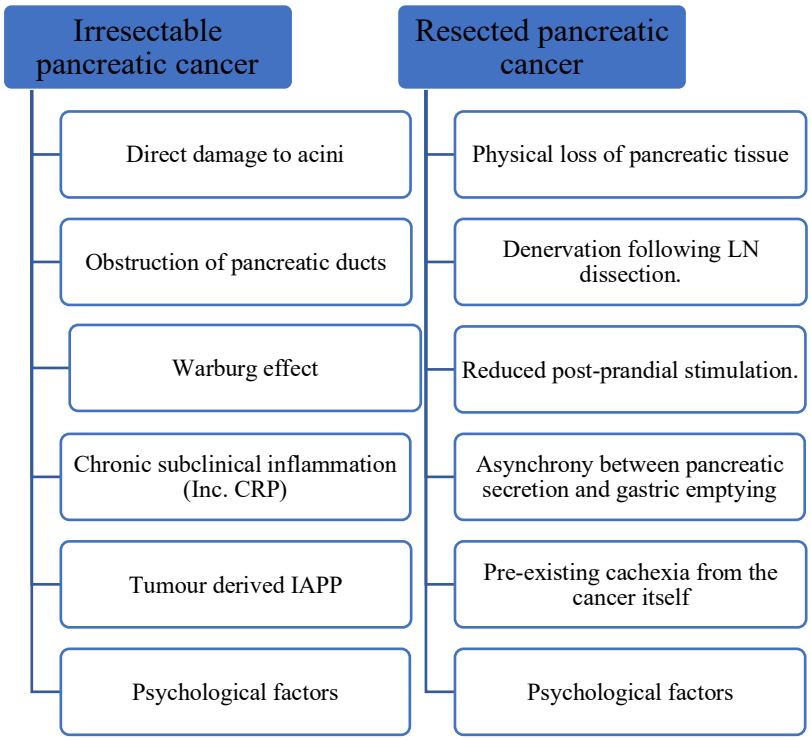
The majority of studies looking at PEI after pancreatic resection show a significant increase in PEI post-operatively; Sato et al found an increase in the prevalence of PEI from 46% pre-

operatively to 75% at 2 months post-operatively, Halloran et al found PEI in 67% of patients at 6 months post resection [46] and Nordbeck et al found that 100% of patients had PEI at a median post-operative time of 52 months. [47] The exception to this is in studies looking at just resection for ampullary cancer where PEI was shown to improve, the theory being that PEI in these patients was secondary to complete pancreatic duct obstruction which was relieved by resection. Sato et al compared DP to PPPD showed a significant increase in PEI in those post PPPD but not in those undergoing DP and concluded that the important factor was actually the thickness of the pancreatic duct at the transection line and showing a negative correlation between post-operative PABA excretion rate (used to assess PEI) and the duct-parenchymal ratio. [48, 49]

To summarise, studies looking at PEI following PD and PPPD show a prevalence of between 70-100%, this is significantly lower following distal pancreatectomy (30-66%, explained by the preservation of the duodenum). Unfortunately, this data is limited by small and heterogenous cohorts (not all limited to pancreatic cancer), the use of a wide variation of non-standardised testing for PEI and the frequent use of FE-1 testing which is not the gold standard and has many limitations. [50] Looking at just cancer related resections, a 2016 systematic review by Tseng et al evaluated PEI in patients with peri-ampullary or pancreatic cancer before and after resection; they found that before PD the median prevalence of PEI was 44% (range 42-47%) and after PD the median prevalence of PEI at least 6 months post-operatively was 74% (range 36-100%.). Again, the results of this systematic review are limited by small studies, variation in follow up timing and the use of a non gold-standard test (FE-1) in the majority of studies (which tends to underestimate PEI after pancreatic resection thus making the results a likely conservative estimate).[51] Since this systematic review Hartman et al 2023 performed the ^{13}C MTGT on 197 patients following PD for oncological indication and found an incidence

of PEI in 68% of patients at 6 weeks post-operatively. Fundamentally, one can infer that PEI following pancreatic resection for pancreatic cancer is a significant problem, but more studies are needed to study the long-term course of PEI after pancreatic resection.

Figure 4 Contributors to PEI and malnutrition in both resectable and irresectable pancreatic cancer.



1.3.4 PEI in patients with rarer causes of PEI

An increasing amount of evidence supports the existence of PEI in other, less recognised disease states. For example, our group is leading research into PEI in patients with neuro-endocrine tumours starting somatostatin analogues.

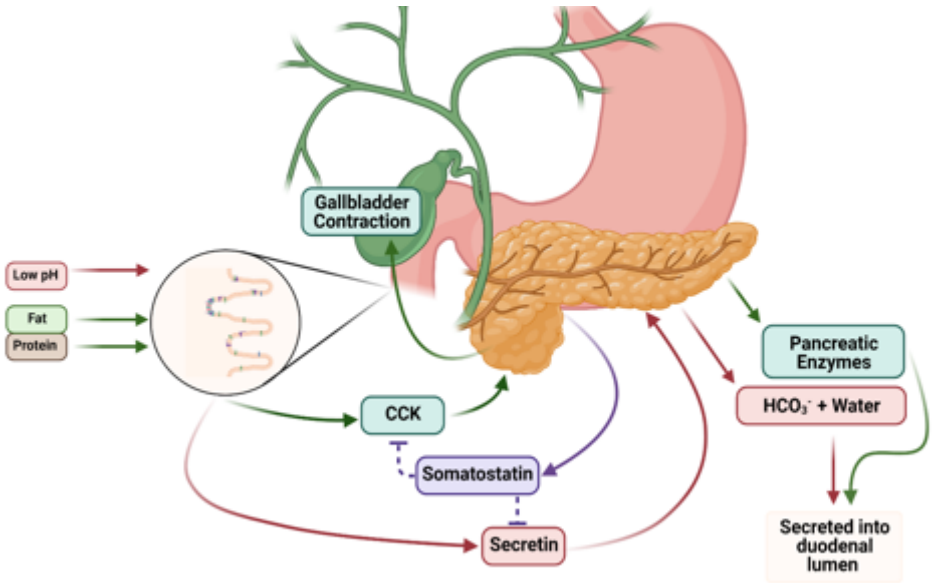
1.3.4.1 Neuroendocrine tumours on Somatostatin Analogues

Neuroendocrine tumours (NETs) are a rare, heterogenous group of malignancies of the diffuse neuroendocrine system.[52, 53] These neoplasms have an indolent disease course and a unique

potential to secrete monoamines and peptide hormones.[54] Tumour behaviour depends on histopathological features, including tumour location, stage and grade, and presence of the somatostatin receptor (SSTR).[55] Somatostatin analogue (SSA) therapy is used to combat the unpleasant symptoms associated with NETs.[56, 57] SSA therapy has three rationales in NET patients: Firstly, the overexpression of somatostatin receptors on certain NETs provided a molecular target for SSA therapy, secondly, SSAs inhibit receptors that potentiate pathological hormone secretion, and finally somatostatin can mediate the oncogenic potential of NETs, limit their growth and reduce tumour size.[58-60]

Endogenous somatostatin is crucial to the delicate balance of the exocrine pancreas, when released it triggers a negative feedback loop to prevent continued pancreatic secretion and auto digestion.[61] Exogenous somatostatin (in the form of SSA therapy) uses this anti-secretory function to ameliorate neuroamine hypersecretion in functioning NETs.[62] Unfortunately, like endogenous somatostatin, SSAs also suppress pancreatic secretion which leads to the development of PEI.[63] (*See **Figure 5)***

Figure 5 Negative feedback mechanism of somatostatin (emulated by somatostatin analogues, thereby inhibiting pancreatic exocrine function (Created by Lewis Hall (permission given) on Biorender ®)



The incidence of SSA-related PEI is hard to quantify, the current literature reports a range of 12-48% which is likely to be an underestimate owing to diagnostic limitations.[54, 63-66] Prophylactic PERT in this group of patients has been shown to improve patients' weight, suggesting a more widespread degree of exocrine insufficiency.[67] Diagnosis of PEI in this cohort is particularly challenging. Symptomatic assessment is not useful owing to the significant overlap between NET symptoms and PEI.[68] The use of faecal elastase (the most widely available diagnostic test) is contentious as FE-1 is affected by diarrhoea, a common symptom of functional NETs.[52, 69]

1.4 Consequences of PEI and introduction of the PEI-Q tool

For recognisable symptoms to occur in patients with PEI the ingested food must overwhelm the enzymatic capacity of exocrine pancreatic secretion. Owing to the unpleasant nature of the symptoms, patients with PEI may automatically avoid or reduce the intake of food that exacerbates them. This makes it difficult to accurately assess the presenting symptoms of PEI. The key defining symptom of PEI is steathorrhoea, and other symptoms include diarrhoea, weight loss, vitamin deficiency symptoms, abdominal distension, and flatulence. In the process of developing a patient-reported outcome (PRO) instrument, Johnson et al. performed a thorough literature review to identify concepts associated with PEI and conducted expert physician-led interviews with patients to explore the key symptoms and their impact on quality of life. Their literature review identified key symptoms, 'impact concepts' and coping strategies. (See **Table 2**)

Table 2 Pancreatic exocrine insufficiency concepts elicited from qualitative literature review.

Concepts	Sub-concepts
Symptoms	Painful gastrointestinal sensations, other gastrointestinal sensations, trapped wind, changes in appearance of stools, changes in bowel movements, symptoms related to eating
Impacts	Psychological, family, occupational, and eating-related impacts; fatigue/tiredness/lack of energy; loss of physical strength; and PEI treatment-related impacts
Coping strategies	Altering administration of enzymes, denial, socializing with people who know about the condition, relying on others for support, balancing benefits and risk, modifying diet, and performing upright gentle activities
Triggers	Altering administration of enzymes, denial, socializing with people who know about the condition, relying on others for support, balancing benefits and risk, modifying diet, and performing upright gentle activities Triggers Eating-related

Analysing the patient interviews the group identified six primary symptom concepts: pain, bloating symptoms, bowel movement/stool symptoms, nausea/vomiting, eating related symptoms, and tiredness. From the interviews they identified a conceptual framework and collated symptom reporting within these concepts and sub-concepts. The reported symptoms and their frequencies are presented in **Table 3**. [1]

Table 3 Pancreatic exocrine insufficiency, frequency of concepts and sub concepts identified in patient interviews. [1]

Concepts	Frequency	Sub-concepts	
Pain	80%	Abdominal	84%
		Non-abdominal	16%
Bloating symptoms	64%	Stomach noises	82%
		Flatulence	33%
		Trapped wind	15%
Bowel movements and/or Stool symptoms		Constipation	48%
		Increased frequency	18%
		Urgency	33%
		Diarrhoea	75%

		Fatty stool	49%
		Change in stool colour	48%
Nausea and vomiting	64%	Nausea alone	44%
		Vomiting alone	21%
		Nausea and vomiting	21%
Eating-related symptoms		Weight loss	67%
		Loss of appetite	33%
Tiredness	41%		

Following this body of work Johnson et al. went on to develop and validate a 26-item PEI-specific PRO instrument, the PEI-Q. [70]

Other than poor quality of life and the symptoms described above, the main consequence of PEI is malnutrition. Deficiencies of various proteins such as pre-albumin, apolipoproteins, transferrin, and lipoproteins develop. [71] Long term fat malabsorption leads to deficiencies of fat-soluble vitamins (A, D, E and K) in addition to deficiencies in calcium, magnesium, zinc, thiamine, and folic acid. [72] The impact of these deficiencies is broad ranging, PEI has been shown to increase the risk of osteoporosis (and the associated fractures [73]), cardiovascular events and sarcopenia (even in those who have maintained their subcutaneous fat and visceral fat). [74, 75] For those with CP, PEI has been shown to be an independent factor associated with high mortality. [75] [76] For those with pancreatic cancer, PEI is a major cause of weight loss and malnutrition and has been associated with shorter survival and a poorer quality of life. Post pancreatic resection, PEI has been associated with increased costs, higher post-operative complications and longer inpatients stays.[77-80]

1.5 Treatment of PEI with Pancreatic Enzyme Replacement Therapy (PERT)

The treatment of PEI revolves around the exogenous replacement of the required enzymes with the administration of PERT. The focus is on normalising fat absorption as there are other physiological mechanisms of protein and carbohydrate absorption. The aim is to restore normal fat absorption by delivering enough *active* lipase to the *right place* at the *right time*.

1.5.1 Treatment dosing

The level associated with *sufficient* (not normal) absorption and digestion of fat is 10% of normal lipase (consistent with clinically evident PEI manifesting at greater than 90% loss of pancreatic function). [81] Units of lipase can be confusing, either noted as USP (United States Pharmacopeia) or IU (international units), with 1 IU being 3 USP, (as CREON ® uses USP and is the most widely used form of PERT, all dosing will be referred to as USP in this work). In absence of disease, with a meal, the pancreas produces more than 900,000 units (USP) of lipase. [82] Thus, theoretically PERT aims to provide at least 90,000 USP alongside a meal (this doesn't precisely translate as there are other, small sources of lipase such as gastric lipase). PERT dosing recommendations depend on the underlying disease process, these will be addressed separately.

1.5.2 Gastric barrier and ingestion timing

The first challenge is getting active lipase to the right place at the right time; the acidic gastric environment inactivates pancreatic enzymes. Lipase is inactivated at a pH of 4 or less, trypsin and other enzymes are more acid stable but are destroyed by pepsin in an acid environment thus most preparations are enteric coated, if not, acid suppression is required. The question of whether to try and pharmacologically counter the gastric pH with antacids or anti-secretory

agents when giving enteric coated PERT is an interesting one, though aluminum hydroxide and sodium bicarbonate have both been shown to reduce steatorrhea when given alongside PERT, overall most studies do not show a significant benefit to adding an antacid or anti-secretory agent when using an enteric coated preparation, except in those with a poor response to PERT due to high gastric acid secretion. [83] Of note, in pancreatic cancer, pancreatic bicarbonate secretion can be severely limited hence these patients may benefit from the addition of a proton pump inhibitor to enable the dissolution of the enteric coating (which requires a pH of more than 5.5 to dissolve). There is also evidence that proton pump inhibitors maintain pancreatic volume after pancreatoduodenectomy, thought to be via a mechanism that involves increased levels of gastrin.[84]

Not only must the enzymes overcome the acidic gastric environment, but they must also be able to pass unhindered into the duodenum to mix with nutrients at the correct time. Most nutrients pass into the duodenum as <1mm particles suspended in a liquid layer. If the enzyme microspheres are sufficiently large enough, they will be separated out from the bulk of the nutrients and emptied later thus impeding their action. [85-88] Recently, a Cochrane review demonstrated a higher efficacy for enteric coated microspheres in comparison to enteric coated tablets for the treatment of PEI in CP [83], further to this, it has been shown that mini-microspheres (1.0-1.2mm diameter) are emptied simultaneously with the meal and are associated with higher efficacy in comparison to microspheres (1.8-2.0mm), though the microspheres still work satisfactorily. [83] In the light of the above, the preparations of choice are enteric coated mini-microspheres <2mm in size. Many other preparations are available and comparative clinical trials are lacking.

It is difficult to pinpoint the optimal time to give pancreatic enzymes, ideally it should be timed to co-ordinate gastric emptying with delivery of pancreatic enzymes into the duodenum. One suggestion for continued fat malabsorption is that a significant amount of ingested fat is emptied within the first post-prandial hour, yet the enzyme microbeads tend to remain in the proximal stomach within this time. [88, 89] The effectiveness of PERT assumes the mixing of enzymes and chyme. Therefore, enzyme preparations should be taken alongside meals. Dominguez-munoz et al performed a randomised, three-way cross over study to optimise an administration schedule and suggested that PERT should be given either just after or distributed along the meal rather than before but concluded that enteric coated mini-microspheres are highly effective in improving fat maldigestion regardless of the administration schedule.[90] This study was limited by strict exclusion criteria, however, it's findings are reinforced by a retrospective survey of 262 patients showing better weight gain and fewer symptoms in patients who consumed PERT throughout their meals rather than all before or all after.[91] The advice that patients should try and spread out their capsule ingestion across the course of a meal is supported by a position statement from the International Study Group on Pancreatic Surgery.[92]

1.5.3 PERT in Chronic Pancreatitis

A recent systematic review and meta-analysis by Inglesia-Garcia et al found PERT to significantly improve fat and protein absorption in CP with marked increases in co-efficient of fat absorption (CFA) compared to baseline and to placebo. In addition, significant improvements in GI symptoms were observed. They also found that high dose (>60,000 USP/day) and enteric coated PERT appeared to be more effective than low dose or non-enteric coated PERT.[93]

Although longer term studies on survival benefit of PERT in CP is lacking, PEI has been shown to be an independent risk factor for mortality in this cohort and a lack of PERT on discharge for those undergoing surgery for CP has been identified as an independent risk factor for survival.[94, 95]

The United European Gastroenterology evidence-based guidelines for the diagnosis and therapy of chronic pancreatitis (HaPanEU), advised an optimal dose of 40,000 to 50,000 units with main meals, and half with snacks, distributed along the meal with an increased dose or a PPI added in cases of unsatisfactory response. [83] A U.K Consensus statement advises at least 50,000 units with meals and 25,000 with snacks.[96]

1.5.4 PERT in Cystic Fibrosis

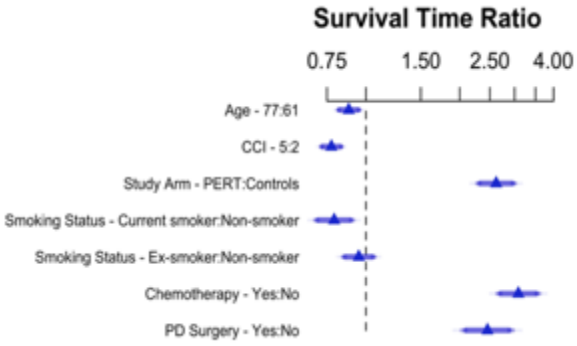
Lifelong PERT use is the cornerstone of nutritional treatment for patients with CF and is required by most. PERT has been well proven in CF to improve nutrient absorption which in turn improves overall prognosis and survival. [97-99] Perhaps the most dramatic example of PERT improving outcomes is in the 1988 comparison of Boston's Vs Toronto's CF patients. Toronto advocated a high-fat diet with a strong emphasis on PERT where Boston advocated a low-fat diet with a lesser emphasis on PERT. The height, weight and survival of Toronto's patients were significantly higher.[100] A 2016 systematic review looking at PERT for CF patients was limited by small scale studies with significant potential for bias. Their data supported the use of enteric coated micro-spheres but was not able to support well evidence-based timing or dosing and called for further studies to be developed to clarify.[101] Dosing in children is more complex than adults, weight based and targets the lowest effective dose, to prevent tackle clinical symptoms and poor weight gain.[102]

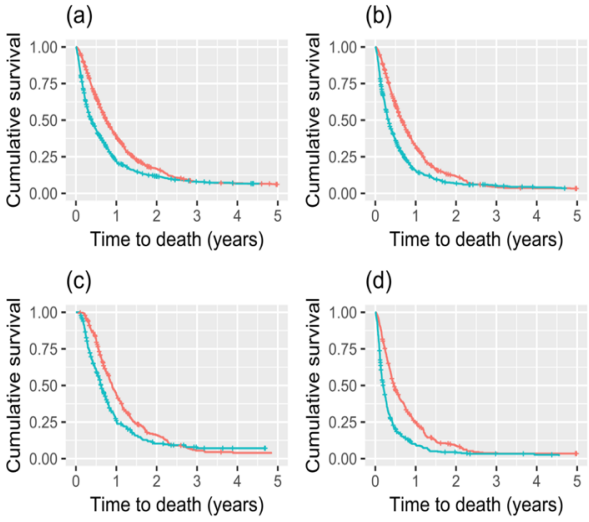
1.5.5 PERT in pancreatic cancer

As discussed above, PEI is a key contributor to the malnutrition experienced by most patients with resectable or irresectable pancreatic cancer. PERT has been shown to significantly improve symptoms and quality of life in pancreatic cancer. Landers et al showed PERT to be associated with a statistically significant improvement in symptoms with both general and pancreatic cancer specific quality of life questionnaires. In their study, within one week of starting PERT there was a reduction in diarrhoea scores and pain, after three weeks a significant improvement was also seen in bloating/gas symptoms. [2] A 2013 RCT comparing PERT to placebo for PEI post pancreatic surgery showed significant improvements in body weight, BMI, stool frequency and CFA at the end of one year.[103] There is also emerging evidence of survival benefit among patients with pancreatic cancer and PERT. In fact, the magnitude of survival benefit among those patients with pancreatic cancer is equivalent to that of potentially curative surgery or chemotherapy, see **Figure 6**.

Figure 6 Survival benefit of PERT - Reproduced from Roberts et al 2019

Adjusted analysis of a population-based study showing survival benefit of PERT is similar to that of chemotherapy





Kaplan-Meier curves comparing PERT-treated PDAC patients (red line) with their matched non-PERT treated control (green line) group (a) overall, (b) only patients who did not undergo surgery, (c) only patients who did not undergo surgery and received chemotherapy, and (d) only patients who did not undergo surgery and did not receive.

Despite the high prevalence of PEI and the evidence that PERT treatment has the potential to improve symptoms, quality of life, nutritional markers and even survival, there is widespread undertreatment. Landers et al. found that of 129 patients referred to a specialist Australian unit with metastatic pancreatic cancer only 21% received PERT despite over 70% manifesting symptoms attributable to malabsorption. [8] Subramaniam et al. looked at all patients with pancreatic cancer coming through a large London hospital MDT and found that 61.5% of patients were recorded to have symptoms of PEI, yet only 19.5% were receiving PERT and many on PERT were insufficiently dosed.[104] A large northern European survey found that most patients suffering from PEI after pancreatic surgery are undertreated. [7] Data from the 2018 prospective national audit of pancreatic cancer, RICOCHET, confirms that most patients with pancreatic cancer in the UK do not receive PERT.[5, 6] Reasons for undertreatment are poorly defined but in part relate to the lack of a diagnostic test that is both accurate and easy to perform.

1.6 Summary of the problem – Inadequacies of current diagnostic tests for PEI

An effective mechanism of diagnosis and quantifying PEI is essential to assess the need for, correct dosage of and efficacy of pancreatic enzyme replacement therapy (PERT) to prevent malabsorption/malnutrition and the associated complications. Functional tests can be divided into direct (determining the secretory capacity of the pancreas) or indirect (measuring the digestive effect of pancreatic enzymes). One can also classify them as invasive or non-invasive. The ideal test is readily available, cost effective, non-invasive, and specific to diagnose PEI. Furthermore, if the test can quantify the severity of PEI, it could potentially guide treatment and dose response to PERT. This is lacking in current available tests. This section will provide an overview of PEI diagnostic tools.

1.6.1 Direct testing for PEI

Direct testing can be invasive (involving measurement of enzymes via a duodenal tube) or non-invasive. (See **Table 4** for current and historic methods of direct testing)

Table 4 Invasive and non-invasive, direct testing of pancreatic function

Direct – Invasive	Direct – Non-invasive
Secretin test	Serum trypsin assay
Secretin-cholecystokinin test	Faecal chymotrypsin
Secretin – caerulin test	Amino acid consumption test
Secretin – bomesin test	Faecal elastase-1
Intraductal secretin test	
Serum pancreatic polypeptide	
Lundh test	

Invasive, direct pancreatic function testing is based on the principle of stimulating pancreatic output followed by collecting and measuring pancreatic secretions. The most accurate direct test is the measurement of enzyme output via collection of stimulated duodenal secretions from a naso/oro duodenal tube. These tests were originally described by Langerloef over 70 years ago [105] and have undergone a variety of modifications since. Owing to the variable nature of basal pancreatic secretion, enzyme secretion must be stimulated either by cholecystokinin or secretin (or an analogue of either). The literature on direct testing is broad and difficult to summarise, and several different methods and timings of stimulation and collection have been described. The Japanese Society of Gastroenterology reached a consensus and recommended a standardised format of testing [106], however, The European Pancreatic Club could not reach a consensus and neither the UK nor the USA has a standardised test. Unfortunately, these tests are not translatable into routine clinical use as they are invasive, costly, time and resource consuming, require specialist equipment and training, and carry some risk of harm. All involve the stimulation of the pancreas via a secretagogue or test meal. The Lundh test was based on a 300 ml test meal containing 5% protein, 6% fat and 15% carbohydrate in liquid form, followed

by aspiration of duodenal contents for 2 hours via a tube sited in the duodenum. The enzyme measured was trypsin. [107] The Lundh test was abandoned after comparative studies showed that stimulation with a secretagogue (such as secretin or cholecystokoinin) was more sensitive and specific. [108, 109] The use of a secretagogue instead of a test meal has generated much debate regarding which secretagogue (or combination), how much, which route of administration, what dose, length of duodenal juice collection and what to use as the test outcome parameter (total secretion volume, bicarbonate secretion, enzyme output etc). The Secretin Stimulation test (SST) is based on a similar principle to the Lundh test, with the fluoroscopic siting of a duodenal tube (a Dreiling tube) but involves the intravenous administration of Secretin followed by the collection of duodenal fluid over an hour and analysing the output of bicarbonate. The SST has a reported sensitivity of 97% for late-stage chronic pancreatitis but only 75% for early. [110, 111]

Faecal elastase 1 (FE-1); Pancreatic elastase 1 is a stable pancreatic enzyme produced by pancreatic acinar cells. It binds to bile salts and undergoes negligible degradation as it travels the GI tract. It can be measured in faeces using an enzyme-linked immunosorbent assay (FE-1) on a spot stool sample and acts as a direct marker for pancreatic exocrine secretion. It is less cumbersome that CFA as does not require 72-hour faeces collection but as with faecal chymotrypsin cannot reliably diagnose the earlier stages of PEI and is not reliable in patients post pancreatic resection. There are a limited number of studies looking at the use of FE-1 to diagnose PEI directly (though there are many evaluating the use of FE-1 for the diagnosis of CP). Benini et al looked at FE-1 in both patients post pancreatic resection and patients with CP and drew three important conclusions, firstly that severe PEI can be detected with high sensitivity and specificity in CP patients, secondly that intermediate FE-1 values are difficult to interpret making it less reliable in mild to moderate PEI and thirdly that FE-1 is unreliable

in patients post pancreatic resection. [112] FE-1 has the benefit of not requiring discontinuation of PERT during testing, it is reasonably easy to perform, does not require specialist testing facilities and is more cost-effective than most direct tests. [113] With severe PEI, the reported sensitivity ranges between 73 and 100% and with mild insufficiency between 0 and 63%. [114, 115] The accuracy is also affected by the selected cut off value; Many studies use a value of <200μ/g stool, but this level confers a high false positive rate, if the cut off rate is lowered to <100μ/g stool the specificity is much better but the sensitivity drops.[116] The three main limitations of FE-1 testing are; its limited sensitivity in mild PEI, its limited specificity in watery stools and that it cannot be used to assess PEI in patients after pancreatic surgery (as there are other physiological and anatomical factors that contribute to PEI after surgery making the assessment of just pancreatic secretion less useful). [115]

Measuring pancreatic enzymes in serum is unreliable with amylase and lipase serum levels bearing an inconsistent relationship to pancreatic function. Amylase studies found low serum levels in patients with PEI, however the sensitivity proved very poor. Serum trypsin has been correlated to deranged pancreatic function, with low levels of serum trypsin being found in advanced chronic pancreatitis with steatorrhea (for which it is reasonably specific)[117]. It is a widely available, low risk, inexpensive test but as an overall marker of PEI serum trypsin is not particularly useful with a sensitivity ranging between 33-65% it has also shown false positive results in pancreatic ductal obstruction, recent acute pancreatitis, pancreatic pseudocysts, renal failure, and diabetes mellitus. [117-119]

1.6.2 Indirect testing for PEI

Table 5 Indirect tests for PEI – all non-invasive

Faecal fat quantification Faecal fat analysis (spot faecal fat) Breath tests (^{13}C-labelled substrates) Dual label schilling test	Serum glucose level Pancreolauryl test Bentiromide (NBT-PABA) test

The gold standard indirect testing of PEI is faecal fat quantification requiring 72-hour collection of faeces and determination of the coefficient of fat absorption (CFA). More than 7g of fat per 100g stool per day diagnoses fat malabsorption and more than 15g diagnoses severe steatorrhea.[10] This test is unpleasant and time consuming for both patients and laboratory staff alike, requiring strict adherence to 100g of fat per day diet for 5 days (inconsistent as an outpatient test making the calculation of the coefficient of fat unreliable) and all faeces to be collected over 3 days. It is also not pancreas specific and requires the patient to not have simultaneous enzyme replacement therapy (PERT). Again, it is of limited use in mild or moderate PEI. It also has a significant drawback in the lack of specificity for pancreatic disease (all other causes of steatorrhea). Thus this test is now largely reserved for research purposes.[120] Spot faecal fat analysis has also been investigated using Sudan staining or near infrared reflectance analysis of a random stool sample for faecal fat, though correlating well with steatorrhea in CP (sensitivity 88.8% and specificity 97% in a study by Benini et al)[121] it is of limited use in the broader quantification of PEI as it suffers from similar problems to the 72-hour faecal fat quantification (patient inhibitions, the need to stop PERT, high fat diet before collection and false positives) but without the sensitivity.

Several breath tests using ^{13}C labelled fatty substrates have been studied in the evaluation of pancreatic exocrine function. The most promising and evaluated is the ^{13}C mixed triglyceride breath test ($^{13}CMTGT$). ^{13}C labelled fatty substrates are digested by lipolysis (using pancreatic secretion of lipase) in the proximal small bowel. The free fatty acids (FFAs) and monoglycerol released in this process are then absorbed and oxidised by the liver to $^{13}CO_2$ which is then exhaled. The most variable step along this process is the enzymatic breakdown of the substrate in proximal small bowel. The increase in the concentration of $^{13}CO_2$ in the breath thus correlates with the secretion of pancreatic lipase. [122] It is non-invasive, relatively easy to perform, the test meal is easy to standardise/control (unlike when performing CFA testing) and the result is not affected by watery stool, making it more widely acceptable and more specific than some of the previously described indirect techniques for PEI diagnosis.

Another advantage of the $^{13}CMTGT$ is that unlike many of the direct tests for PEI it reflects the entire process of digestion and absorption, not just the secretion of enzymes and thus could be useful after pancreatic resection (after which even if enzymatic secretion is maintained many other physiological and anatomical factors affect overall exocrine sufficiency.) When used to evaluate PEI in diabetes mellitus (DM) Keller et al found that although the $^{13}CMTGT$ could detect mild to moderate PEI it had a low specificity owing to confounding factors contributing to decreased lipolysis in these patients. [122] Dominguez-Munoz et al proposed an optimal breath test with the administration of 250mg of $^{13}CMTG$ together with a solid test mean containing 16g of fat after an overnight fast, they found that for diagnosis PEI in chronic pancreatitis the $^{13}CMTGT$ correlated well with CFA testing (the gold standard of PEI diagnostics) with a sensitivity of 92.9% and a specificity of 91.77%. [123]

As the ^{13}CMTG breath test will act as the comparative diagnostic test for this body of work and required a discrete body of work to set up locally, adjusted for use alongside metabolomic testing it will be discussed more extensively in the following chapter.

Table 6 Advantages and disadvantages of currently available tests of pancreatic function.

	Advantages	**Disadvantages**
Secretin-pancreozymin test	Most sensitive test for the diagnosis of PEI Can classify severity	Costly, time consuming, Invasive Not widely available Poorly standardised
72-hour faecal fat quantification	Gold standard for quantifying steatorrhea	Unpleasant/time consuming Must stop PERT Limited sensitivity in mild/moderate PEI
Faecal Chymotrypsin	Cost effective Easy to perform	Must stop PERT Limited sensitivity for mild/moderate PEI Affected by watery stool
Faecal elastase (FE-1)	73-100% sensitivity for severe PEI Cost effective Easy to perform	Limited use post surgery 0-63% sensitivity for mild PEI Limited specificity with watery stool
^{13}C-mixed triglyceride test	>90% sensitivity for detecting fat maldigestion Good for estimating the effect of PERT	Limited sensitivity in mild pancreatic dysfunction Time consuming
Serum trypsin	Cost effective Easy to perform Widely available	Insensitive: range 33-65% sensitivity False positives in non-pancreatic steatorrhea

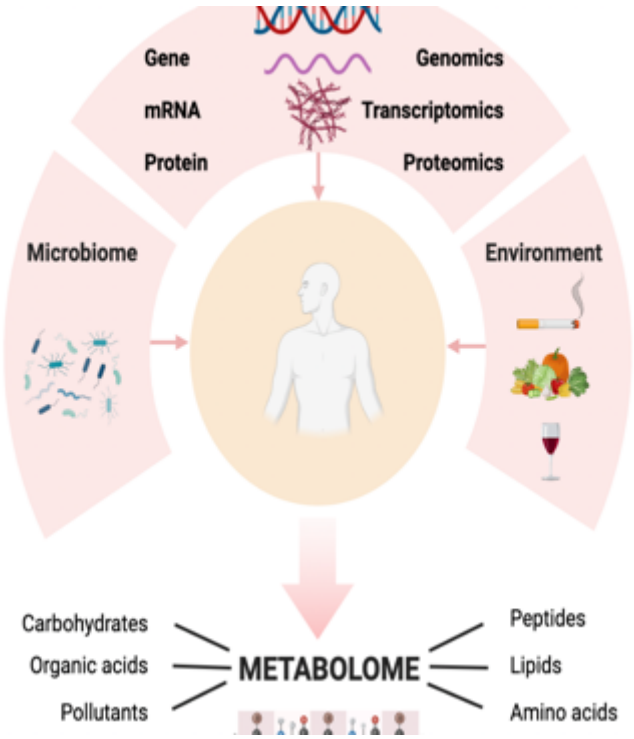
1.7 Metabolomics as a potential diagnostic tool for PEI.

To date, no group has used a metabolomic marker as a diagnostic tool for PEI. However, given that exocrine function is required to digest macronutrients we hypothesise that the products of digestion will be detectable after a meal, will be quantifiable as metabolites and their concentrations will be affected by PEI.

1.7.1 The Human metabolome and metabolomic biomarkers

The definition of a biomarker is “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”.[124] Most biomarkers in clinical use are related to genes, transcripts, and proteins. Metabolomics looks at biochemical activity during cellular metabolism by analysing the presence and changes in concentration of small molecules (low molecular weight, 50-1000 Da) in blood, saliva, urine and other human biofluids and tissues.[125] Metabolomic analysis has the potential to identify metabolite biomarker panels reflective of health and disease in a far more dynamic fashion than the other ‘Omic’ categories as it is not fixed by inherent human genetics but reflects current phenotype, altered constantly by disease state and external factors including food intake. (See **Figure 7**).

Figure 7 Different 'Omics' and factors influencing the human metabolome. (Created using BioRender ®)



The ‘food metabolome’ can be defined as ‘the part of the human metabolome directly derived from the digestion and biotransformation of foods and their constituents’ while the ‘endogenous metabolome’ includes ALL the metabolites produced by the host, both are influenced by dietary intake. [126] More than 25,000 compounds come from the food ingested by humans and most of these are metabolised further within the body creating an enormous and diverse range of metabolites which in this age of ‘omics’ technologies and data driven approaches are now available for analysis. ‘Metabolomics’ is the analysis and characterisation of the metabolome using high-throughput analytical chemistry technologies such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, these allow the measurement of thousands of metabolites at a time. With the vast and diverse nature of metabolites available for study there has emerged the need for a comprehensive database to catalogue and understand the origins and end results of food metabolites. The Human Metabolite Database (HMDB) is an online database of all known (or predicted) human metabolites, (https://hmdb.ca/statistics) others are listed below (See **Table 7**)

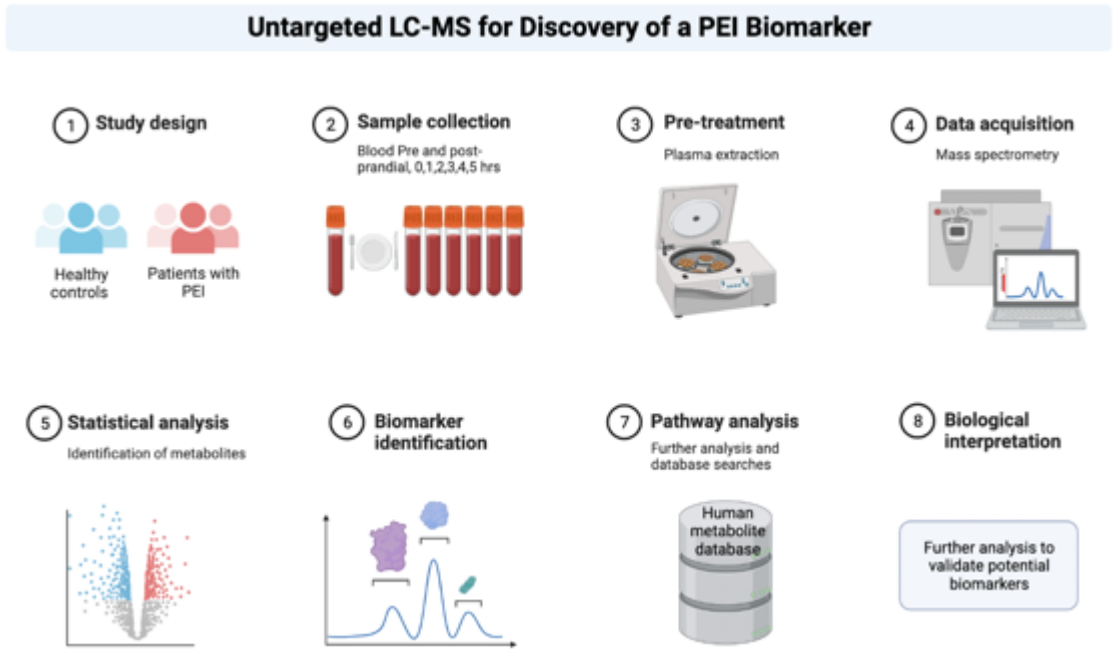
Table 7 Available metabolite databases.

Database	Metabolites	Number of metabolites
HMDB	Endogenous, microbial, biotransformed, and exogenous/ xenobiotic compounds identified in humans .	40,000
ECMDB	Escherichia coli metabolites	2750
YMDB	Saccharomyces cerevisiae metabolites	1730
FooDB	Food constituents and food additives	28,000
Phenol-Explorer	Dietary polyphenols and their metabolites	502
PhytoHub	Dietary phytochemicals and their metabolites	1500

The metabolome is a functional picture and can change quickly and measurably with changes in the state of the individual, some are co-related, and patterns can be representative of a distinct pathophysiological state. The dynamic and temporal nature of the metabolome is of particular interest to research as it is a real time reflection of that person's pathophysiological state. It is important to consider that a metabolomic biomarker is not just a single metabolite that may vary with disease but rather a 'comprehensive biochemical fingerprint', the relationships and interdependence of metabolites result in a metabolic profile or 'signature' (as described by Marchand et al).[127] Therefore, a metabolomic biomarker panel has the potential to monitor changes in an individual's pathophysiological state.

The process of metabolomics begins with the extraction of metabolites from the biological sample (i.e with a blood test) from comparison groups (for example diseased and healthy controls), and an analytical chemistry process (typically either NMR or MS) is employed to obtain a spectral profile of the samples. Next multivariate statistical analysis techniques (e.g Orthogonal Projections to Latent Structures (OPLS) or Principal Component Analysis (PCA)) or univariate statistical tools (e.g. one-way ANOVA) are used to assess whether there is a difference between the metabolomes and if there is, determine the metabolites (spectral features) that define the difference. (See **Figure 8** for an overview of metabolomic discovery workflow)

Figure 8 Untargeted LC-MS for the discovery of a PEI Biomarker (Created using BioRender ®)



There are three approaches to metabolomics, targeted, semi-targeted and untargeted. The three approaches differ in metabolite quantitation (absolute or relative), complexity of sample preparation and accuracy of the results. Targeted, or semi-targeted uses a preliminary hypothesis to pre-define the groups of metabolites (usually fewer than 200) under scrutiny, this enables absolute quantification of metabolites within a sample. However, the metabolites analysed are limited, reducing the potential for generating novel hypotheses. Untargeted metabolomics is a much more comprehensive analysis of metabolites (within the scope of the analytical platform, usually thousands for LC-MS) and has much greater potential for hypothesis generating research. However, the volume of complex data produced by untargeted metabolomics is huge and requires extensive data reduction, processing, and analysis before any biological understanding can begin.[131, 132] To ensure that metabolomic data can be translated into clinically useful information, each step of an untargeted metabolomics workflow needs careful consideration when designing a study (**Figure 8**).

1.7.2 Analytical chemistry platforms

The most frequently used technologies for this are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. MS is commonly coupled with liquid chromatography (LC-MS), gas-chromatography (GC-MS) or capillary electrophoresis (CE-MS). Each of these four (NMR, LC-MS, GC-MS, and CE-MS) have their pros and cons (see **Table 8**). The benefits of NMR are simpler sample preparation, excellent long-term reproducibility, lower per analysis cost, no need for chemical derivization and non-destructive sample analysis, but it has lower sensitivity than MS, only detecting the more abundant compounds whereas MS can detect compounds of much lower concentration.[128] MS, rather than NMR spectroscopy will be used for this work, an in depth assessment of the pros and cons of NMR are beyond the scope of this work, it can be complementary and its role in metabolomics has been covered in depth in several reviews.[128-130]

Table 8 Analysis platforms for metabolomics.

Analytical platform	Advantages	Disadvantages
NMR	Minimal sample handling. No need for chromatography. Easily quantitative. Multiple means of metabolite identification.	Limited to detecting the most abundant metabolites
LC-MS	Can measure very low concentrations of metabolites. Higher resolution and dynamic range. Wide range of metabolites measurable	Long-term signal reproducibility is poor
GC-MS	Can measure very low concentrations of metabolites. Higher resolution and dynamic range.	Can introduce unwanted chemical artefacts Long-term signal reproducibility is poor

Put very simply, mass-spectrometry provides data on the mass-to-charge (*m/z*) ratios of the metabolites (or fragments of metabolites) within a sample. Coupling chromatography beforehand provides partial or complete separation of metabolites prior to MS detection, this can either be in the form of gas chromatography (GC-MS - using flow of nitrogen or helium to push the metabolites through the column) or liquid chromatography (LC-MS – using the flow of liquid solvent to push metabolites through the column). Application of chromatography prior to MS has a number of advantages, it can provide separation of metabolites with the same *m/z* so both metabolites can be separately detected, it reduces the complexity of the sample entering the MS which in turn prevents ionisation suppression (where the ionisation of a high concentration metabolite reduces the ionisation efficiency of a lower concentration metabolite) and it also provides data on retention time (time between injection of a sample and metabolite detection) which can aid metabolite chemical identification.

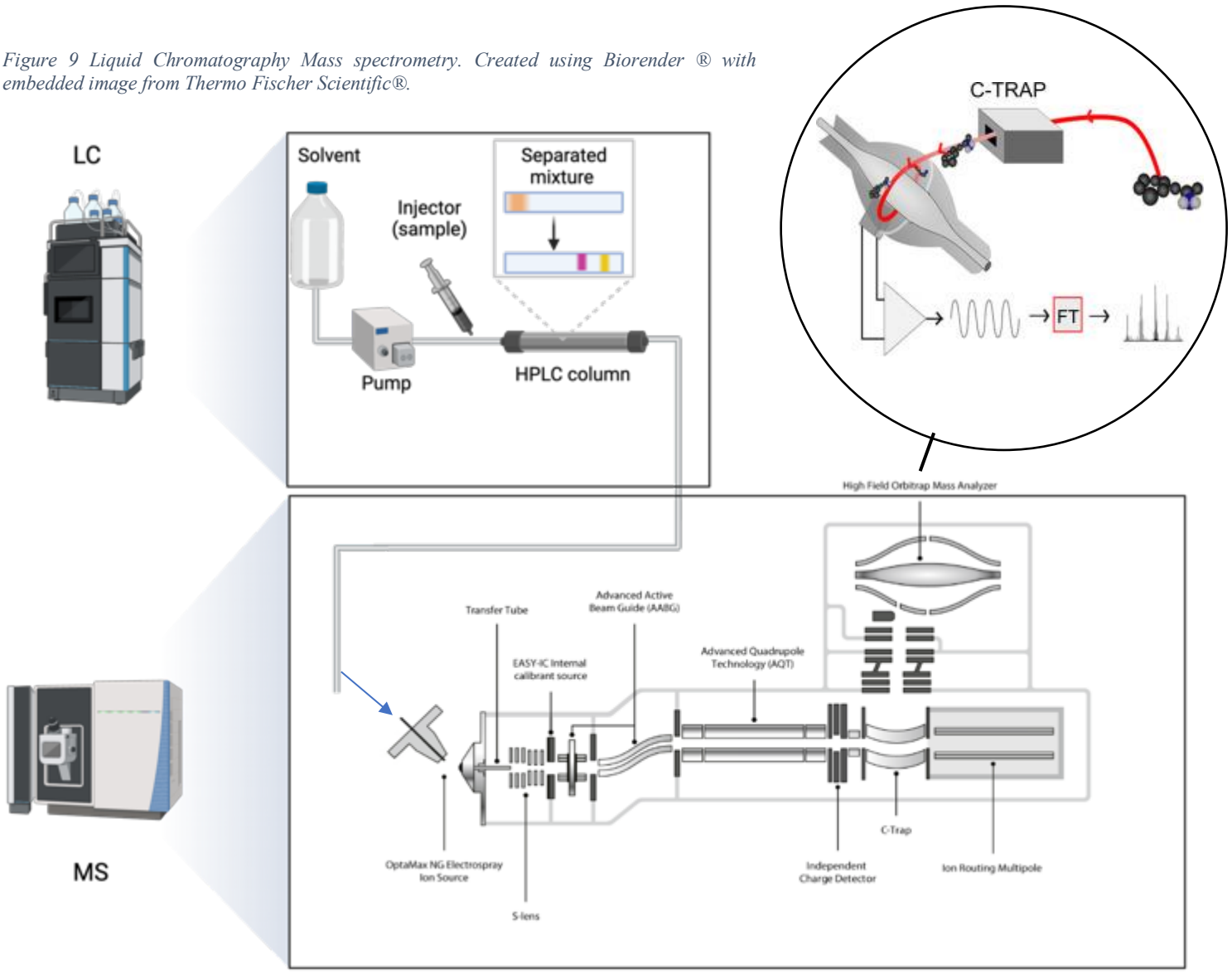
1.7.2.1 Liquid Chromatography Mass Spectrometry

LC-MS couples the physical separation of metabolites provided by liquid chromatography to the mass analysis provided by mass spectrometry. An entire LC-MS system as used in this work provides an interface between the two, which transfers the separated components eluting from the LC column into the ion source of the mass spectrometer.

High Performance Liquid Chromatography (HPLC): HPLC is the separation of sample compounds in a column packed with a 'stationary phase'. The 'mobile phase' refers to the solvent (or eluent) that flows through the column, carrying the liquid sample. The liquid sample is introduced by an autosampler and then a pump pushes the 'mobile phase' through the column at a set pressure. After elution from the column, the mobile phase transports the separated compounds to the ionisation source for transfer into the mass spectrometer. (See **Figure 9**)

The specific mass spectrometer used for this study was the Thermo Scientific Orbitrap Exploris 240 Mass Spectrometer ®. MS steps can be broken down into ionisation, acceleration, deflection, and detection. Electrospray ionisation (ESI) takes the liquid eluting from the LC column and creates gaseous ions required for MS analysis. The sample passes through a narrow, high potential electrical nozzle that results in an electrostatic spray composed of multiple charged droplets. These then pass through a heated source at atmospheric pressure where the solvent evaporates into microdroplets, and the gaseous charged ionized analytes are formed. After which they are accelerated through the quadrupole (which allows selection of specified *m/z* ranges) then towards the C-trap (effectively an external ion storage device). From the C-trap they are sent into the Orbitrap using a high voltage pulse across the trap. Unlike earlier MS technology that utilised magnets to deflect ions dependent on their *m/z* ratio, the Orbitrap uses electric fields instead. In an Orbitrap, the outer electrodes cause the ions to orbit around the central electrode and oscillate back and forth. The frequency of oscillation is dictated by each ion's *m/z* ratio. The oscillation of these ions on the central electrode induces a current that can then be detected and mathematically converted into a mass spectrum for analysis (See **Figure 9**).

Figure 9 Liquid Chromatography Mass spectrometry. Created using Biorender ® with embedded image from Thermo Fischer Scientific®.



1.7.2.2 Processing of LC-MS data

There are three dimensions to the full-scan raw data produced by LC-MS: retention time (RT), mass to charge ratio (*m/z*), and signal intensity. A feature is a molecular body that has a unique *m/z* and RT. A single metabolite can form many features, different features related to the same metabolite will have a different *m/z* ratio but the same RT. The output from untargeted mass-spectrometry results is a large number (typically up to 15,000) of 'features' for each sample.

Before analysis the three-dimensional raw data undergoes pre-processing to identify/quantify features and create two-dimensional numeric data.

Processing LCMS data is done in three parts, Peak picking or deconvolution, data alignment and chemical identification of metabolites, and can be done with several different software packages such as the open-source package XCMS®. Peak picking is performed on each sample separately and every possible *m/z* or *m/z* range is plotted to look for chromatographic peaks, if there is a chromatographic peak, the area of that peak is calculated, and the *m/z* ratio, retention time and peak area are reported providing a list of metabolite features in each sample. Next, the samples are combined into a single dataset and peaks are matched across all samples and peak areas reported. A metabolomics data table can be created with rows corresponding to individual samples and columns containing the ion intensity values of a specific metabolite feature. Most metabolomics work results in high dimensional data matrices, with a very high volume of information from each sample.

Quality control in studies with larger numbers of samples must be considered. Multiple analytical batches are run, unavoidably the samples physically interact with the machine itself creating changes in the responses measured over time. Quality control (QC) and process blank (PB, containing no biological material at all but processed in the same way as biological/QC samples) samples are periodically analysed, algorithms can use this data to correct the signal attenuation, remove peaks with poor reproducibility, report data quality and link batch data together. As this signal correction and batch integration is not perfect, sample order within each batch should be random but stratified by exposure group to reduce the potential for systematic bias (from sample preparation or injection order).

1.7.2.3 Data reduction and visualisation

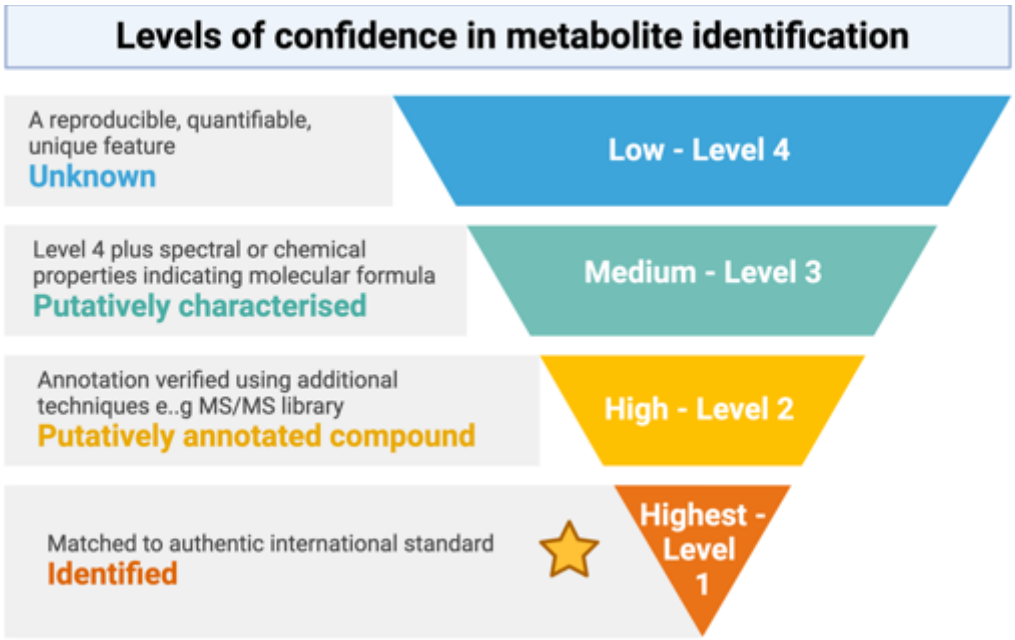
High dimensional data matrices make traditional statistics and visualisation techniques challenging. Dimension reduction whilst retaining as much information as possible is essential to being able to understand the data. Principle component analysis (PCA) is the commonest form of dimension reduction, this modelling reduces a complex three-dimensional dataset to show only the largest differences between samples, thus enabling more traditional data visualisation and statistical methods to be applied to a much narrower data table.

1.7.2.4 Identifying metabolites

The end goal is being able to correlate this complex data output to relevant biological knowledge, to do this the metabolites detected must be chemically identified. *m/z* values from features can narrow the possibilities down to a limited number of potential metabolites and then this can be further narrowed down, or a single metabolite identified using the retention time (RT) and MS/MS fragmentation mass spectrum of that compound through comparison of experimental data to data collected for authentic chemical standards. The use of RT and MS/MS data can be limited due to the relatively low availability of chemical standards required to construct RT and MS/MS libraries.

There are four levels of confidence in metabolite identification, level 1 compares the data acquired to equivalent data for a pure chemical standard analysed using the same scientific method, if they match then there can be high confidence in metabolite identification. If this cannot be done then, with reducing confidence, compounds can be putatively annotated (level 2), putatively characterised (level 3), or classed as unknown (level 4).[133] (**See Figure 10)** To enable annotation and characterisation, data output can be matched to metabolite databases such as those described in section 1.7.1 (**Table 7)**.

Figure 10 Annotating metabolites, levels of confidence. Figure created in Biorender @.



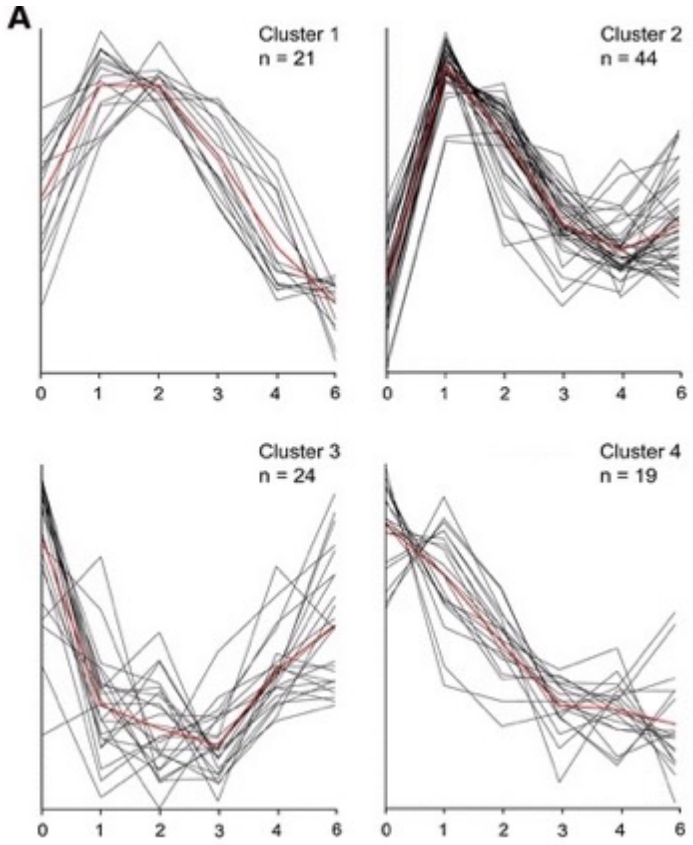
1.7.3 The post-prandial metabolome

Metabolomics has been proven to be of use in reflecting physiological changes to nutritional intake, both short and long term. Measuring post-prandial responses to meal challenges has been used to investigate the fate of different food types, the interplay of biological mechanisms involved and their response to different dietary intake. Several studies have proved that different dietary intakes are associated with identifiable metabolomic signatures. Anderson *et al* successfully differentiated between two differing dietary plans (new Nordic diet vs average Danish diet) using untargeted metabolomic profiling. Cheung *et al* could identify a dose dependent change in metabolites related to meat and fish using a metabolomic study of 24-hour urinary samples. [134, 135]

Of greater interest to this body of work is the immediate (within 6 hours) response of the metabolome to dietary intake (with the hypothesis that *'those with PEI will have a significantly different metabolomic profile in response to a test meal when compared to healthy controls'*). Several studies have successfully investigated the short term metabolic response to different diets.[136-138] A systematic review of the literature (as detailed later, in test meal development methods, **section 2.1.2**) highlighted preceding work on the post-prandial metabolome. Of most relevance to our proposed study is the extensive work by Pellis *et al* on plasma metabolomic profiling after a postprandial challenge.[139] Pellis *et al* gave fasted subjects a high fat dairy shake (300 ml custard, 150 ml cream cheese and 50 ml whipping cream) and took blood samples at 0hr (fasted), 1hr, 2hr, 4hr and 6 hours and used GC-MS metabolomic profiling. The results revealed major metabolic changes following the test meal. Their work identifies the changes in plasma concentrations of 145 metabolites and identified six discrete post-prandial time courses ranging from rapid response (with maximum change within 1-2 hours) to slow (maximum change in plasma concentration at 6 hours). For metabolites associated with carbohydrate metabolism there was a classical absorption profile with values peaking at 1-2 hours post-prandially the same was shown for amino-acid metabolism showing a very rapid increase, returning to baseline in 4-5 hours. Lipid metabolism was a little more varied; Total triglycerides, C10:0 free fatty acid, monoglycerides C16:0 and C18:1 levels rose immediately after intake with maximum values at 1-2 hours, followed by slow increase in C12:0 and C14:0 free fatty acids and longer free fatty acids (C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, arachidonic acid (C20:4)) rising in values all the way up to 6 hours. (See **Figure 11**) Pellis *et al.* then went on to instigate a dietary intervention in a group of patients, repeated their 'post-prandial challenge test' in those having undergone dietary intervention and a control group and proved identifiably different post-prandial responses in the intervention versus control group.

This sets the precedent not only for the validity of a test meal creating significant, measurable, short-term changes in the metabolome, but also that these changes can be identifiably different in those with differing dietary intakes/absorption/metabolism. [139]

Figure 11 Time cluster profiles of plasma metabolites following a challenge meal (reproduced from Pellis et al 2012).



Red line represents average cluster time profile. Discrete post-prandial time courses: Metabolites associated with carbohydrate metabolism peaked at around 1-2 hours, similar for amino acid metabolism. Lipid metabolism more varied, total triglycerides, C10:0 FFA, monoglycerides C16:0 and C18:1 levels rose immediately after intake with maximum values at 1-2 hors, followed by a slow increase in C12:0 and C14:0 FFAs s and longer FFAs rising in values all the way up to 6 hours

1.8 Hypothesis, Aims & Objectives of the DETECTION trial

1.8.1 Hypothesis

A test meal will create a dynamic change in the measurable blood metabolome. The post-prandial dynamic metabolome of those with PEI will be different to those with healthy exocrine function.

1.8.2 Aim

To determine the post-prandial metabolome among those with and without PEI and use this to differentiate between the cohorts to propose a novel diagnostic test.

1.1.1 Objectives

Primary

- To define the metabolomic profile of patients with PEI in the fasting state and following a test meal and compare these with healthy controls.

Secondary

- To define an optimal time for assessing the metabolic profile after a test meal.
- To compare patient's metabolomic results to their ^{13}CMTG breath test results.
- To identify whether changes in the metabolome correlate to severity of PEI.
- To determine the accuracy and specificity of a metabolomic test for diagnosis PEI (using ROC curves).
- To identify whether this test could be used to dose patients with PERT.

1.1.2 Ethical consideration

Ethical approval was obtained for this study from the West Midlands Research Ethics committee and approval was granted by the University Hospital Birmingham. The purpose of the study was explained and written informed consent was obtained from all subjects taking part in the study. See methods for full protocol and **Appendix 1** for HRA approval letters.

Chapter 2 Methods

There are three discrete bodies of work covered here; setting up and refining the ^{13}C MTGT to use as "gold standard" for establishing PEI status in trial recruits, designing/delivering the DETECTION trial, and finally analysing the acquired samples using liquid chromatography-mass spectrometry. Therefore, this methods chapter is divided into 3 sections:

Section 2.1: Development of the ^{13}C MTGT to use as a "gold standard" reference test for PEI.

Section 2.2: Development of the DETECTION study protocol.

Section 2.3: Acquisition, processing, and analysis of samples for metabolomics.

Summary of chapter 2

Section 2.1 covers local set up, refinement and validation of the ^{13}C MTGT for use as a reference test for PEI. The ^{13}C MTGT was the most appropriate choice for a reference test but not in widespread use, locally there was no existing expertise and it required set up from scratch. Training and guidance were provided by Professor Dominguez-Munoz (a world expert in breath test methodology), Dr. Afolabi and Dr. Slater (both of whom have used the ^{13}C MTGT in their own research) and I conducted my own systematic review of the literature on ^{13}C MTGT methodology and accuracy. Setting it up to be used alongside metabolomic testing required a few modifications (especially regarding the challenge meal), as such series of experiments were designed to develop and validate my protocol using a novel challenge meal. Also included in this section is sample (breath) acquisition for the ^{13}C MTGT, measurement of ^{13}C isotope abundance and determining cumulative percent dose recovery of ^{13}C.

Section 2.2 covers development of the DETECTION study trial protocol and includes pilot work, PPIE work, the development of standard operating protocols and a summary of the final study protocol (the full protocol and all amendments as approved by the West Midlands Research Ethics committee and University Hospital Birmingham are in **Appendix 1**.). This protocol was designed and developed by me with advice and input from Professor Roberts (Consultant HPB surgeon at UHB) and Professor Dunn (Professor of Metabolomics at the University of Birmingham at the time), between whom there is a wealth of expertise on PEI and metabolomics.

Section 2.3 covers the acquisition, processing, and analysis of metabolomic samples. Included here is study design, subject selection, and recruitment to ensure adequate quality sample acquisition in both pathological cohorts and healthy controls. Also covered is metabolomic sample storage, transfer, processing, and analysis,

2.1 Development of the 13C MTGT to use as a "gold standard" reference test for PEI

2.1.1 Systematic review and meta-analysis of methodology and accuracy of the 13CMTGT

Methods covered here, for results see section **3.2.** *Full review published in Pancreatology. 2023 Feb 16:S1424-3903(23)00040-6. doi: 10.1016/j.pan.2023.02.004. PMID 36805050 (1st Author)*. (See appendix 8)

The ^{13}C MTGT, as discussed above was the most appropriate choice for a reference test to ascertain PEI status, unfortunately it was not in widespread use, locally there was no existing expertise and no accepted protocol is in use in the U.K. It is in more routine use in Europe (although still limited to specialist or research centres). Before using the ^{13}CMTGT as a reference test within the DETECTION study it was essential to ensure a robust, reproducible protocol that could produce reliable results. To establish current practice and variation, a systematic review of the literature on ^{13}C MTGT methodology and accuracy was performed.

This review was prospectively registered with the PROSPERO database (CRD42020199944) and reported according to PRISMA guidelines.[140]

2.1.1.1 Search strategy.

A structured literature search was conducted of MEDLINE, EMBASE, PubMed and COCHRANE online databases. Free search terms, MeSH terms and EMTree terms (where relevant) were used for the two components, pancreatic exocrine insufficiency (or pancreatic function), and the ^{13}C MTGT, these were combined using the Boolean operator 'AND'. Database searches included all results from inception of the database to 1st July 2022.

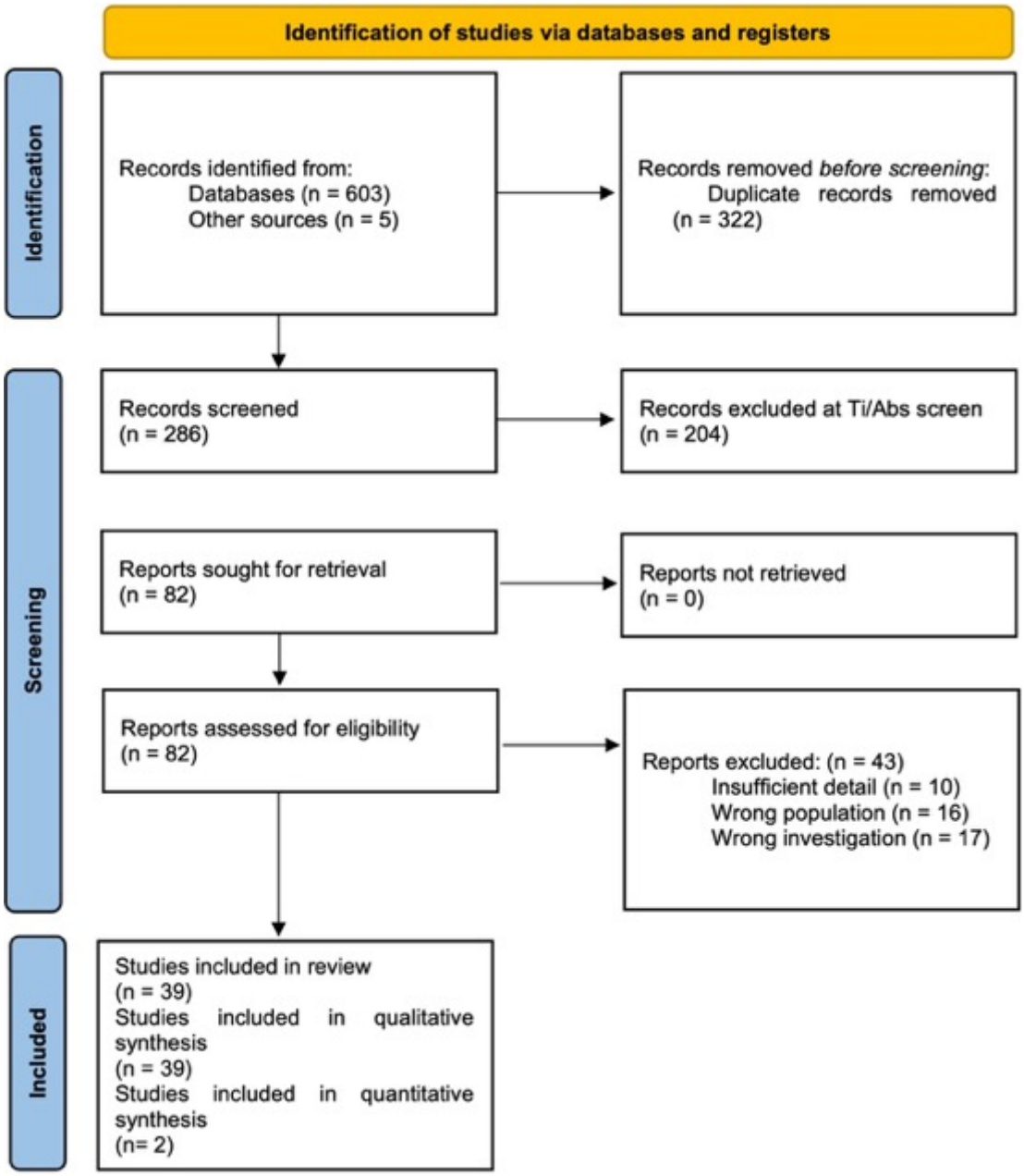
2.1.1.2 Selection criteria.

Following the removal of duplicates two independent reviewers (SPB and ME) screened by title and abstract and then by full text review. Any disputes were resolved by a third, independent, reviewer (LH). Selection was limited to full articles unless the abstracts contained enough detail to meet the rest of the inclusion criteria. Translations were sought for full texts that had the potential for inclusion. Primary studies reporting on adult patients or healthy controls undergoing the ^{13}C MTGT for the evaluation of pancreatic exocrine function were considered eligible for inclusion. Studies included had to give a description of the methodology of their ^{13}C MTGT used (at least four of the data extraction points for methodology had to be described). Studies which included adults and children were included but interrogated based on their adult population only. Studies reporting on children alone were excluded. See **Figure 12** for PRISMA flow diagram of study identification.

2.1.1.3 Data extraction and outcome definitions

Data extraction elements were defined in advance for both the quantitative and qualitative elements. Two reviewers independently extracted data from each eligible study using a pre-piloted extraction template (SPB and ME). A third reviewer assessed the extracted data to ensure accuracy and completeness (LH). The core themes extracted for qualitative review were pre-test control measures, dose of ^{13}C MTG, test meal constituents, in-test control measures, breath collection (frequency, method, and total timeframe), and analysis platform. For quantitative synthesis, the following were extracted: reference standard, number of participants (patients and controls), diagnostic cut-off values, sensitivity, specificity, true positive (TP), false positive (FP), false negative (FN), and true negative (TN) rates.

Figure 12 PRISMA flow chart for 13C MTGT methodology and accuracy review.



2.1.1.4 Study quality assessment.

The QUADAS-2 tool was used to assess the quality of primary diagnostic accuracy studies.[141] Two independent reviewers assessed the risk of each relevant paper (SPB and ME) and disputes were settled by a third reviewer (LH).

2.1.1.5 Statistical analysis

All papers meeting the eligibility criteria were included in the narrative synthesis (n=39) a thematic analysis was undertaken and summarised in a narrative fashion to address each of the method steps under scrutiny. As per the meta synthesis of qualitative data described by Thomas and Harden in 2008, themes were identified, coded, compared, and merged to allow for higher order data abstraction.[142] Quantitative synthesis of diagnostic accuracy was limited owing to the heterogenous nature of the reference tests applied, studies comparing the ^{13}C MTGT to the current gold standard of PEI diagnosis (CFA) and the old gold standard (duodenal intubation and enzyme collection) were included in quantitative synthesis. Sensitivity, specificity, accuracy, positive and negative predictive values, of the ^{13}C MTGT in the diagnosis of PEI were obtained from the individual studies, and forest plots were used to calculate and graphically display pooling of the data. Statistical analyses were conducted using Stata 16.1.

Results are shown in **section 3.2,** in summary, this systematic review enabled the creation of an evidence-based protocol to use in the DETECTION trial and showed the ^{13}C MTGT to be an accurate enough measure of PEI to use as a reference test. To use the ^{13}C MTGT alongside metabolomic sampling I needed to create a test meal that adheres to the test meal criteria outlined in this review and that could be used in any environment without complex storage or re-constitution, as such, the next step in trial development was to design and validate an appropriate test meal (see methods **section 2.1.2** and results section **3.3**).

2.1.2 Test meal development.

To test the core hypothesis that '*A fatty test meal will create a dynamic change in the metabolome that is significantly different in those with PEI compared to healthy controls*', the DETECTION study delivers a test meal that must elicit pancreatic stimulation for both the reference test (the ^{13}C MTGT) and for metabolomic testing. To this effect the test meal must be compatible with both. The ^{13}C MTGT as highlighted in the systematic review (**Section 2.1.1 and 3.2**) has no universal protocol, and the test meal has very little consensus in published literature. Blood sampling for metabolomics is not a novel field, however looking for post-prandial metabolites that reflect pancreatic exocrine insufficiency is and as such there is no precedent for what test meal to use. A review of the existing evidence and theory behind test meal constituents for metabolomic sampling was performed (**methods described below, results in section 3.3.1**). Also covered will be what other factors are required for a practical test meal to be used within the DETECTION study and the potential test meals reviewed.

There is no precedent for examining the post-prandial metabolome of patients with exocrine dysfunction, and the idea of using this as a diagnostic marker for PEI is entirely novel therefore the test meal must be developed from scratch based on wider literature on the post-prandial metabolome and the physiology of pancreatic secretion. The post prandial metabolome has been the subject of thorough investigation and there exists a very wide range of challenge meals most targeting a specific biology system. I undertook a structured literature search of MEDLINE, EMBASE, PubMed and COCHRANE online databases with the aim of identifying existing challenge meals that elicited an identifiable post-prandial response in the blood metabolome. Free search terms, MeSH terms and EMTree terms (where relevant) were used for two components, metabolomics (or metabolome), and test meal/challenge meal, these were combined using the Boolean operator 'AND'. Database searches included all results from

inception of the database to 1st Jan 2020. Following the removal of duplicates two independent reviewers (SPB and ME) screened by title and abstract and then by full text review. Any disputes were resolved by a third, independent, reviewer (LH). Selection was limited to full articles unless the abstracts contained enough detail to meet the rest of the inclusion criteria. Primary, human studies reporting on the blood/plasma metabolome of adults in the 6 hours following a dietary challenge (mixed macro-nutrient) were considered eligible for inclusion. Those looking at longer term changes to the metabolome after a sustained dietary change were excluded. Studies using challenge meals targeting a single macronutrient were excluded, these include OGTT, OLTT, and OPTT (oral glucose OR lipid OR protein tolerance test), as pancreatic enzyme secretion is targeted mainly at lipid and protein digestion, challenge meals based on a single macronutrient alone would be inappropriate.[151]

Data extraction elements were defined in advance. Two reviewers independently extracted data from each study using a pre-piloted extraction template (SPB and ME). A third reviewer assessed the extracted data to ensure accuracy and completeness (LH). The core themes extracted for review were test meal constituents, sample collection time points, analysis platform and metabolite responses (focus on lipid and protein metabolism). The aim of this review was to identify requirements for a challenge meal that would result in an early, identifiable response in the post-prandial blood metabolome (specifically metabolites related to protein and fat metabolism), and to identify a timeframe for sampling that would capture the dynamic responses of fat and protein metabolism. Results of this are covered in **section 3.3.1,** this review alongside the review of ^{13}C MTGT methodology and practical requirements for the DETECTION trial resulted in a challenge meal of a **Nutillis® Complete Crème Level 3 and a Fortisip® 2Kcal .**

Following test meal design, a short series of experiments were performed to ensure that this meal would be a suitable medium for carrying the ^{13}C MTG tracer, that the novel test meal produced comparable results to commonest test meal for the ^{13}C MTGT and that these results were reproducible, the results of these experiments are in **section 3.3.2, 3.3.3 and 3.3.4** and the methods are presented below.

2.1.3 Validating a novel test meal for use with the 13C MTGT.

This sub-study has been published in a peer reviewed specialist journal, See Appendix 9. *'A standardised nutritional drink as a test meal for the 13C mixed triglyceride breath test for pancreatic exocrine insufficiency. A randomised, 2-arm crossover comparative study. Powell-Brett, S. Hall, L. Halle-Smith, Journal of Human Nutrition and Dietetics* 10.1111/jhn.13237.' Methods are presented here and results in **section 3.3.3.**

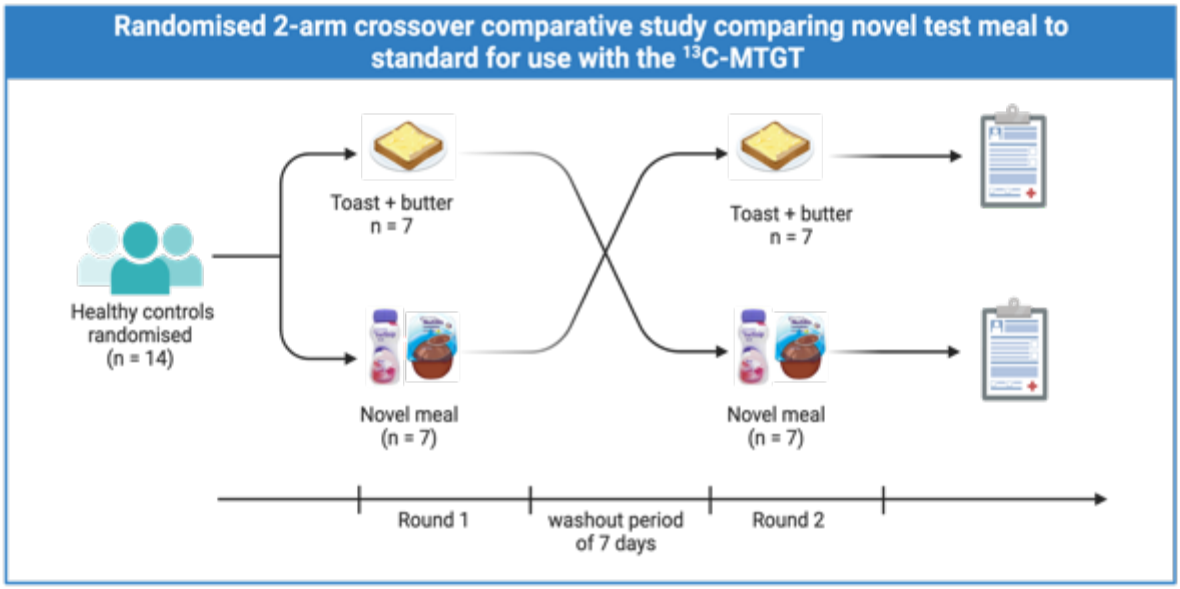
Before using this challenge meal in conjunction with the ^{13}C MTGT, a prospective, randomised, 2-arm crossover comparative study comparing it to the most widely used challenge meal (toast and butter) as used by the Domínguez-Muñoz group was performed to ensure that the results of the breath test could be correctly interpreted when using this novel meal. This was conducted according to the guidelines laid down in the Declaration of Helsinki and all study procedures were approved by the Black Country Research Ethics Committee and all subjects completed a written consent form.

Ethics*:* As per the DETECTION study (See **Appendix 1**)

Inclusion and exclusion criteria: As per the DETECTION study protocol for healthy controls.

Study protocol: Each subject was randomly assigned to receive either the novel or the standard test meal at their first attendance and then they repeated the test with the alternate meal at their second attendance. The second test day had to be at least 7 days after the first to allow for adequate ^{13}C washout. (**Figure 13**)

Figure 13 Meal validation study infographic, (created using Biorender ®.)



At the initial screening interview, subjects were given pre-test instructions. They had to refrain from ingesting foods rich in ^{13}C (corn products, cane sugar, pineapple, kiwi, broccoli, or sweetcorn) for 48 hours preceding the start of the test and they were asked to fast (except for small volumes of water) and refrain from smoking for 12 hours preceding the test. Breath samples were collected by blowing through a straw into an inverted exetainer (Exetainer®; Labco Limited, High Wycombe, UK) and immediately capping. Subjects were asked to attend 30 minutes before the test start time to allow heart rate and temperature to settle, baseline breath samples were taken then the assigned test meal with 250mg of ^{13}C MTG (weighed on a five-figure balance) carefully incorporated into it was ingested over a maximum of 5 minutes at around 8am. Repeat samples were then taken every 30 minutes up to a total of 6 hours. For the

duration of the test subjects had to remain sedentary and fasted. Small amounts of water were allowed for comfort and a short trip to the loo at a gentle pace if needed. The $^{13}CO_2$ content of the breath samples was determined by GC-IRMS (Gas chromatography isotope ratio mass spectrometry). Enrichment of $^{13}CO_2$ in the post-test meal samples was calculated by subtracting the abundance of $^{13}CO_2$ in the baseline sample from that of each post-test meal sample. The percentage dose of ^{13}C recovered (PDR) for each sample was calculated using the equation below (**Figure 14**). The cumulative PDR (cPDR) was calculated by adding individual PDR values averaged over the time interval.

Figure 14 Calculation for the percent dose recovered (PDR) at each time point.

$$PDR\ h^{-1} = \frac{VCO^2\ (mmol.h^{-1}) \times Breath\ CO^2\ enrichment\ (ppm\ ^{13}C\ excess) \times 100}{mmol\ excess\ ^{13}C\ in\ dose \times 10^6}$$

Statistics:

Data were summarised as either median and interquartile range (IQRs) for non-parametric continuous variables, mean and standard deviation (S.D) for parametric continuous variables, or by percent for categorical variables. The Shapiro-Wilk test was used to test for normality. ^{13}C enrichment of exhaled breath was determined with an isotope ratio mass spectrometer (IRMS). The primary outcome was cumulative percent dose recovery of ^{13}C (cPDR) at 6 hours. A one-way repeated ANOVA was run to look for a difference in cPDR between the two test meals. A one-sample mean T-test was used to determine minimal detectable difference. Stata 16.1 was used for all statistics.

Results: In summary, 14 healthy controls were recruited and performed both meals adhering to the strict 13C MTGT protocol, no detectable difference was found between the cPDR for the two test meals, (***for full results see Chapter 3, results, section 3.3.3).***

2.1.4 Preparation and consumption of substrate and test meal

Precise weighing and incorporating of the ^{13}C labelled MTG into the chosen test meal is paramount to obtaining accurate results from the ^{13}C MTGT reflective of a trial recruits' true exocrine status. On the first healthy control pilot patient the recovery of ^{13}C in exhaled breath was worryingly low, this recruit had no history or findings indicative of PEI and had consumed the entire test meal across the correct timeframe whilst adhering strictly to the test day protocol criteria. 3 repeated measures reflected similar results and therefore I examined the substrate incorporation steps in detail and investigated for potential losses.

Original process:

- Weighing ^{13}C MTG powder onto a small plastic square tray on a 5-figure balance
- Tipping this into the test meal, stirring to distribute and replacing the lid.
- The recruit would then use a spoon to ingest the meal over 5 minutes.

On close inspection there were several points of substrate loss, the corners of the small plastic tray when tipped into the meal had small amounts of powder left in them, the spoon used to stir the substrate into the mousse had mousse and powder particles stuck to it. Weighing of all containers, implements and cutlery confirmed discrepancies pre and post meal assembly making this a likely source of substrate loss. Weighing onto a small square of foil was tried but it was evident macroscopically and via pre and post assembly weighing of the foil that small amounts of ^{13}C MTG powder were stuck in the creases. A revised process was devised to minimize potential points of substrate loss.

Revised Process:

- Weighing ^{13}C MTG powder **directly** into the mousse
- Same spoon used to fold substrate into the mousse and for ingestion so any powder stuck to the spoon would be ingested.
- Folding not stirring the substrate into the meal minimizing losses to the edge of the pot.
- Asking the recruit to lick the foil lid of the mousse to catch any powder stuck to it.
- Ensuring recruit took a small volume of water after the meal to wash down any residue.

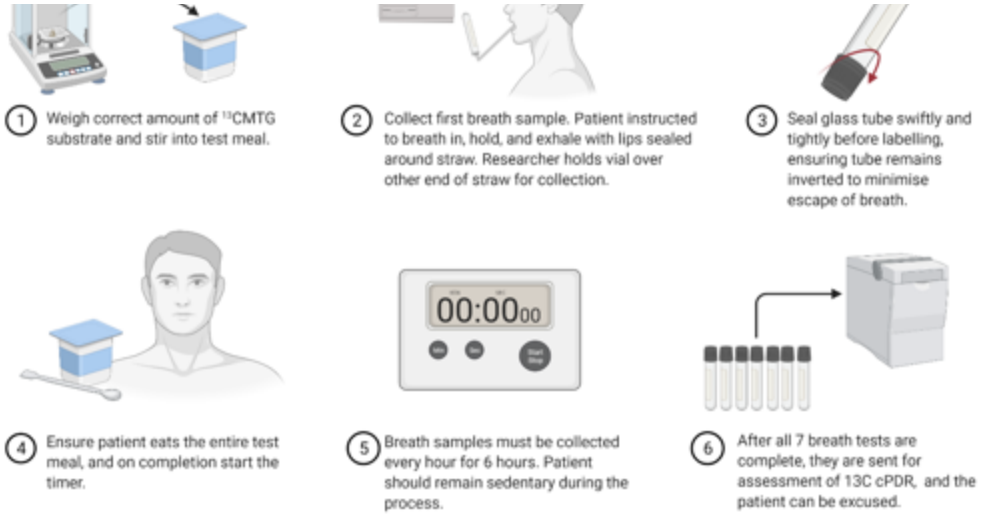
Using the revised process, the recovery of ^{13}C MTG was much more aligned with expected for a healthy control of that age group. **(**For full results **see chapter 3, results section 3.3.4)**

2.1.5 Sample acquisition – Exhaled breath for the ^{13}C MTGT.

Breath samples for analysis of ^{13}C recovery were obtained hourly after the test meal ingestion, finalised protocol for ^{13}C MTGT sample acquisition for determining PEI status was as follows (work-flow diagram in **Figure 15**):

- Arrive 7.30am, starved overnight, consumption of diet low in ^{13}C the previous day.
- Baseline breath taken after 20min sedentary.
- Entire test meal with 250mg 13C MTG incorporated ingested over 5 minutes.
- Spoon and lid licked clean and 50ml of water taken to rinse mouth.
- Breath samples taken every hour until 6 hours after test meal ingestion:
    - Inverted labco® exetainer and cap held by researcher.
    - Breath in, hold for 2sec, seal lips around straw and asked to steadily exhale through straw until the end of their breath.
    - Exetainer capped whilst inverted, labelled with date, study number and time.
- Remain sedentary and fasted for duration of test.

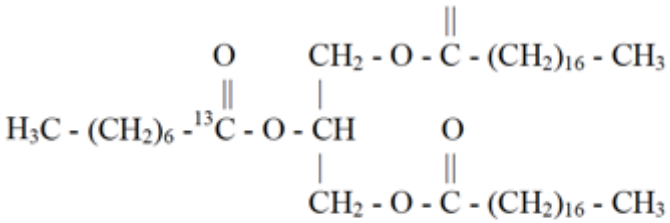
Figure 15 Sample acquisition for the 13C MTGT.



2.1.6 Measurement of ^{13}C isotopic abundance.

^{13}C and ^{12}C are stable isotopes present in all organic materials, The 'Major' isotope (i.e. that of greatest abundance) is ^{12}C, with ^{13}C accounting for only 1.1% of atoms naturally present in the environment. ^{13}C mixed triglyceride is a stable isotope labelled tracer, the major isotope (^{12}C) has been replaced by the minor isotope (^{13}C). The compound used is referred to as 'highly enriched' where 99% of the atoms in a specified position on the molecule (MTG) are the minor isotope (^{13}C rather than ^{12}C). The ^{13}C labelled MTG used in this work was obtained from CK isotopes® (2-OCTANOYL-1,3-DISTEARIN(OCTANOIC-1-13C,99%), microbiological & pyrogen tested, molecular formula C46H90O6) in batches, kept in a dark refrigerated room, protected from light in 1g vials (used within 4 weeks once open). **Figure 16** molecular structure.

Figure 16 Structure of the synthetic triacyglycerol used for the 13C MTGT (2-OCTANOYL-1,3-DISTEARIN).



For the ^{13}C MTGT it is the enrichment of ^{13}C in exhaled breath that is used as a surrogate marker for pancreatic function. Enrichment is the abundance of ^{13}C above baseline, therefore sample acquisition and processing is aimed at measuring ^{13}C isotope abundance in exhaled breath of subjects at baseline and after consuming the ^{13}C labelled mixed triglyceride.

Gas chromatography isotope ratio mass spectrometry (GC-MS) is the analysis platform used to measure isotopic abundance in the breath samples. Subject samples and references are flushed from their exetainers via a double holed needed through a packed column GC at 70° C. The chromatographic peak passes into the isotope ratio mass spectrometer (IRMS; Europa Scientific Hydra ® 20-20) and the ion beams at *m/z* 44, 45 and 46 (for CO_2) are measured and thus the value of ^{13}C abundance is measured. The abundance of ^{13}C is calculated in terms of delta (δ) notation in units of $^{o}/_{oo}$ per mil. Delta is defined as the relative difference in parts per thousand between the sample isotope ratio and the ratio of the international standard.

The original international standard was Pee Dee Belemnite (PDB) a cretaceous marine fossil established as $\delta\,^{13}C$ zero, as none of that original standard exists it has been superseded by the convention that NBS 19, (a carbonate material) has a value of + 1.95% versus PDB. This new scale is termed Vienna-PDB (VPBD). The international atomic energy agency (IAEA)

distributes several secondary standards including graphite (USGS24), oil (NBS-22), and calcium carbonate (NBS-18).

The reference gas used to determine sample gas δ ^{13}C for this work was IA-CO2-8 (δ ^{13}C = -39.97 ‰ vs. V-PDB) which was prepared to 4.2 % CO2 by volumetric dilution. IA-CO2-8 is traceable to NBS-18 Calcite (δ ^{13}C value of –5.01 ‰ vs. V-PDB) and IA-EA-CO-1 Marble (δ ^{13}C value of +2.49 ‰ vs. V-PDB), which are distributed as isotope reference standards by the IAEA, Vienna. Samples of IA-CO2-8 were measured as check samples, flushed in turn along with the subject samples for quality control. Rather than δ notation, for reporting of tracer studies, atom percent (atom %, AP) is preferred, this measures absolute isotope concentration (the atom fraction expressed as a %) with very small abundances expressed in units of parts per million (ppm)

$$\text{atom \% } (AP)\ 13C\ = \frac{[13C]}{[12C] + [13C]}\ x\ 100$$

$$\text{parts per million } (ppm) = atom\ \%\ x\ 10^4$$

As discussed above it is the enrichment of ^{13}C in exhaled breath samples above the baseline sample that is required, i.e the abundance above baseline, expressed as atom percent excess (APE).

$$\text{Atom \% excess (APE)} = (atom\ \%)_E\ -\ (atom\ \%)_B$$

- $(atom\ \%)_E$ is the abundance of the enriched sample.
- $(atom\ \%)_B$ is the abundance of the baseline sample.

AP is calculated for the baseline exhaled breath sample prior to tracer ingestion and APE is calculated for each subsequent exhaled breath sample up to 6 hours following tracer ingestion.

2.1.7 Expressing diagnostic results of the ^{13}C MTGT as PDR and cPDR

To express the diagnostic results of the ^{13}CMTGT, the dose recovered in exhaled breath is calculated (percent dose recovered, PDR) at each timepoint as per the original Vantrappen formula and then the sum of PDR is used for a diagnostic value (cumulative percent dose recovered, cPDR).[170] To calculate PDR the CO_2 production rate (VCO_2) and the breath CO_2 enrichment (calculated as above) is required. VCO_2 can be predicted using the formula by Shreeve et al. (below), Body surface area (m^2) for the Shreeve equation can be predicted using the Haycock equation (below).[185, 186]

$$PDR\ h^{-1} = \frac{VCO_2(mmol.h^{-1})\ x\ Breath\ CO_2\ enrichment\ (ppm\ 13C\ excess) x\ 100}{Tracer\ dose\ (mmol)\ x\ Tracer\ enrichment\ (atom\ \%\ excess\ x\ 10^4)}$$

- $VCO_2(mmol.h^{-1}) = 300\ x\ body\ surface\ area$
    - $BSA\ (m^2) = (weight\ (kg)^{0.5378})\ x\ (height(cm)^{0.3964})\ x\ 0.024265$

- $Tracer\ dose\ (mmol) = \frac{weight\ substrate\ (mg)}{molecular\ weight\ substrate}$
    - Molecular weight of C46*CH90O6 = 751
    - When using 250mg, tracer dose = 250/751

Atom	No.	Mass (g/mol)
C	47	565.50
H	90	90.71
O	6	96
	Total	751.21

- $tracer\ atom\ \%\ excess = abundance\ 13C\ substrate - natural\ abundance\ 13C$
    - With 99% enriched ^{13}C MTG Tracer APE = 99-1.11 = 97.9

With the above knowns, the equation for each enriched breath sample becomes:

$$PDR\ h^{-1} = \frac{(300\ x\ BSA)\ \ x\ Breath\ CO_2\ enrichment\ (ppm\ 13C\ excess)\ x\ 100}{(\frac{13CMTG(mg)}{751})\ x\ \ (97.9\ x\ 10^4)}$$

Therefore, for each subject, height and weight must be taken to calculate the BSA as per the Haycock formula above and the breath CO_2 enrichment in each sample must be measured and the ppm calculated. These are then plumbed into the formula above to obtain the percent dose recovered at each time point. The cumulative percent dose recovered (cPDR) is then calculated as the sum of each PDR value averaged over the time interval between samples. To minimize errors, an excel spreadsheet with auto calculate formulae was set up into which subject height & weight (as measured on the day), substrate weight (as measured on a 5-figure balance, directly into the test meal) and breath CO_2 enrichment values for each time point could be entered and the PDR for each hour, cPDR for the total 6 hours could be calculated. As this is not used in the U.K outside of specialist research setting, this was sent to an external expert to be cross checked using their own dataset of raw results from previous work. (See **Appendix 5** for auto-calculate spreadsheet with P1 as a worked example and **Appendix 6** for a summary of formula)

Having established the ^{13}C MTGT locally to ascertain PEI status, adjusted it for use alongside metabolomic analysis and confirmed reproducibility, trial development could begin. The next section (2.2) covers design and delivery of the DETECTION trial and covers pilot work, PPIE work, protocol development, and acquisition of breath samples for the ^{13}C MTGT.

2.2 Development of the DETECTION trial protocol.

2.2.1 Pilot work

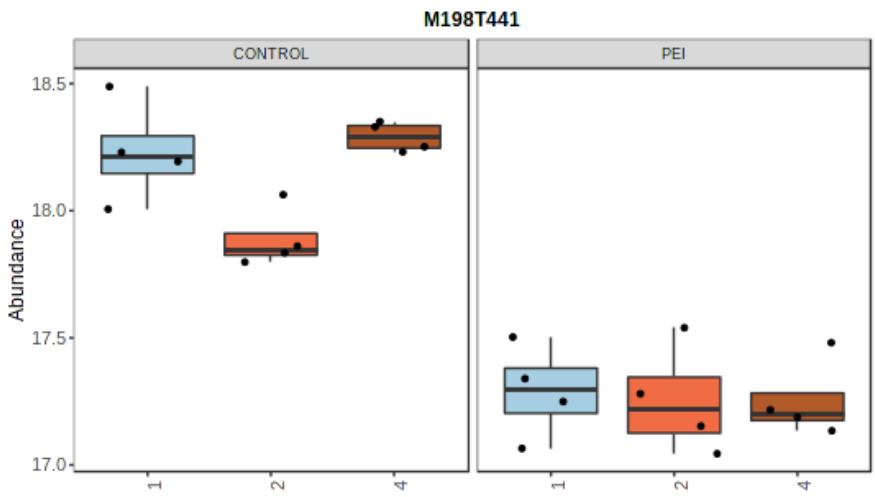
With the starting hypothesis that '*A fatty test meal will create a dynamic change in the metabolome that is significantly different in those with PEI compared to healthy controls*' a short protocol was developed to obtain small volume pilot work. The aim of this pilot work was to prove feasibility, enable a robust trial design and obtain funding to support much larger work. No work had been done regarding the post-prandial metabolome of patients with PEI but Pellis et al. had published a body of work defining the post-prandial metabolome in healthy patients, proving that it responds dynamically to different nutritional intake. Ethics were in place through an amendment to a local tissue and blood bank study (IRAS ID 199000 NSA03) to enable blood sampling for metabolomics, but not for any other form of PEI testing, hence no reference test for PEI was used, just clinical and radiological suspicion from existing investigations. Funding was applied for and received from the University of Birmingham (UoB) ECMC seed grants for preliminary data collection.

Five patients, who were very likely to have PEI were selected by reviewing the inpatients list of the Hepatopancreatobiliary (HPB) team at University Hospitals Birmingham (UHB). Each of them provided written, informed consent to participate in the study. This was a highly selected cohort of inpatients at UHB who had obstructing pancreatic head tumor with symptoms suggestive of PEI and not on any form of enzyme replacement therapy. After a period of starvation, each were given a fatty drink (an off the shelf nutritional drink that these patients were already prescribed as part of their standard care) followed by post-prandial blood samples, hourly for 4 hours.

No reference test for PEI was used and 5 controls from healthy volunteers (no existing medical conditions, no previous gastrointestinal surgery, and no symptoms suggestive of PEI) were used as comparison. They were not age or sex matched and again did not undergo a reference test for PEI.

Samples were quenched, spun and frozen (see section 2.3.4 and 2.3.5 for sample acquisition methods) in preparation for processing via LC-MS (See section 2.3.6 and 2.3.7 for sample and data processing). Results are discussed at length **section 5.2**, in summary, a significant difference between healthy controls and patients with PEI was found across multiple metabolites at multiple time points. Although there were significant limitations to this work (no matching of healthy controls and no formal reference test for PEI) the results gave enough weight to the original hypothesis to start full trial design, ethics, and funding applications. **See Figure 17** for an example of one such metabolite.

Figure 17 Example metabolite showing a significant difference in abundance between patients with PEI and healthy controls across all 3 time points.



2.2.2 PPIE work

Having completed the pilot work phase and created a rough plan of what the DETECTION study would look like, a local expert patient group, CRAG (Clinical Research Ambassador Group) was engaged to assess trial expectations, worth to the patient population, study day acceptability, barriers to recruitment, and review of patient facing documents.

The presentation and patient feedback questionnaire were developed using Pancreatic Cancer U. K's patient involvement toolkit and 'INVOLVE' national standards (an NIHR funded group who developed to set the standards for PPIE work in the U.K.).[205] A Likert scale was used for key question responses along with short answer questions for other feedback. Results are presented in **section 3.4**. See **Appendix 2** for summary answers feedback form from the CRAG group.

2.2.3 Ethics

Ethical approval for this study was obtained from the West Midlands Research Ethics Committee and approval was granted by the University Hospital Birmingham. The purpose of the study was carefully explained and written informed consent was obtained from all subjects taking part in the study. **See Appendix 1.** For HRA approval letters. Several amendments were required throughout the course of the study. These are mentioned in each relevant section and approval letters are included in **Appendix 1.** All IRAS submissions for the original protocol and subsequent amendments were done by me. This study was also registered with the international trials database Clinicaltrials.Gov, NCT0598022.

2.2.4 Final DETECTION trial protocol (Summary only)

2.2.4.1 Trial design

Statement of design

This is a single centre, prospective cohort study investigating the use of metabolomic analysis to identify a metabolic test able to diagnose and quantify PEI.

Patients of interest

For the main study: Adult patients with known PEI and pancreatic cancer. The study aims to recruit 45 patients with pancreatic cancer and PEI, 10 patients with pancreatic cancer and no evidence of PEI and 45 age relevant healthy controls. PEI status will be considered a clinical diagnosis with consultant and dietician agreement (based on symptoms, FE-1 and ^{13}C MTGT results). For secondary outcomes: 15 patients with CP and 15 patients with CF will be recruited.

Intervention

The only invasive procedure in the initial phase will be peripheral venous cannulation and blood sample collection at baseline and at 1, 2, 3, 4 and 5 hours following a test meal.

Comparison

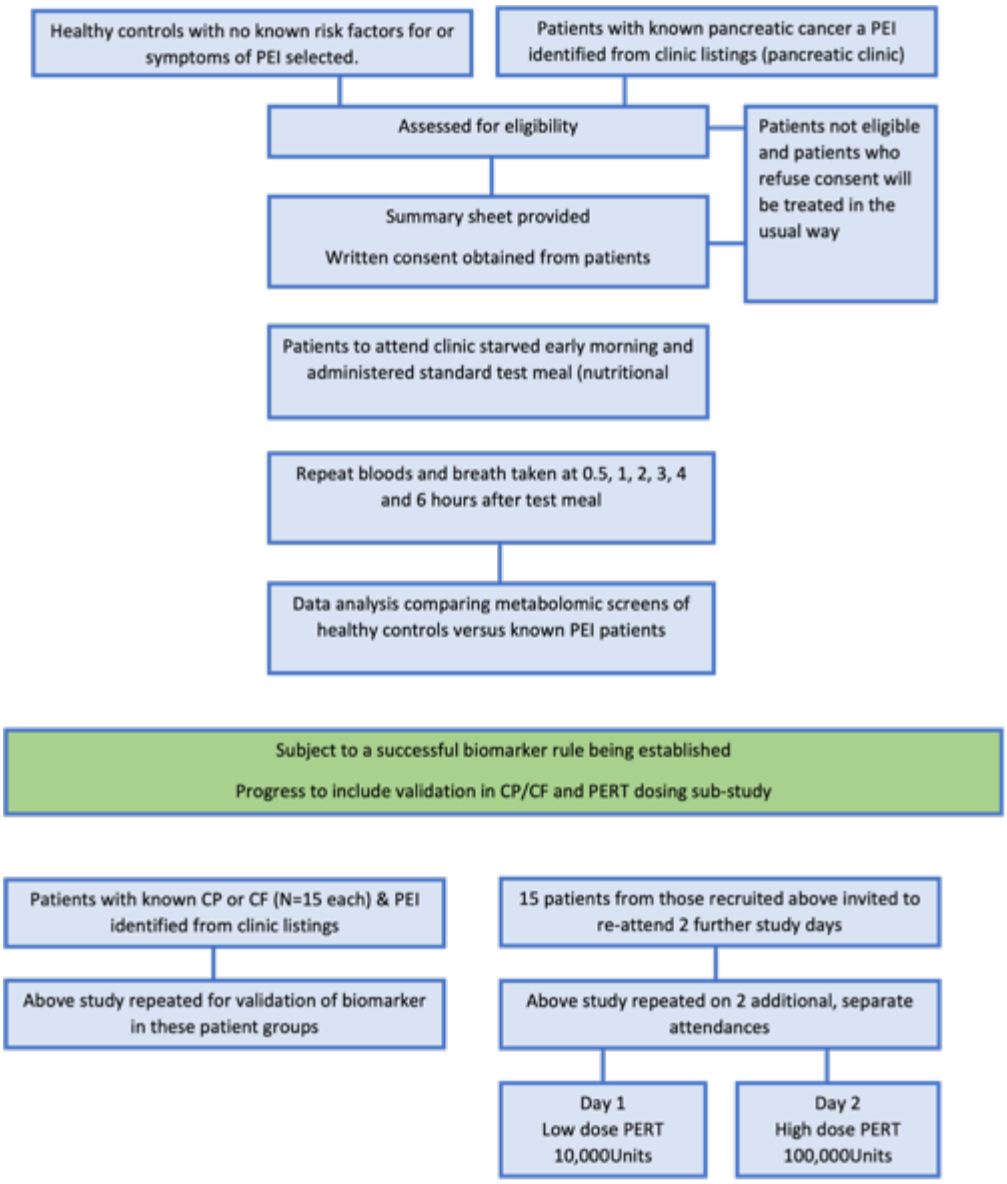
Primary comparison is of the metabolic profile of patients with pancreatic cancer and PEI vs healthy controls following a test meal. The ^{13}C-MTGT and FE-1 will be performed in parallel to establish disease state. In addition, PEI-Q sample questionnaires will be used to compare results to symptom reporting and QOL.

Should the results of the initial test development show an identifiable metabolic profile consistent with PEI then we will repeat the test comparing low and high dose PERT. Those subjects with PEI will be invited to repeat the above tests after the test meal together with a low (10,000u) or high dose of PERT (100,000u) with the test meal to test the hypothesis that PERT will correct maldigestion and malabsorption and further, that the metabolic test can be

used to identify when an adequate dose of PERT has been given. A total of 15 repeat measures will be targeted (with 5 patients each with severe, moderate, and mild PEI).

An adequate dose of PERT will be defined as the ability to correct an abnormal metabolic fingerprint to one that is within 33% of that of a healthy control. The metabolic profiles in the state without PERT will be compared to that with the low and high dose of PERT to assess the stepwise change in repeated measures.

Figure 18 DETECTION study flow chart.



2.2.4.2 Participant eligibility criteria

Inclusion criteria all arms: > 16 years old

Inclusion criteria (Patient arm)

Diagnosis of PEI (see criteria for diagnosis on page 23)

Diagnosis of pancreatic cancer, (CF or CP for secondary analysis patients)

Clinical features consistent with PEI

Inclusion criteria control arm PDAC without PEI

Diagnosis of pancreatic cancer

No formal diagnosis of PEI AND no significant symptoms consistent with PEI

Inclusion criteria healthy controls

No formal diagnosis of PEI AND no significant symptoms consistent with PEI

No diagnosis of pancreatic cancer, CF or CP

Exclusion criteria all arms

< 16 years old

Unable to consent

Unable to travel to UHB for testing

Prognosis < 2months

Performance status 2+

Exclusion criteria healthy controls

Any current or prior diagnosis of pancreatic cancer, CF or CP

Any current or prior diagnosis of PEI

2.2.4.3 Study procedures

Recruitment

Members of the clinical team will identify potential participants for the main study through pancreatic and dietetic clinic at UHB. (For the sub-groups of CF and CP clinic listings for CF clinic and CP clinic respectively, both based within the trust). The research team will approach the potential participant only once eligibility has been confirmed through a review of the medical notes and clinic letters and the parent team consultants have been consulted.

Patients will not be recruited by publicity such as posters or adverts etc. Eligibility will be confirmed by a medical practitioner. To confirm a diagnosis consistent with pancreatic cancer and PEI, clinical team members will review the QEHBs online notes system, Clinical Portal to ensure they meet the criteria for PEI and Pancreatic cancer.

2.2.4.4 Consent

Where potential participants fulfil eligibility criteria, they will be approached by a member of the research team who will provide the patient information sheet and clarify any information from the patient/relatives that may prevent recruitment.

Informed consent will be obtained from the patient at clinic assessment and baseline data collected. Due to the nature of the study patients without the capacity to consent will not be considered eligible. The consent form has been reviewed by the clinical team members (Clinical consultants and dieticians) and by both a generic PPIE group (the CRAG group) and by a specialist gastrointestinal PPIE group based at UHB. At this time, a CRF will be started, and the patient added to the screening log. The screening log will be kept ensuring that there is minimal selection bias during recruitment of participants. Anonymised information will be entered into the screening log, including:

2.2.4.5 Baseline data

All participants will have the following recorded in the Case Report Form (CRF):

Standard: Demographics, Co-morbidities, medications, smoking status

Study related data: Cancer diagnosis, Resection status, Neo-adjuvant or Adjuvant therapy, PEI diagnosis and PERT treatment.

2.2.4.6 Study assessments.

Baseline (on morning of test):

Blood for metabolomic analysis, breath for ^{13}CMTGT, stool for FE-1, BMI, PEI-Q

At 0.5, 1, 2-, 3-, 4- and 5-hours following test meal.

Blood test for metabolomic analysis: See section 2.3.4 for SOP for plasma collection.

Breath for ^{13}CMTGT: See section 2.2.4 for SOP for breath test.

2.2.4.7 Adverse event and serious adverse event reporting (AE and SAE)

AE and SAE reporting

AEs and SAEs in this study relate to events that occur during the test meal and fasting period. All AEs and SAEs should be recorded in the AE and SAE log. Reports of SAEs should be made to the CI. All SAEs will be reported to the Sponsor within 15 days of receipt by the CI.

Likely AE and SAEs that may occur during the study.

Events surrounding blood test or cannulation, such as significant pain or bruising.

Events surrounding the fasting period, such as a hypoglycaemic episode.

Notification of deaths

Only deaths assessed to be caused by the period of fasting or administration of the test meal will be reported to the Sponsor. This report will be immediate.

2.2.4.8 Sample size calculation.

The primary analysis, comparing pancreatic cancer patients with PEI to healthy controls will be performed using a two-step process (as described below). In short, N=15 per arm will be used in the initial screening to identify candidate metabolites, with the remaining N=30 per arm used to quantify the predictive accuracy of these metabolites. As such, the study was powered based on a ROC curve analysis of N=30 patients per arm. This identified minimal

detectable area under the ROC curve of 0.68, with 80% power and 5% alpha. However, this does not account for the raised false positive rate that will result from the multiple comparisons being made, since all the metabolites identified by the initial screening will be considered in the analysis. As such, it was assumed that around 50 metabolites will be identified after initial screening (i.e. approximately 5% of those tested), and so the power calculation was repeated after Bonferroni correction, giving an alpha level of 0.1% (i.e. $p<0.001$). This returned a minimal detectable area under the ROC curve of 0.75 at 80% power. A precedent from Pellis *et al* which shows a significant difference in post-prandial metabolomic profiles in a group of 36 test subjects concurs with the above estimate.[139]

2.3 Metabolomics methods

Training for sample acquisition, processing and analysis was undertaken at several stages during my PhD; prior to protocol development I completed a UoB online course in metabolomics, for processing Professor Dunn and his team provided training on how to perform LC-MS and for analysis I received training and guidance from Professor Dunn alongside the standard Metabolanalyst training exercises.

2.3.1 Sample acquisition.

The process of metabolomics begins with the collection of the biological sample from comparison groups, it is imperative that this stage is done consistently, and the biological sample is 'quenched' as soon as possible to prevent ongoing enzymatic metabolic reactions. The aim in this study was to get an accurate picture of the post-prandial food metabolome and compare it between patients with PEI and healthy controls. Based on previous work (outlined in chapter 1) the aim was to collect samples at baseline, then hourly, up to 5 hours following the test meal.

The protocol for sample acquisition was developed with the following in mind:

- Patient acceptability (as determined by early PPIE work and feedback from pilot subjects
    - 6 individual blood tests were deemed unacceptable, so a cannula was sited at the start of each study day to back bleed from.
- Patient safety. To minimise the potential risks associated with cannulation (discussed in the adverse event section of study protocol in methods section 2.1.4)

- Sample truly reflective of the food metabolome at that time point. Steps taken to minimise contamination (from flushes and dead space), and to urgently quench the sample.

The following protocol was adhered to for every recruit**:** Strict Aseptic measures maintained throughout, flush pre and post sampling to prevent clotting.

- On arrival site cannula, (20G, Pink), (ACF preferable, forearm or back of the hand also acceptable), standard hospital protocol.
    - Withdraw 10ml sample using 5ml Plastipak syringe with low pressure.
    - Attach BD Connecta (Pre prepped with 5ml 0.9% N Saline flush)
    - Flush via BD Connecta with 10ml 0.9% Saline
- Repeat sample collection at 1hr, 2 hr, 3 hr, 4 hr and 5 hr. For each repeated sample:
    - Flush via BD Connecta with 2ml 0.9% Saline (wipe end of BD connecta with alcohol wipe first and allow to air dry for >30secs)
    - Withdraw and discard 4ml blood using Plastipak syringe
    - Withdraw 5ml blood for sample with 5ml Plastipak syringe
    - Flush Via BD Connecta with 10ml N saline

2.3.2 Sample processing and storage prior to UHPLC-MS

After sample collection as described above:

- Sample immediately transferred to Lithium heparin plasma collection tubes.
    - Greiner, cat. no. 455084 (green top)
    - Mix with the anticoagulant by inverting the tube three times.
    - Straight onto wet ice to transport to centrifuge.

- Plasma fraction prepared ASAP (<10mins): centrifugation at 3,000 x *g* for 20 min at 4°C. (Eppendorf centrifuge model 5810R)
- Divide samples immediately into aliquots (0.5ml) in cryovials (Greiner, cat. no. 122261/122263) and freeze by placing in a -80°C freezer.
- Use wet ice for any transport or whilst awaiting processing and avoid any exposure of samples to ambient temperatures after collection.

2.3.3 Ultrahigh performance liquid chromatography mass spectrometry (UHPLC-MS)

2.3.3.1 Chemicals

Acetonitrile, methanol, isopropanol and water (HPLC grade) were purchased from Fisher Scientific (Loughborough, U.K.). Formic acid and acetic acid (≥98.0% purity) was purchased from VWR International (Lutterworth, U.K.), and ammonium formate and ammonium acetate (≥98.0% purity) was purchased from Sigma-Aldrich (Poole, U.K).

2.3.3.2 Sample preparation

Plasma samples for UHPLC-MS HILIC (hydrophilic interaction liquid chromatography) analysis: Samples were prepared by dilution of 50µL of plasma with 150µL of solvent (1:1 acetonitrile/methanol) followed by vortex mixing (10 seconds) and centrifugation (13,000g, 20 minutes). The supernatants (100µL) were transferred to 300µL glass vials ready for analysis. All biological samples were randomised for sample preparation. A pooled plasma QC sample was prepared by combining 50µL aliquots of all biological samples and vortex mixing for one minute. Separate QC samples were extracted as described above (94 QC samples in total). A process blank sample was prepared for each assay by performing sample extraction as described above with no biological sample present.

Plasma samples for UHPLC-MS lipidomics analysis: Samples were prepared by dilution of 50µL of plasma with 150µL of solvent (isopropanol) followed by vortex mixing (10 seconds) and centrifugation (13,000g, 20 minutes). The supernatants (100µL) were transferred to 300µL glass vials ready for analysis. A pooled plasma QC sample was prepared by combining 50µL aliquots of all biological samples and vortex mixing for one minute. Separate QC samples were extracted as described above (94 QC samples in total). A process blank sample was prepared for each assay by performing sample extraction as described above with no biological sample present.

2.3.3.3 UHPLC-MS Assays

Each biological sample, QC sample and blank sample were analysed applying two complementary UHPLC-MS assays; a HILIC assay to study water-soluble metabolites and a lipidomics assay to study lipids.

HILIC assay: The samples (maintained at 4°C) were analysed applying a Vanquish binary pump H system coupled with a heated electrospray Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, MA, USA). Sample extracts were analysed using an Accucore-150-Amide-HILIC column (100 x 2.1 mm, 2.6 µm, Thermo Fisher Scientific, MA, USA). For positive ion mode mobile phase A was 95% acetonitrile/water (10 mM ammonium formate, 0.1% formic acid) and mobile phase B was 50% acetonitrile/water (10 mM ammonium formate, 0.1% formic acid). For negative ion mode mobile phase A was 95% acetonitrile/water (10 mM ammonium acetate, 0.1% acetic acid) and mobile phase B was 50% acetonitrile/water (10 mM ammonium acetate, 0.1% acetic acid). The gradient elution applied was t=0.0, 1% B; t=2.1, 1% B; t=4.6, 15% B; t=7.6, 50% B; t=10.1, 95% B; t=11.0, 95%B; t=11.5, 1%B, t=15.0, 1% B. All changes were linear (curve = 5) and the flow rate was 0.50 $mL.min^{-1}$. Column temperature was 35°C and injection volume was 2 µL. Data were acquired in positive and

negative ionisation modes separately in the *m/z* range of 76 –1050 with a mass resolution of 120,000 (FWHM at *m/z* 200). Ion source parameters applied were: sheath gas = 40 arbitrary units, aux gas = 8 arbitrary units, sweep gas = 1 arbitrary units, spray voltage = 3.2kV (positive ion mode) and 2.7kV (negative ion mode), vaporizer temperature = 320°C and ion transfer tube temperature = 250 °C. All samples were collected as MS1 data in the profile mode applying scan time = 100ms, microscans = 1, RF lens = 50% and normalised AGC target=100%. For peak annotation purposes, MS/MS data were collected in the "Data dependent mode" setting for five QC samples analysed as injections 7-11 in each batch over different *m/z* ranges (76−210 *m/z*; 200−310 *m/z*; 300−410 *m/z*; 400-510 *m/z* and 510-1010 *m/z*) using stepped normalized collision energies of 20/50/130% (negative ion mode) and 20/40/100% (positive ion mode). MS/MS data were applied with the number of dependent scans = 3, a mass resolution of 15,000 (FWHM at *m/z* 200) and an isolation width = 3 *m/z*. Orbitrap Exploris 240 Tune application software controlled the instrument.

Subjects were randomised for data collection to ensure appropriate distribution of age, gender, BMI and disease/control status ranges across the analysis order. The different timepoint samples for each subject were then randomised so that all samples for a subject were analysed sequentially but in a random order. The analysis was separated in to two batches of 201 biological samples, 47 QC samples and 2 process blank samples with instrument cleaning (UHPLC column and ion source) performed between each batch. Each batch was started with 12 QC samples with a process blank sample analysed between QC5 and QC6. A QC sample was analysed after all samples for one subject were analysed (typically every 7th injection after six biological samples). Two QC samples and then a process blank were analysed at the end of each batch.

Lipidomics assay: The samples (maintained at 4°C) were analysed applying a Vanquish binary pump H system coupled with a heated electrospray Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, MA, USA). Sample extracts were analysed using a Hypersil GOLD C_{18} column (100 x 2.1mm, 1.9 µm; Thermo Fisher Scientific, MA, USA). For positive and negative ion modes mobile phase A was 60% acetonitrile/40% water (10 mM ammonium formate, 0.1% formic acid) and mobile phase B was 85.5% propan-2-ol /9.5% acetonitrile/5% water (10 mM ammonium formate, 0.1% formic acid). The gradient elution applied was: t=0.0, 20% B; t=1.5, 20% B; t=3.0, 25% B; t=9.2, 100% B; t=10.5, 100% B; t=12.5, 20% B; t=15.0, 20% B. All changes were linear (curve = 5) and the flow rate was 0.40 $mL.min^{-1}$. Column temperature was 55°C and injection volume was 2 µL. Data were acquired in positive and negative ionisation modes separately in the *m/z* range of 150 –2000 with a mass resolution of 120,000 (FWHM at *m/z* 200). Ion source parameters applied were sheath gas = 40 arbitrary units, aux gas = 8 arbitrary units, sweep gas = 1 arbitrary units, spray voltage = 3.2kV (positive ion mode) and 2.7kV (negative ion mode), vaporizer temperature = 320°C and ion transfer tube temperature = 250 °C. All samples were collected as MS1 data in the profile mode applying: scan time = 100ms, microscans = 1, RF lens = 60% and normalised AGC target=100%. For peak annotation purposes, MS/MS data were collected in the "Data dependent mode" setting on five QC samples analysed as injections 7-11 in each batch over different *m/z* ranges (150-500 *m/z*; 500-710 *m/z*; 700-860 *m/z*; 850-1010 *m/z* and 1000-2000 *m/z*) using stepped normalized collision energies of 20/50/130% (negative ion mode) and 20/40/100% (positive ion mode). MS/MS data were applied with the number of dependent scans = 3, a mass resolution of 15,000 (FWHM at *m/z* 200) and an isolation width = 3 *m/z*. Orbitrap Exploris 240 Tune application software controlled the instrument.

Subjects were randomised for data collection to ensure appropriate distribution of age, gender, BMI and disease/control status ranges across the analysis order. The different timepoint samples for each subject were then randomised so that all samples for a subject were analysed sequentially but in a random order. The analysis was separated in to two batches of 201 biological samples, 47 QC samples and 2 process blank samples with instrument cleaning (UHPLC column and ion source) performed between each batch. Each batch was started with 12 QC samples with a process blank sample analysed between QC5 and QC6. A QC sample was analysed after all samples for one subject were analysed (every 7^{th} injection after six biological samples). Two QC samples and then a process blank were analysed after each batch.

2.3.4 Data processing

2.3.4.1 Raw data processing

Data files (in the .RAW file format) were converted to mzML file formats applying msConvert in Proteowizard. Data (mzML format) for each sample were processed applying the R package XCMS to construct a single data matrix for all samples (metabolite features as rows and samples as columns) for quality assessment and statistical analysis. XCMS applied three steps. Step1: Peak detection ("findChromPeaks") using the "centWave" algorithm was employed with parameter settings of *m/z* deviation = 25 ppm, peakwidth = 5 ~ 20, snthresh = 10, prefilter = 3 ~ 100, mzCenterFun = "wMean") and mzdiff = 0.001. Step 2: Alignment ("adjustRtime") was applied to perform retention time correction (alignment) between chromatograms of different samples using the Obiwarp method with parameters of binSize = 1, gapInit = 0.4) and gapExtend = 2.4. Step 3: Peak grouping ("groupChromPeaks") was performed to group the chromatographic peaks within and between samples. The sample/replicate category group, such as "sample", "QC" and "blank" was used as peak group information.

2.3.4.2 Metabolite annotation

MS1, MS/MS and chromatographic retention times were applied for metabolite annotation/identification. All annotations match to level 2 (using matches to retention time and/or MS/MS libraries) or level 3 (matches of MS1 data to metabolite databases) of the MSI reporting standards. Putative metabolite annotation using MS1 data and based on the peak list and data matrix returned by XCMS was performed by the Python package BEAMSpy (https://github.com/computational-metabolomics/beamspy) applying three steps. Step 1: Feature grouping performed correlation analysis between pairs of mass spectral features using retention time difference =1 second, correlation method = spearman rank, correlation coefficient threshold = 0.5 and correlation analysis p-value = 0.05. Step 2: Peak annotation was performed for metabolite features within a correlation group being annotated as different adducts, isotopes and neutral losses. The parameters are the libraries of adducts, isotopes and neutral losses and mass error tolerance = 5 ppm. Step 3: Compound annotation queried two compound databases (HMDB (https://hmdb.ca) and LIPIDMAPS (https://www.lipidmaps.org)) within mass error tolerance = 5 ppm. Putative metabolite annotations applied retention times of metabolites in biological samples and matching to metabolites in an in-house retention time library; matching was confirmed with a retention time difference between biological sample and library of < +/- 10 seconds. Putative metabolite annotations also applied MS/MS data of metabolites in biological samples and matching to MS/MS data available in mass spectral libraries. MS/MS data collected for 5 QC samples was processed in Compound Discoverer v3.3 using 'Identification only' as the sample type and three MS/MS libraries (in-house, mzCloud (https://www.mzcloud.org/) and LipidBlast). A match was reported if the match score was >70%.

2.3.4.3 Data quality assessment and filtering

Data for each assay was visualised using Principal Components Analysis in MetaboAnalyst. KNN (feature-wise) missing value imputation (using remove features with >50% missing values), no data filtering, normalisation by sum, log transformation (base 10) and Pareto scaling were applied. Scores plots for PC1/PC2 were visually assessed to identify QC data quality (through tight clustering of QC samples compared to biological samples) and QC sample or biological sample outliers (sample present outside the 95% confidence region). Outlier samples were removed from the dataset. Outlier samples were also identified by calculating the sum of all peaks areas for each sample and comparing to all other samples; any sample with a total peak area <50% or greater than 200% of the mean peak area across all samples were removed from the dataset. Subsequently, filtering of data based on QC and process blank sample data was applied. Metabolite features were removed from the dataset if the relative standard deviation (RSD) for QC sample 11 to QC sample 47 in each batch was greater than 30%. Metabolite features were also removed if the mean process blank peak area was greater than 10% of the mean QC sample peak area. This constructed a quality-filtered data matrix for each assay.

2.3.5 Statistical analysis

Data for each assay were normalised to total peak area (normalisation by sum) and log transformed (base 10) for each sample. Statistical analysis applied repeated measures two-way ANOVA in MetaboAnalyst. Pathway enrichment analysis for water-soluble (non-lipid) metabolites was performed in MetaboAnalyst and lipids were classified according to LipidMaps classification system (https://www.lipidmaps.org/databases/lmsd/browse). Fold changes were calculated by dividing the mean of pre-class A samples by the mean of class B samples.

Results chapters

There are three discrete bodies of work covered here; refining the ^{13}C MTGT to use as "gold standard" for establishing PEI status, results of PEI assessment (using established tests) in recruits for the DETECTION trial, and finally analysing the acquired plasma samples using mass spectrometry to evaluate novel markers of PEI. Therefore, there will be 4 results chapters:

Chapter 3. Results: Refining the 13C MTGT for use alongside metabolomic testing.

Chapter 4: Results: PEI status and demographics of recruits for the DETECTION trial.

Chapter 5: Results: Biological interpretation of metabolomic analysis.

Chapter 6: Results: Developing a biomarker panel for diagnosing PEI.

Chapter 3 Results: Refining the ^{13}C MTGT for use alongside metabolomic testing.

3.1 Summary of chapter 3

PEI is a much under-investigated disease, with significant challenges regarding diagnosis, grading of disease state, and evaluating treatment. One of the biggest challenges of this PhD was to accurately define the disease state. Work done to set up the ^{13}C MTGT in the recruiting hospital and then refine it for use alongside metabolomic testing was extensive and entirely novel. This section includes:

3.2: ^{13}C MTGT systematic review results

3.3: Test meal development and validation

3.4: PPIE work

For breath test refinement work, healthy controls were recruited with written consent as per REC approved protocol (**Appendix 1**), to be eligible they had to be over 18, be able to give informed, written consent and have no history of or symptoms suggestive of pancreatic pathology. Those with prior gastro-intestinal or hepato-pancreato-biliary surgery (except for appendicectomy or cholecystectomy) were not eligible.

3.2 Systematic review and meta-analysis of the accuracy and methodology of the ^{13}C MTGT – Results

Before setting the ^{13}C MTGT up locally there were several protocol elements that were poorly described and not standardised. I attended a European centre for initial training, then performed a systematic review and meta-analysis of methodology and accuracy to develop a detailed protocol that contained the most widely used and accepted steps. The methods of this review are covered already in section **2.1.1**., the key results are below in section **3.2.1** and the manuscript has been published in Pancreatology *Powell-Brett S, Hall L, Edwards M, Roberts K. A systematic review and meta-analysis of the accuracy and methodology of the 13C mixed triglyceride breath test for the evaluation of pancreatic function. Pancreatology. 2023 Apr;23(3):283-293.. PMID: 36805050 doi: 10.1016/j.pan.2023.02.004.* (See appendix 9)

3.2.1 Studies included.

Study characteristics and demographics.

39 studies were included in total, 38 from the initial database searches and a further 1 from reference screening of review articles.[90, 122, 123, 143-178] Included studies were published between 1989 and 2022 and encompassed 2774 subjects. All were undergoing the ^{13}C MTGT for assessment of pancreatic function. Some 32 studies were conducted in Europe and 7 further in Japan. 6 of these studies, encompassing a total of 368 subjects compared the ^{13}C MTGT to another, validated measure of PEI and were therefore included in quantitative analysis. Study characteristics are presented in **Table 9**.

Table 9 ^{13}C MTGT SR + MA study details and demographics

Study	Country	Study type	Population (disease status, *n*)	Breath test accuracy assessed	Total *n*
Vantrappen (1989)	Belgium	Prospective, comparative cohort study	Chronic pancreatitis, 21 Pancreatic cancer, 8 Healthy control, 25	Yes, Duodenal intubation	54
Kalivianakis (1997)	Netherlands	Prospective, comparative cohort study	Healthy volunteers, 11	No	11
Swart (1997)	Netherlands	Prospective, comparative cohort study	Cystic fibrosis, 8 Healthy Controls, 17	No	25
Loser (1998)	Germany	Prospective, comparative cohort study	PEI, 26 Healthy controls, 27	Yes, Duodenal intubation	53
Perri (1998)	Italy	Prospective, cohort study	Celiac disease, 17	No	17
Boedeker (1999)	Belgium	Prospective, comparative cohort study	Chronic pancreatitis, 10 Healthy controls, 10	No	20
Domínguez-Muñoz (2004)	Spain	Prospective, comparative, crossover study	Chronic pancreatitis, 24	No	24
Schneider (2006)	Germany	Prospective, comparative cohort study	Chronic pancreatitis, 18 Healthy controls, 5	No	23
Domínguez-Muñoz (2007)	Spain	Prospective, comparative cohort study	Chronic pancreatitis, 29	Yes, CFA	29
Nakamura (2009)	Japan	Prospective, comparative cohort study	Pancreatic resection, 95 Chronic pancreatitis, 10 Healthy controls, 7	Yes, Faecal Elastase	112
Nakamura (2009) *2	Japan	Prospective cohort study	Pancreatic resection, 61	No	61
Keller (2011)	Germany	Prospective, comparative cohort study	Chronic pancreatitis, 9 Healthy controls, 10	Yes, Duodenal intubation	19
Lisowska (2011)	Poland	Prospective cohort study	Cystic fibrosis, 24	No	24
Nakamura (2011)	Japan	Prospective, comparative cohort study	Post PPPD, 52	No	52
Yuasa (2012)	Japan	Prospective, comparative cohort study	Post PD, 80 Post DP, 30	No	110
Bozek (2012)	Poland	Prospective, comparative cohort study	Healthy volunteers, 18	No	18
Domínguez-Muñoz (2012)	Spain	Prospective cohort study	Chronic pancreatitis, 128	No	128
Lindkvist (2012)	Spain	Retrospective cohort study	Chronic pancreatitis, 114	No	114
Jonderko (2013)	Poland	Prospective, comparative cohort study	Healthy volunteers, 22	No	22

Rabih (2014)	Spain	Prospective, observational cohort study	Alcoholic liver disease, 129 Non-alcoholic liver disease, 25	No	154
Nakagawa (2014)	Japan	Prospective, observational cohort study	Post PD, 104	No	104
Luaces-Regueira (2014)	Spain	Prospective cross-sectional cohort study	Chronic pancreatitis, 241	No	241
Muniz (2014)	Brazil	Prospective cohort study	Post PD, 15 Healthy controls, 15	No	30
Keller (2014)	Germany	Prospective, comparative cohort study	Diabetes Mellitus, 14 Healthy controls, 10	Yes, Duodenal intubation	14
Keller (2014) *2	Germany	Retrospective cohort study	Symptoms suggestive of PEI, 200	No	181
Drzymała-Czyż	Poland	Prospective comparative cohort study	Cystic Fibrosis, 23	No	23
Domínguez-Muñoz (2015)	Spain	Prospective observational study	Chronic pancreatitis, 115	No	115
Hirono (2015)	Japan	Prospective, observational cohort study	PD with PJ, 99 PD with PG, 90	No	189
Okano (2016)	Japan	Prospective, observational cohort study	Post PD, 227	No	227
Domínguez-Muñoz (2016)	Spain	Prospective, crossover, comparative study	Chronic pancreatitis, 20 Healthy controls, 10	Yes, CFA	30
González-sánchez (2017)	Spain	Prospective, comparative cohort study	Chronic pancreatitis, 54	Yes, CFA	54
Alfieri (2018)	Italy	Prospective, comparative cohort study	Post PD with PJ, 16 Post PD with PDO, 16	No	32
Søfteland (2019)	Denmark	Prospective, observational cohort study	Diabetes Mellitus, 12	No	12
Domínguez-Muñoz (2021)	Spain	Prospective randomised controlled	Unresectable pancreatic cancer	No	
Dozio (2021)	Italy	Prospective comparative cohort study	T1 Diabetes, 31	No	56
Malczyk (2021)	Poland	Prospective case control study	Anorexia nervosa, 31	No	69
Uribarri-Gonzalez (2021)	Spain	Prospective comparative cohort study	Post Bariatric Surgery, 95	No	105
Hedstrom (2022)	Sweden	Prospective cohort study	Sjogrens syndrome, 57	No	139
Afolabi (2022)	U.K	Prospective cohort study	Pancreatic cancer, 25 Chronic pancreatitis 10	No	60

*CFA = Coefficient of fat analysis, PEI=Pancreatic Exocrine insufficiency, PPPD = Pylorus preserving pancreaticoduodenectomy, PD = Pancreaticoduodenectomy, PJ = Pancreaticojejunostomy, PG = Pancreaticogastrostomy, PDO = Pancreatic duct occlusion. *2 = 2nd paper, same 1st author same year.*

Review aims

13/39 directly investigated the ^{13}C MTGT and 24/39 used the ^{13}C MTGT to evaluate pancreatic function for the purpose of other study outcomes. Of those directly investigating the breath test 7/13 were investigating different study protocols and 6/13 were comparing the breath test to other diagnostic tests of PEI to quantify sensitivity and specificity.

Quality assessment and risk of bias.

6 studies directly assessed the accuracy of the breath test and were therefore eligible for risk of bias scoring. The QUADAS-2 tool for evaluating the risk of bias and applicability of primary diagnostic accuracy studies was used. See ***Table 10***

.

Table 10 QUADAS-2 tool for risk of bias and applicability of studies reporting on diagnostic accuracy.

Study	RISK OF BIAS			APPLICABILITY CONCERNS		
	Index test	Reference standard	Flow and Timing	Patient selection	Index test	Reference standard
Vantrappen 1989						
Loser 1998						
Nakamura 2009						
Keller 2011						
Dominguez-munoz 2016						
Gonzalez-Sanchez 2017						

3.2.2 Results – Breath test methodology

14/39 of the included studies directly investigated the breath test, 6 of these reported on diagnostic accuracy in comparison to another test of PEI. 4 of the 6 reporting on diagnostic accuracy (Vantrappen, Nakamura, Domínguez-Muñoz and Keller) described a robust protocol that has been reproduced by other groups. All included studies are reported on here, those 6

that have reported diagnostic accuracy will be described separately (referred to as 'Group A') where relevant. The protocol described by Domínguez-Muñoz reports excellent sensitivity and specificity, is the most widely reproduced and as highlighted by the QUADAS tool above appears to be at the least risk of bias and will therefore be individually referred to if relevant.

- *Pre-test control measures - Dietary intake and fasting period:*

Exhaled 13 Carbon (^{13}C) is expressed as enrichment above baseline level, dietary control of ^{13}C before the test is done to minimise baseline variation.[179] Only 7/39 (including 1 'Group A' study)[180] explicitly describe the control of pre-test dietary intake to limit the intake of foodstuffs naturally rich in ^{13}C, all for 48 hours preceding test start time. These measures included avoidance of: 'corn products, cane sugar and pineapple'[152], 'maize, cane sugar, pineapple or Kiwi'[145, 151], 'corn and fibre-rich products'[166] 'Corn, pineapple, broccoli, sugarcane.'[180] 35/39 studies enforced an overnight fast from between 10-12 hours, 3 did not specify and one described an 8-hour fast.

- *Pre-test control measures - Smoking:*

4/39 studies specified smoking cessation for the 12 hours preceding. The Domínguez-Muñoz protocol specifies no smoking from the night before until after the test.

- *Pre-test control measures - Metoclopramide:*

Some 8/39 studies (those following the Domínguez-Muñoz protocol) advocate 10mg Metoclopramide 20-30 mins pre ingestion of test meal, theoretically to negate the effect of delayed gastric emptying. 2/7 of the Group A studies support the use of metoclopramide, both using the Domínguez-Muñoz protocol. Keller et al specifically evaluated the effect of gastric emptying and found no significant association between delayed gastric emptying and the

results of the ^{13}C MTGT.[181] Considering that gastric emptying does not affect 6-hour ^{13}C recovery and the layer of complexity metoclopramide adds (it has a variable onset/duration of action with both clearance and half-life affected by renal function and requires a prescription for administration), the addition of metoclopramide is unnecessary for a ^{13}C MTGT with a 6 hour timeframe, but should be re-visited if the test were to be shortened to 4 hours.

- *Pre-test control measures - Cessation of PERT pre-test:*

Some 2/39 studies evaluated PERT usage and 7/39 did not include study subject on PERT; therefore, the duration of PERT cessation prior to the test was relevant in 30 studies. 19/30 studies did not specify when or whether PERT should be stopped pre-test, of those that did specify, PERT was stopped at 2 days (2/30), 3 days (4/30), 4 days (4/30) or 5 days (1/30) before the breath test, there was no consensus between Group A studies. In the absence of evidence for prolonged PERT cessation and considering that untreated PEI for longer than necessary is unpleasant and potentially harmful, especially for those with severe disease, 48 hours should be long enough to evaluate untreated pancreatic function, even allowing for slow transit time.

- *Dose of ^{13}C MTG Administered*

21/39 studies used 250mg, 10 used 200mg, 2 used 300mg and 3 used 150mg. 1 study dosed by body weight using 4mg ^{13}C MTG per kilogram, 2 studies dosed by test meal fat content, using 16mg of ^{13}C MTG per gram of butter. 2 studies [155, 180] directly compared different doses of ^{13}C MTG: Loser et al compared 200mg to 250mg on the same patients, under identical test conditions after a 2 week wash out period, the results showed near identical courses and cumulative $^{13}CO_2$ excretion with no significant differences, concluding that 200mg is as effective as 250mg. Dominguez-Munoz et al compared 125mg to 250mg after a 1-week

washout period and found that with 125mg the area under the ROC curve of the breath test with 125mg of ^{13}C MTG was lower than obtained with 250mg.

- *Test meal*

There is much heterogeneity in the constituents of the test meal. The original Vantrappen test meal comprised of 100g bread with 0.25g/kg body mass of butter. Most studies, 22/39, used some variation on bread and butter with fluid to create a solid-liquid test meal with a specified amount of fat, however this appears to be the least standardised aspect and indeed shows huge variance. All group A studies use a solid-liquid test meal but with varying amounts of fat in the form of butter, chocolate cream and/or cheese, see **Table 11** for details.

Table 11 Summary of test meal constituents.

Group A study	Test meal components	Other notes on composition
Vantrappen 1989	100g toast, 0.25g butter per Kg	
Loser 1998	100g toast, 15g butter, 10g chocolate cream (Nutella)	1st spread on butter then homogenised with nutella (repeat + water)
Nakamura 2009	90g toast, 15g margerine, 200ml Milk	
Keller 2011	100g white bread, 20g butter, 30g chocolate cream (Nutella).	26g fat 420kcal. (Modified from 16g) 13CMTG mixed into nutella + 200ml Water
Keller 2014	100g white bread, 20g butter, 30g chocolate cream (nutella).	13CMTG mixed into nutella + 200ml Water
Dominguez-Munoz 2016	16g vs 32g vs 48g of fat (butter or butter + cheese) with 40g bread	Final decision 16g butter 200ml water
Gonzalez-sanchez 2017	16g fat. 2 slices white toast, 20g butter	Substrate spread on to the butter, 200ml water.

Keller et al modified the test meal described by Loser to increase the fat content from 16g to 26g and found excellent sensitivity and specificity that was repeated in their subsequent 2014 paper using the same test meal. The Domínguez-Muñoz group compared 16g, 32g and 48g of fat in a prospective, randomised, 3-arm crossover comparative study and found 16g of fat resulted in the greatest area under the ROC curve though with negligible difference between the 3 different fat contents. Bozek et al (2011) examined four test meals: 300mg ^{13}C MTG a)

Contained in 2 wafers; b) with a 50g wheat roll; c) wheat roll + 10g butter and d) wheat roll + 30g butter. They found that the addition of unlabelled fat is essential for ^{13}C recovery and found that the peak ^{13}C recovery occurred earlier and the cumulative ^{13}C recovery was greater with the lower fat load of 10g.

- *Method, frequency, and total time of breath collection*

There are two described methods of sampling: exhaling through a straw into an inverted exetainer or exhaling into a specifically designed valved collection bag. The latter is considerably more expensive and bulkier to transport but does eliminate the potential for procedural errors when capping the exetainer. Some 14/39 studies use the exetainer method and 9/39 use a collection bag, the remaining do not specify. Of the group A studies there is equipoise between the 2 methods. The frequency of sampling ranged from every 15 minutes (9/39), every 30 minutes (21/39) and every hour (7/39), (2 did not specify). Group A studies use 30-minute sampling (3/6), 15- minute sampling (2/6) and 60-minute sampling (1/6). Exhaled air has variable CO_2 content depending on whether it is at the beginning or end of exhalation, specific training for all executing or supervising the ^{13}C MTGT is needed to ensure this is accurate and consistent.

Some 21/39 studies used a 6-hour testing timeframe, the others ranged from 4 to 9 hours. 4 studies specifically investigated the duration of breath collection. Keller et al (2011) found a significant drop in sensitivity if under 6 hours and no further improvement in sensitivity when extending to 8 hours. In their 2014 study they found that reasonable sensitivity and specificity (88% and 94% respectively) could be maintained with a 4-hour test, shortening this was affected by gastric emptying and found to yield unreliable results. Domínguez-Muñoz et al found a decreasing correlation coefficient between CFA and breath test from the 6-hour test to

1-hour test, and a decreasing area under the ROC curve of breath test for the diagnosis of PEI from 6 hours down to 1 hour but also suggested that reasonable sensitivity and specificity (90.5% and 80.9% respectively) could be achieved with breath collection at 4 hours.

- *In-test control measures*

The aim of in-test control measures is to prevent external influences on substrate digestion, absorption, metabolism, and exhalation, ensuring that the step under investigation is pancreatic lipolysis only. Anything that could influence pancreatic enzyme secretion/action or increase the basal metabolic rate and affect CO_2 metabolism must be controlled. There is good consensus (25/39) that patients should remain seated and fasted with 2/39 allowing black tea or coffee at 6-hours if using an extended timeframe and 1/39 allowing black tea or coffee throughout, the others did not specify. The study by Kalivianakis et al directly investigated the effect of exercise on ^{13}C recovery and found that even moderate exercise had a significant impact.[152] 5/6 group A studies kept subjects fasted for 6 hours, 4 specify remaining seated, the others did not clarify. There is good evidence from other ^{13}C breath tests that smoking affects ^{13}C recovery,[182-184] but no-smoking is only specified in 2 studies, (both Group A).

- *Analysis platform*

The measurement of $^{13}CO2/\ ^{12}CO2$ ratio in breath samples can be done by isotope ratio mass spectrometry (IRMS) or isotope selective nondispersive infrared spectrometry (NDIRS). IRMS for the measurement of $^{13}CO2/\ ^{12}CO2$ ratio is very sensitive but also complex and expensive, NDIRS is simpler and cheaper. 16/39 used NDIRS, 22/39 IRMS and 1 study compared the two. Boedeker et al directly compared NDIRS to IRMS and found that with NDIRS there was moderate to good correlation but with cPDR discrepancies of up to 18.9% concluding that using NDIRS could compromise validity.[144]

- *CO_2 production calculation*

The results of the ^{13}C MTGT are expressed as the cumulative percent of ^{13}C recovered in breath over a certain amount of time: cPDR (cumulative percent dose recovery). PDR in each sample is calculated from rate of CO_2 production (VCO_2) and breath. Only 5/39 studies mentioned how CO_2 production was calculated. 3 assumed CO_2 production to be 5 mmol.min-1 .m-2 (Shreeve calculation with BSA estimated by the Haycock formula).[185, 186] 1 study estimated production at 9mmol/hr/kg and 1 study measured CO_2 output by indirect calorimetry. Vantrappen et al compared both the 'simplified test' of CO_2 estimation and the 'quantitative test' with measurement of CO_2 production and found similar excretion patterns between the two groups.

3.2.3 Results – Breath test accuracy

6/39 evaluated the sensitivity and specificity of the ^{13}C MTGT in comparison to another diagnostic test, see **Table 12**.

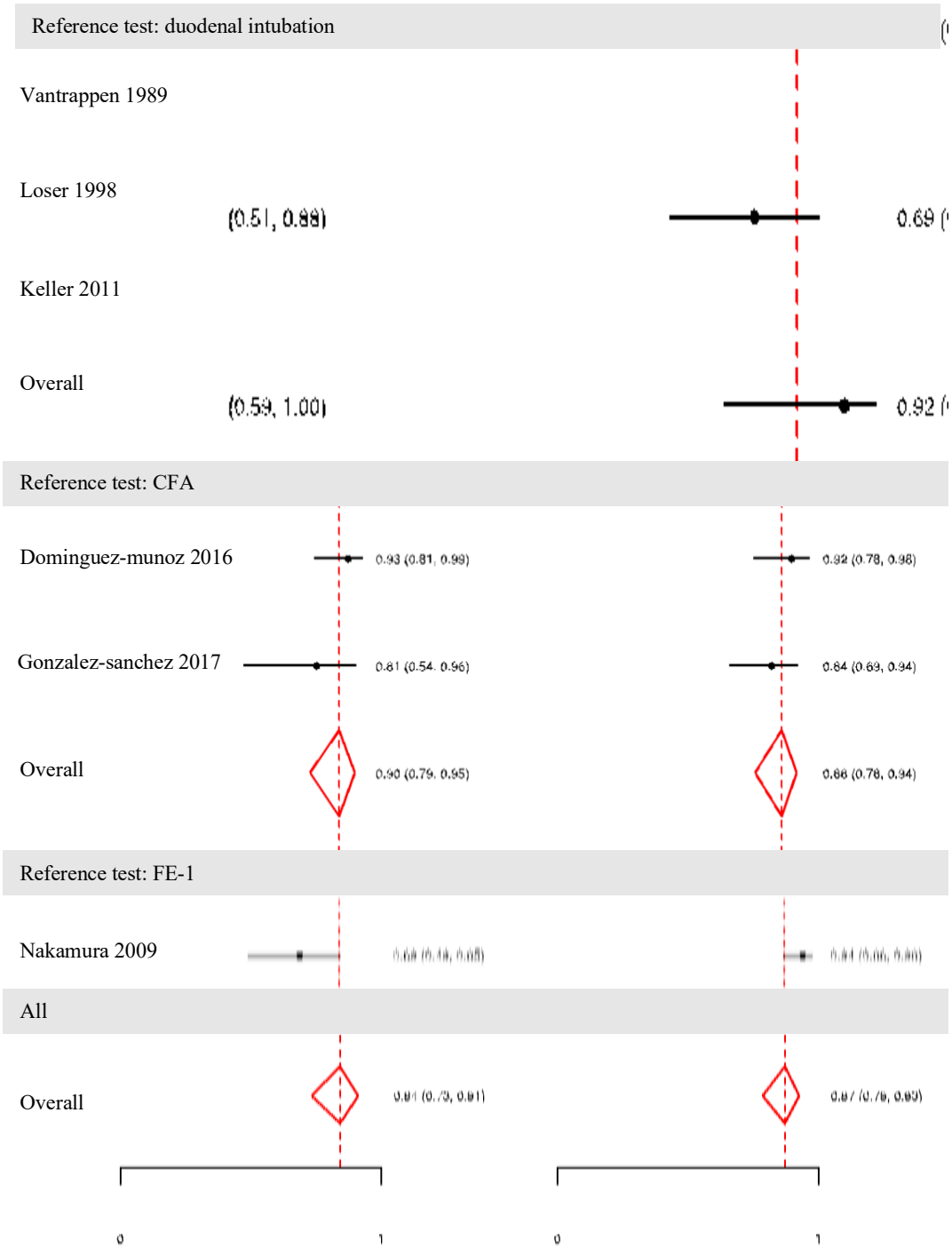
Table 12 Studies evaluating the sensitivity and specificity of the 13C MTG breath test.

Author	Population	Reference test	Ref standard	Index test	Index standard (cPDR)	Sensitivity	Specificity
Vantrappen 1989	29 patients 25 controls	SC-T	<90kU lipase/Hr	^{13}C MTGT	<22% at 6 hrs	89%	81%
Loser 1998	51 patients	SC-T	<90KU lipase/Hr	^{13}C MTGT	AUC at 5 hrs	73%	69%
Keller 2011	10 controls 9 patients	S-T	<400U lipase/min	^{13}C MTGT	<28% at 6 hrs	100%	92%
Dominguez-Munoz 2016	78 patients	CFA	<92.5%	^{13}C MTGT	<29% at 6 hrs	93%	92%
Gonzalez-sanchez 2017	54 patients	CFA	<93%	^{13}C MTGT	<29% at 6 hrs	81%	84%
Nakamura 2009	105 patients 7 controls	FE-1	<200	^{13}C MTGT	<5% at 7 hrs	69%	94%

Abbreviations: S-T=Secretin test, SC-T=Secretin-caerulin test, CCK=Cholecystokinin test, CFA=Coefficient of fat analysis, FE-1=faecal elastase.

3/39 used the previous gold standard of direct collection of enzymes using duodenal intubation (the cholecystokinin test, the secretin test or secretin-caerulein test (CCK-T/S-T/SC-T)) and 2/39 used the current gold standard reference test (CFA), therefore limited inference can be drawn from meta-analysis. However, for interest we have looked at the pooled sensitivity and specificity of all diagnostic studies and those comparing the ^{13}C MTGT to the old and new gold standard separately. The pooled sensitivity and specificity of ^{13}C MTGT for PEI for all studies assessing diagnostic accuracy were 0.84 (95% CI 0.73-0.91) and 0.87 (95%CI 0.79-0.93) respectively. The pooled sensitivity and specificity of ^{13}C MTGT for PEI for studies comparing diagnostic accuracy to the current gold standard (CFA) were 0.90 (95% CI 0.79-0.95) and 0.88 (95%CI 0.78-0.94) respectively. The pooled sensitivity and specificity of ^{13}C MTGT for PEI for studies comparing diagnostic accuracy to a duodenal intubation test (S-T or SC-T) were 0.87 (95%Ci 0.65-0.96) and 0.80 (95%CI 0.64-0.90) respectively. See **Figure 19.**

Figure 19 Pooled sensitivity and specificity of studies comparing 13C MTGT to another recognised diagnostic test.



3.2.4 Discussion

This is a systematic review with narrative synthesis which describes the methodology and conduct among studies of the ^{13}C MTGT breath test with an additional meta-analysis of data comparing the accuracy of this test to other tests of PEI. The aims of this review were to clarify the finer points of methodology where consensus or good evidence exists and identify areas where uncertainty remains (summarised in **Table 13**).

Table 13 Summary table of quantitative and qualitative results.

Summary of Qualitative synthesis	
Areas with good supporting evidence or majority consensus	**Areas of controversy in need of further investigation**
Pre-test control measures	
Limitation of dietary ^{13}C for 48 hours Fasting for 12 hours (except for water) No smoking for 12 hours	PERT cessation duration - authors recommend 48hrs
Test meal and substrate dose	
250mg ^{13}C MTG accurately incorporated into test meal – weigh ^{13}C MTG on a 5-figure balance minimal utensils and transfers to avoid substrate losses. 10g Fat minimum for test meal	Ideal test meal - authors currently recommend 2 slices of toast & butter (to total 16g of fat) + 200ml of water. Metoclopramide - authors do not currently recommend routine use but may be more important if a shortened time frame were used.
In-test control measures	
Remain seated for the duration Remain fasted for duration except for water for comfort	smoking – authors recommend no smoking
Breath sampling and analysis	
At baseline + every 30 minutes for a total of 6 hours Exetainers or breath bags (individual set up) Analyse with IRMS not NDIRS Estimate VCO_2 according to BSA (**Appendix** 6.)	Potential to shorten to 4 hours if further investigated

Summary of Quantitative synthesis		
Reference test	**Pooled Sensitivity (95%CI)**	**Pooled Specificity**
Duodenal intubation test (S-T, SC-T) 3 papers, 124 subjects	87% (0.65-0.96)	80% (0.64-0.90)
CFA 2 papers, 132 subjects	90% (0.79-0.95)	88% (0.78-0.94)
FE-1 1 paper, 112 subjects	69% (0.49-0.85)	94% (0.86-0.98)
All combined 6 papers, 368 subjects	84% (0.73-0.91)	0.87% (0.79-0.93)

13C = 13 Carbon, PERT = Pancreatic exocrine replacement therapy, ^{13}C MTG = 13 Carbon labelled mixed triglyceride, IRMS = isotope ratio mass spectrometry, NDIRS = non-dispersive infrared spectrometry, VCO_2 = CO2 production, S-T = Secretin test, SC-T = secretin caereulin test, CFA = coefficient of fat analysis, FE-1 = Faecal elastase

The ideal test meal is still an area of controversy with much heterogeneity in the literature, the most common is toast with butter and a drink. The meal must be able to incorporate ^{13}C labelled substrate without encountering losses in assembly or ingestion, it must be standardisable, contain at least 10g of fat and be acceptable to patients. For delivery of the test outside a specialised unit, e.g. in a ward, home or clinic-based setting, a move to a set nutritional meal/drink would be preferable over a meal that requires a kitchen/equipment to create. Several studies specifically investigate the ideal test meal but with conflicting results on the quantity of fat and medium for ingestion. With further investigation, a streamlined, standardised test meal could make the ^{13}C MTGT much easier to deliver in any setting.

Overall timeframe is a significant downside of the ^{13}C MTGT and there have been several attempts to shorten it. The work by both Domínguez-Muñoz and Keller suggest that reasonable sensitivity and specificity could be maintained with a four-hour time frame, but no less. However, most of the literature reports to a 6-hour testing time frame and at present there is not enough evidence to shorten this. There is the potential to considerably improve the acceptability of the ^{13}C MTG If more work was done to validate a diagnostic cut off at a 4-hour timeframe. Any future work addressing this should also consider the role of metoclopramide, although the work by Keller et al showed that delayed gastric emptying had no effect on ^{13}C recovery, this was in the setting of a 6-hour timeframe, any new, shorter protocol should undergo similar scrutiny regarding gastric emptying.

The key finding of the meta-analysis was that the ^{13}C MTGT test has a high accuracy when compared to ‘Gold standard’ tests of PEI. This is an important conclusion given how complex, time consuming, invasive, and unpleasant the ‘Gold standard’ tests (namely duodenal intubation or coefficient of fat absorption) are.

In summary, although it has its challenges (cost/availability of the substrate, overall testing timeframe and strict control measures) the ^{13}C MTGT is an accurate, non-invasive test suitable for use as a reference test for my work. This review enabled me to create a set protocol to use in clinical practice but has also highlighted areas of no consensus such as the ideal test meal. To use the ^{13}C MTGT alongside metabolomic sampling I needed to create a test meal that adheres to the criteria outlined above but could be used in any environment without complex storage or re-constitution, as such, the next step in trial development was to design and validate an appropriate test meal (see sections 2.2.2, 2.2.3 and 2.2.4)

3.3 Designing and validating a challenge meal.

The ^{13}C MTGT protocol guidance in **Table 13** reflects the existing evidence base and literature on the ^{13}C MTGT, however, to use the ^{13}C MTGT alongside metabolomic testing the test meal had to be refined. To develop the ideal test meal for the DETECTION study I first reviewed the literature on existing challenge meals used with metabolomic testing and designed a challenge meal based on this and the requirements for compatibility with the ^{13}C MTGT. After which I first performed a series of small experiments to check that the novel test meal could robustly incorporate the tracer compound, then recruited a larger cohort of healthy controls to run a 2 way, randomised cross-over trial comparing the cPDR with the novel test meal to that of the standard test meal and finally performed reproducibility studies on the finalised protocol (under study day conditions). The methods of the review, incorporation and validity studies are described in **Chapter 2.1,** the results are below.

3.3.1 Review of the literature on challenge meals for use with metabolomic testing.

18 studies were eligible for inclusion with a total of 1163 participants, see **Figure 20** for the PRISMA flow diagram of study screening and inclusion and **Table 14** for a list of included studies and their properties.

Figure 20 PRISM flow diagram for challenge meal review.

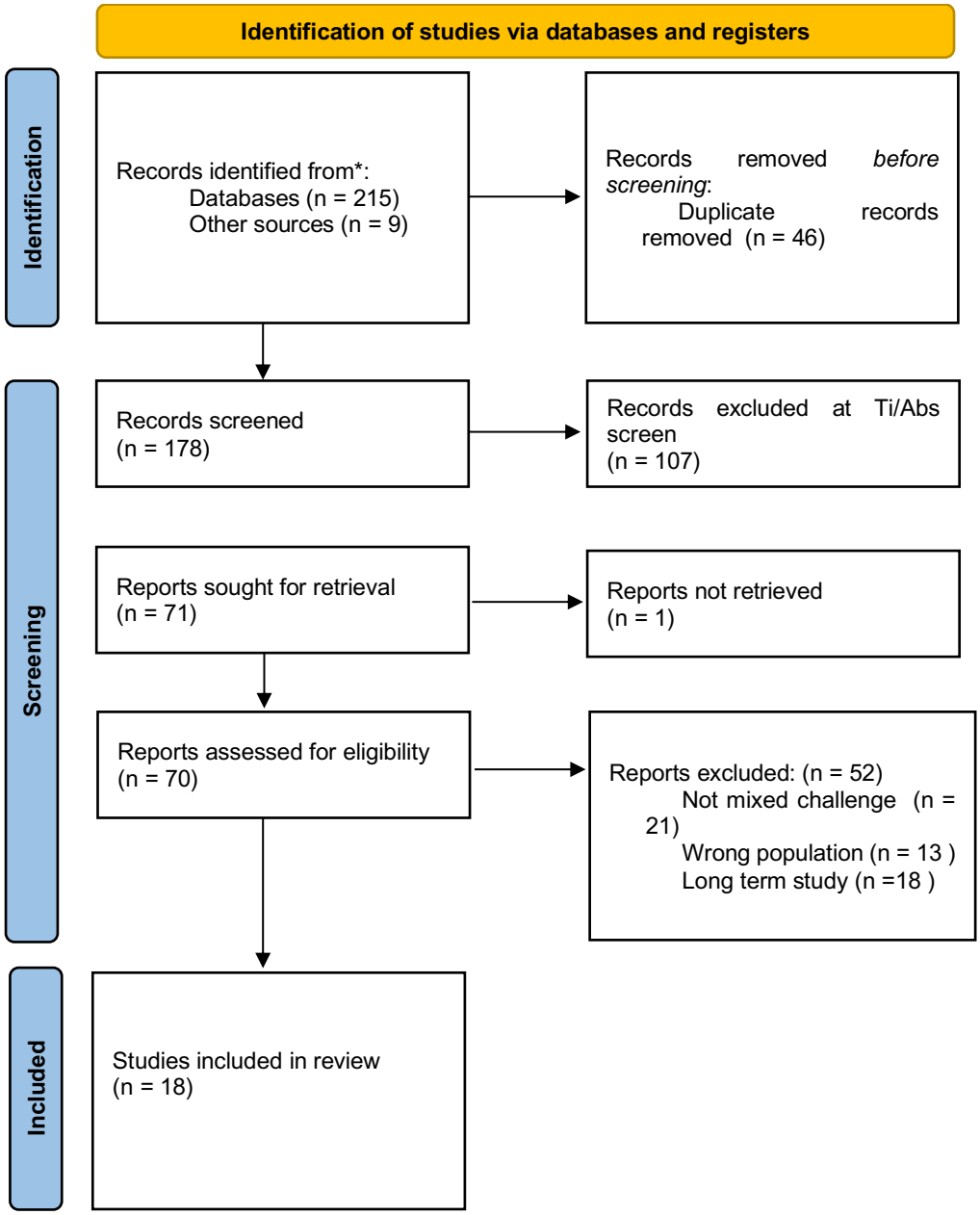


Table 14 Studies evaluating the post-prandial metabolomic response to a mixed challenge meal.

Ref	Author, year	Subjects	Sample timing (hrs)	Meal	Kcal
[188]	Van Den Broek, 2017	Healthy M + F N=100	0, 0.5, 1, 2, 4, 6, 8	400 mL beverage Palm olein: 12.4%, dextrose: 17.25%, protifar (Nutricia)	950 kcal 60g fat, 20 g protein, 75g glucose
[189]	Fiamoncini, 2018	Healthy M + F N=72	0, 1, 2, 4, 6, 8	400 mL beverage Palm olein: 12.4% dextrose: 17.25%, protifar (Nutricia)	950 kcal 60g fat, 20 g protein, 75g glucose
[190]	Shrestha, 2017	Healthy F N=19	0, 30, 45, 60, 90,180 min	Refined wheat bread, cucumber: 40 g, noncaloric orange drink: 300 mL	281 kcal 4.2g fat 9g protein 50g carb
[191]	Krug, 2012	Healthy M N=15	0, .5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8	Fresubin Energy Drink, 200 mL	630 Kcal 11.6g fat 11.2g protein 37g carb (13g*)
[139]	Moriya, 2018	Healthy M + F N=10	-1 and 1	Broiled salmon, pork cutlet, shao mai, Japanese omelet, kamaboko, ganmodoki, rice, vegetables*	763 kcal 24g fat 30g protein 102g carb
[192]	Pellis, 2012	Healthy overweight M, N=36	0, 1, 2, 3, 4, 6	Dairy shake 500 mL: Custard: 300 mL, cream cheese: 150 mL and whipping cream: 50 mL	706 kcal 46g fat 21g protein 52g carb (42g*)
[193]	Schwander 2014	Normal BMI Vs High BMI, N=19 vs 18	0, 1, 2, 4, 6	bread, palm fat, salami, and boiled eggs	500kcal 1000kcal 1500 kcal
[194]	Bastarrachea 2018	Low BMI Vs High BMI F, N=8 Vs 8	0, 0.5, 3, 5	Ensure Plus® (Abbott Nutrition, Lake Buff, Illinois, USA)	600kcal 19g fat 25g protein 80g carb
[195]	Bondia-Pons, 2014	Healthy twins Vs discordant or concordant weight N=50, 32, 18	0, 0.5, 1, 2	McDonald's Big Mac Meal ™	979 kcal 40g fat 32g protein 123g carb
[196]	Adamska 2019	M high T2DM risk, N=8 M low T2DM risk, N=18	0, 0.5, 2	Cubitan (Nutricia): 360 mL OR Nutridrink Fat Free, (Nutricia): 300 mL	450 kcal 13g fat 32g protein 52g carb
[197]	Wopereis, 2017	Healthy M, N=20 T2DM M, N=20	0, .5, 1, 2, 4, 6, 8	400 mL beverage, palm olein: 12.4% dextrose: 17.25%, protifar (Nutricia):	950 kcal 60g fat, 20 g protein, 75g glucose
[198]	Malagelada, 2018	Healthy M, N=32	0, 20min	Warm Ham and cheese sandwich, 38g ham, 38g cheese, 58g bread, 200ml orange juice	425kcal 17g lipid 18g protein
[199]	Badoud, 2015	Healthy and obese M+F N=30	0, 15, 30, 60, 90, 120 min	2 sausage egg English muffins, 1 apple turnover, and 370 mL of orange juice	1330 kcal 66g fat 42g protein
[200]	Thonusin 2017	Healthy and Obese M+F N=51	0, 15, 30, 60, 120 min	237 mL of Ensure original therapeutic nutrition	250 kcal 6g fat 9g protein 41g carb

[201]	Li-Gao 2018	NGT vs IFG vs T2 DM N=533	20, 150min	Fresubin energy drink, chocolate flavor	19g Fat 22g protein 35g carb
[202]	Lopes 2016	RYGB pre- vs post-op N=10	0, 15, 30, 45, 60, 90, 120, 150 min	standard meal	19g fat 22g protein 35g carb
[203]	Fazelzadeh 2018	Healthy vs obese, N=44	0, 30, 60, 120, 180, 240 min	Two muffins and 300 mL 0% fat milk	57g fat 27g protein 121g carb
[204]	Meikle 2015	Healthy M, N=16	0, 1, 2, 3, 4 hrs	cheddar cheese (60 g), butter (20 g), and extra creamy whole milk (300 mL) with toast (50 g) Vs soy based	54g fat 29g protein 37g carb

Meal constituents

Mean total kcal, fat, protein, and carbohydrate content of the test meals under review was 764(±328.9)kcal, 39.9(±25.5)g, 27.2(±13.3)g and 68.8(±32.1)g respectively. N=9(50%) used a liquid component only; the rest used a solid-liquid combination. All allowed a small amount of water in addition. Owing to the inter-related nature of macronutrient metabolism, the ratio of constituents was also reviewed and compared. The mean percentage of fat, protein and carbohydrate content was 42.2(±15.5), 15.1(±5.1) and 40.6(±16.0) respectively.

Testing timeframe

The reported metabolites are too heterogenous to compare between studies, even when broken down into broad categories, those studies investigating the lipidomic and proteomic response in specific relation to the test meal will be discussed individually. The total reporting time frame ranged from 1 to 8 hours, mean 3.9(±2.1).

Take home

The aim of a challenge meal in this context is to stimulate pancreatic secretion and deliver macronutrients available for breakdown by pancreatic enzymes that will then be measurable (via blood sampling and metabolomic testing) within an acceptable testing timeframe. To this

effect, the literature has been examined with specific focus on mixed challenge meals eliciting a measurable response in the food metabolome, specifically lipid and protein metabolism. Based on the available literature, a minimum of 30 % fat is necessary to obtain a response in chylomicron and VLDL-TG production and other processes interrelated to lipid metabolism. The FA species, i.e., MUFA, PUFA and SFA, all have their specific response characteristics. MUFA and PUFA trigger responses in blood pressure regulation, thermic regulation, incretin production and adipose tissue lipolysis. SFA induces a specific strong response in chylomicron production and TG and cholesterol metabolism. In conclusion, a standardized meal targeted at evaluating effects of pancreatic response should contain at least 35 % glucose, 30 % fat and protein with most fat coming from SFA. Of note, the type of fat found in dairy is mostly SFA, milk fat contains on average 70% SFA, this encourages the use of a dairy based test meal. Soybean oil (as used in many vegan options) on the other hand is comprised only 15% SFA and thus unsuitable for this challenge meal.

PhenFlex test

The PFT (PhenFlexTest) was the only reproduced, standardise mixed challenge meal, there are several papers that have utilised this since its conception in 2015 by a group frustrated by the variability in composition of challenge meals. This group designed the PFT with multi-systems approach inclusive of glucose/insulin metabolism, lipid response, and protein metabolism. The 400ml “PhenFlex drink`’ consists of a mixture of 12.40% (w/w) palm olein, 17.25% dextrose, 4.13% Protifar (Nutricia), 0.10% (vanilla flavor), 0.12% (w/w) trisodiumcitrate, 0.08% (w/w) sodiumhydroxide, and 66.12% (w/w) water. This resulted in a drink of 3950 kJ/950 kcal with a macronutrient composition of 60 g fat (of which 39% saturated fatty acids, 47% mono unsaturated fatty acids, 14% poly unsaturated fatty acids), 75 g glucose, and 20 g protein.

Unfortunately, this is not available to buy commercially, also, for the purposes of this work, the fat content would be too high for the ^{13}C MTGT.

In addition to being compatible with the ^{13}C MTGT and being able to elicit pancreatic secretion to enable an early, measurable change in the plasma metabolome the test meal must also fulfil the pragmatic requirements for use in this specific trial setting. See **Table 15** for a summary of these requirements.

Table 15 Summary of test meal requirements to elicit early pancreatic secretion and be compatible with both the 13C MTGT and metabolomic analysis.

For use with the ^{13}C MTGT
An unlabelled fat load ideally between 16-26g
Able to suspend ^{13}C MTG tracer without significant losses during assembly or ingestion,
Able to be ingested completely within 5 minutes
To stimulate pancreatic secretion + elicit early, measurable change in the metabolome
Have a minimum of 30% fat
Preference for SFA (rather than PUFA or MUFA). (e.g., dairy based)
Approximately 750 Kcal
Pragmatic requirements for this specific trial
Transportable
No complex kitchen requirements
Acceptable to Pancreatic cancer, CF and CP patients
Available to purchase 'off the shelf' widely
Vegetarian (Vegan not possible owing to the need for SFA content, may lead to exclusions)

The total fat load of the test meal is difficult to determine and will have to be at the higher end of ideal for the ^{13}C MTGT and lower than the mean of those studies included in the above

review of challenge meals for metabolomic studies. However, when looking specifically at those studies measuring lipidomic and proteomic responses in the short term after a challenge meal, a lower fat load of around 25g still elicits a measurable difference in the metabolome at a reasonable timeframe (Bastarrachea, Lopes, Fazelzahdeh). In addition, the crossover trial performed by the Domínguez-Muñoz group comparing 16g, 32g and 48g of fat although finding an improved AUROC with the lower fat content, identified negligible difference between the 3 different fat content. No single, ready-made nutritional commodity fulfilled all the above requirements, and after reviewing the nutritional constituents of available products, the following combination was chosen (**Table 16**).

Table 16 Selected test meal.

Product	**Medium**	**Fat**	**Protein**	**Carb**	**kCal**
Nutillis® Complete Crème Level 3	125g pot - *mousse*	11.8	12	36.4	306
Fortisip® 2Kcal	200ml bottle, *liquid*	17	20	41.6	400
	Total	28.8g	32g	78g	706
	% total energy	38%	18%	44%	

Although this test meal is novel and the metabolomic response to it is unknown, it fulfils the requirements for pancreatic stimulation and, provides a fat and protein sources that require pancreatic enzymes for breakdown prior to absorption. In addition, Adamska et al used a similar oral nutritional supplement with the same sources of protein and fat and similar macronutrient contributions and found significant differences in the post-prandial lipid profiles of two disease classes. Following test meal development, a short series of experiments were performed to ensure that this meal would be a suitable medium for carrying the ^{13}C MTG tracer, that the novel test meal produced comparable results to commonest test meal for the ^{13}C MTGT and that these results were reproducible, the methods for these experiments are in section 2.1.3/2.1.4 and the results are presented below.

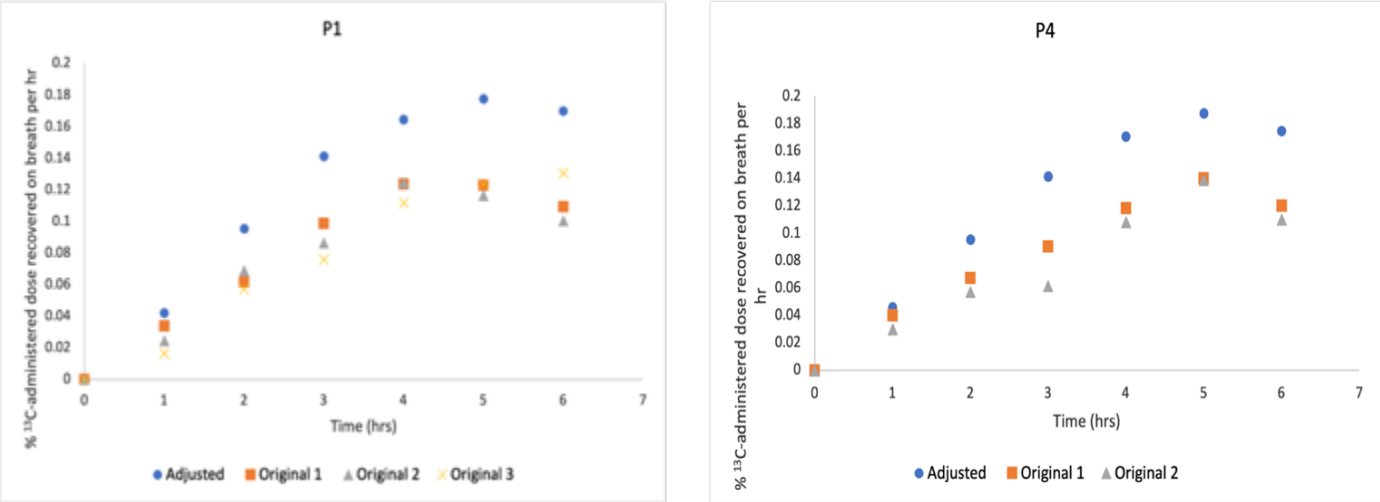
3.3.2 ^{13}C MTG incorporation

As described in methods **section 2.1.4,** a short series of experiments were performed on 2 healthy pilot recruits (P1 and P4) to ensure accurate incorporation and ingestion of the tracer compound.With initially poorer recovery than expected, the process was refined to minimise potential points of substrate loss, after which, cPDR improved significantly. Six-hour cPDR results and breath curves across the original and adjusted processes are shown below (**Figure 21).** With an adjusted process aimed at minimising points of substrate loss the recovery of ^{13}C improved by 12.47% for P1 and 14.83% for P4 (**Table 17**). Both these pilot patients underwent 2 further iterations of the ^{13}CMTGT under study day conditions and were included in the reproducibility studies and test meal validity studies below.

Table 17 P1 and P4 cPDR results across adjusted process of substrate incorporation.

Subject ID	Height (cm)	Weight (kg)	Age	Process	cPDR (%dose)	Mean/IQR	Improvement
P1	162	66	32	Original 1	29.8	28.37 (27.65-29.05)	12.47%
				Original 2	28.3		
				Original 3	27.0		
				Adjusted 1	42.4	40.84 (39.97-42.13)	
				Adjusted 2	37.5		
				Adjusted 3	42.6		
P4	160	68	39	Original 1	30.9	29 (28.05-29.95)	14.83%
				Original 2	27.1		
				Adjusted 1	43.7	43.83 (43.24-44.13)	
				Adjusted 2	45.0		
				Adjusted 3	42.8		

Figure 21 P1 and P4 Tracer incorporation experiment.



3.3.3 Test meal validity

As described in **section 2.1.3** a 2 way, randomised cross-over trial was performed comparing the novel test meal (designed specifically for the DETECTION study) to the most widely used test meal of toast and butter. 14 healthy controls were recruited, receiving both control and test meal at a median of 8 days apart (IQR 8-14.5), cPDR was normally distributed (Tested by Shapiro-Wilk) with no significant outliers (See box plots below). The mean cPDR for Toast and butter was 39.39% (S.D 5.19) for the novel test meal 39.93% (S.D 5.20). A one-way repeated measures ANOVA found no significant difference in cPDR between the 2 meals, $F(1,13)=0.18$, $p=0.68$. See **Figure 22** for paired results for each individual and **Figure 23** for a box plot of the novel versus the standard test meal. With a sample size of 14, there is a minimal detectable difference of 0.8 at 80% power. A one-sample mean T-test was used to determine minimal detectable difference. Subject data, cPDR results, individual comparison cPDR bar charts and box plots below. **(Table 18, Figures 22 and 23)**

Table 18 Pilot cPDR for both the novel and the standard test meal.

ID	Days between	Height	Weight	Age	cPDR Novel	cPDR Standard	% diff
P4	13	1.6	68	43	43.7	45	1.3
P6	15	1.61	49.2	64	49.16	50.77	1.61
P7	15	1.76	84	70	42.99	44.47	1.48
P3	12	1.81	78	34	38.47	38.1	-0.37
P8	19	1.94	92	22	36.33	37.8	1.47
P9	18	1.72	63	23	42.33	35.4	-6.93
P10	8	1.63	67.6	34	41.6	38.7	-2.9
P11	8	1.67	72	47	39.78	41.7	1.92
P12	8	1.58	70	42	33.09	35.1	2.01
P13	8	1.57	62	77	38.4	41.6	3.2
P14	8	1.6	66	25	43.5	39.3	-4.2
P15	8	1.55	62	29	32.3	31.7	-0.6
P16	8	1.64	58	28	43.8	39.9	-3.9
P17	8	1.62	56.25	39	30.7	31.9	1.2

cPDR=Cumulative percent dose recovery, kg=Kilogram, cm=centimetre
Novel meal=Fotisip 2kcal® + Nuttilis complete crème level 3®, Standard=2 slices toast + 15g butter

Figure 22 cPDR of standard vs novel test meal in every subject (test meal validation study).

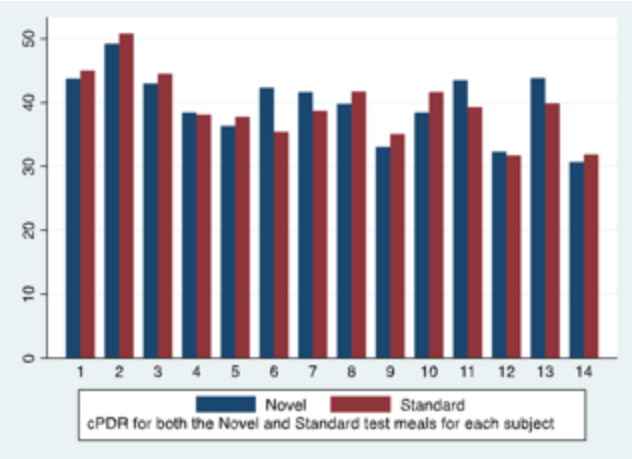
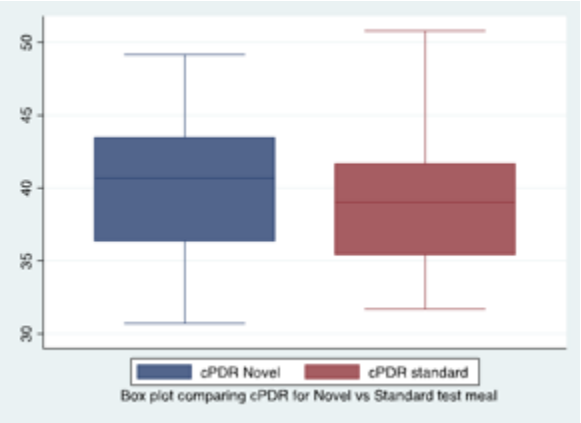


Figure 23 Box plot comparing cPDR for the novel vs standard test meal.



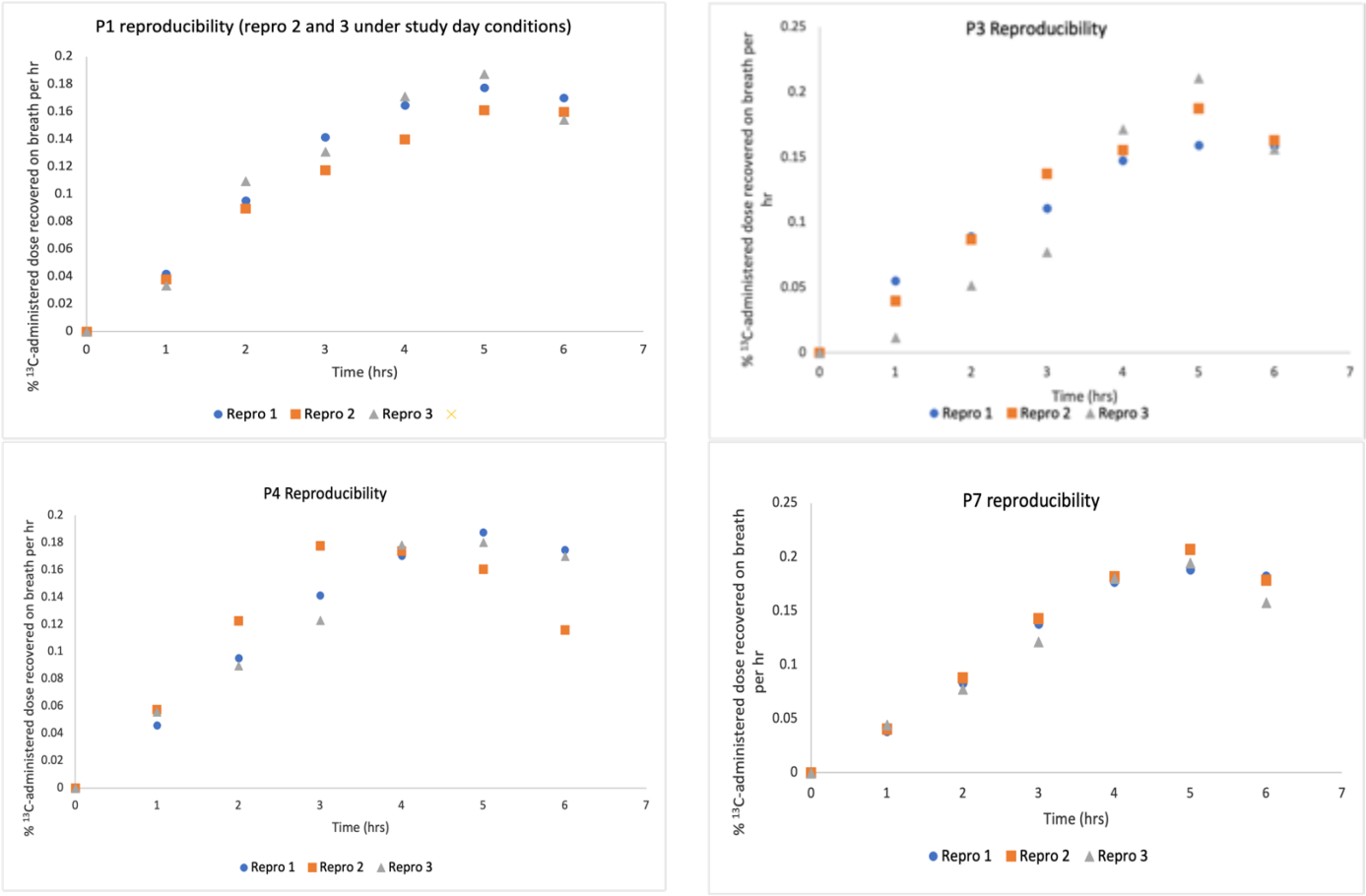
3.3.4 Reproducibility of the ^{13}C MTGT under study day settings results

Having established a robust local protocol for the ^{13}C MTGT, developed a novel test meal, ensured that that test meal could incorporate the tracer compound and give results comparative to a standard test meal, the next step was to ensure that this protocol and test meal produced reproducible results in a study day setting. In addition to the 2 pilot patients who participated in the tracer incorporation studies, 2 male pilot recruits were chosen (male as theoretically a women's BMR can change with the stage of her ovulation cycle which could in turn change her $\dot{V}CO_2$ and cPDR). One of the pilot subjects was picked for his comparable age to our proposed main study cohort. These pilot patients attended together on 3 separate occasions, in the same setting that the study day would be run and with the same information that study subjects would receive. This was to ensure the practical, real-world application of the designed protocol and reproducibility of cPDR using this protocol and the novel test meal. Both pilots were asked to take notes during the day and feedback on how the day was run, the information given, areas of confusion and any potential areas of improvement to ensure subject adherence to the protocol and subject comfort throughout the day. See **Table 19** for cPDR results across the 3 attempts and **Figure 24** for the breath curves.

Table 19 Pilot cPDR results, reproducibility under trial conditions.

Study ID	Height	Weight	Age	cPDR 1	cPDR 2	cPDR 3
P3	1.81	78	43	38.48	41.34	36.39
P7	1.76	84	70	42.99	44.99	41.87
P1	1.62	66	34	42.41	42.36	37.54
P4	1.60	68	43	43.70	45.03	43.70

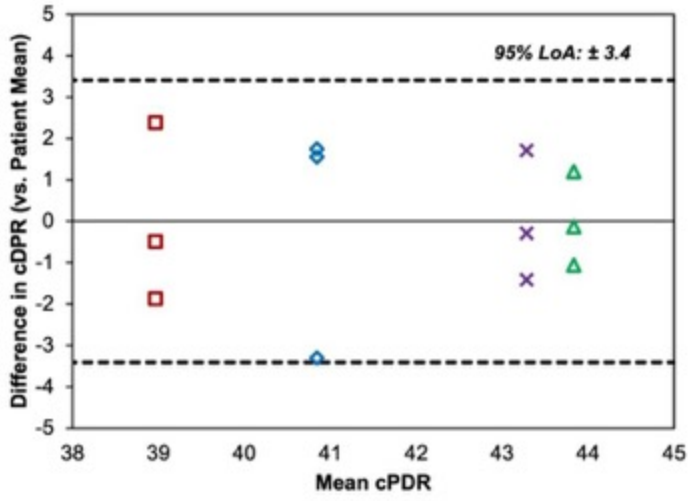
Figure 24 P1, P3, P4 and P7 reproducibility studies.



Of note for P3 the PDR curve sags at 2-3 hours on their 3rd iteration, the subject had actually fallen asleep across this time period which would have reduced their $\dot{V}CO_2$ and consequently also their recovery of ^{13}C, on questioning they had also then had a black coffee (protocol had said water but they assumed as it had no milk and was small volume an espresso would be o.k.), this could account for the sharp spike in cPDR at 4-5 hours. No other protocol deviations occurred across the reproducibility tests. For P4 reproduction 2 the curve peaks early with T-max at three hours rather than 5 hours for reproduction 1 and 3, on examination of their feedback forms, P4 had got lost on the way to the loo and taken a rather brisk walk back to be in time for their next test. It is possible that by raising their heart rate, they increased their $\dot{V}CO_2$ and therefore had an early spike in recovery.

To examine intra-individual variability a one-way repeated measures ANOVA was run (STATA16®), there was no significant difference between the intra-individual measurements, $F(2,6) = 0.27$ ($p=0.64$). To visualise the degree of variability between repeated measurements, a modified Bland-Altman plot for repeated measures was used: the mean of the three cPDR measurements was calculated for each patient, this was then subtracted from each of the three measurements per patient. The mean vs. difference was then plotted for each cPDR measurement, with each patient identified using a different marker style. Broken lines represent 95% Limits of Agreement (95% LoA); defined as ±1.96 times the standard deviation of the differences in cPDR across all twelve data points (**Figure 25**).

Figure 25 Modified Bland-Altman plot of repeated cPDR measurements.

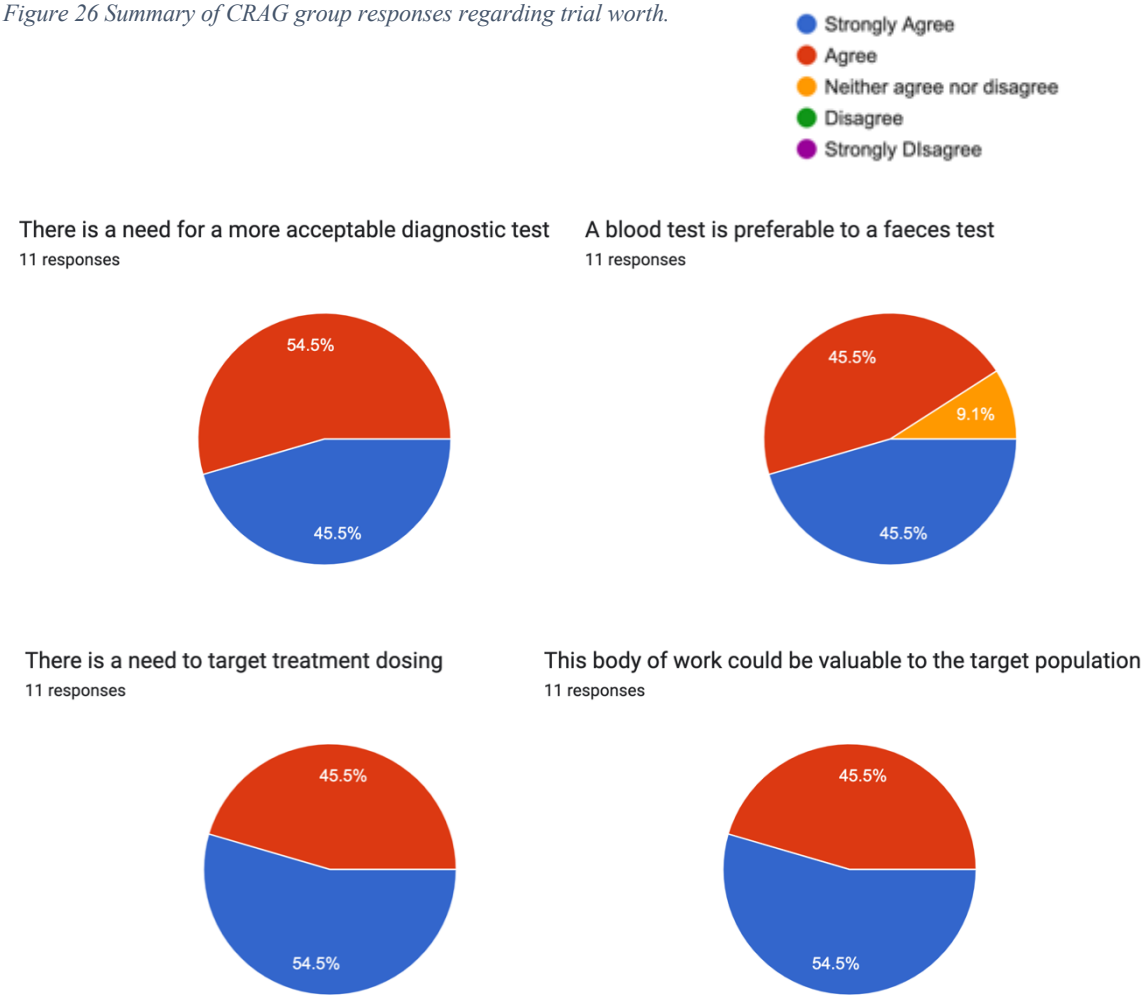


The results of the ^{13}C MTGT reproducibility studies (done using finalised protocol and under study day conditions) show minimal intra-individual variation and the results produced for all pilot patients both within the test meal validity studies and the reproducibility studies are comparable to published European literature on healthy controls. Learning points for the study day were to emphasise clear water only and to ensure the loo was signposted! This work being satisfactory I moved onto recruitment of my main disease study cohort and healthy control comparators.

3.4 PPIE work results.

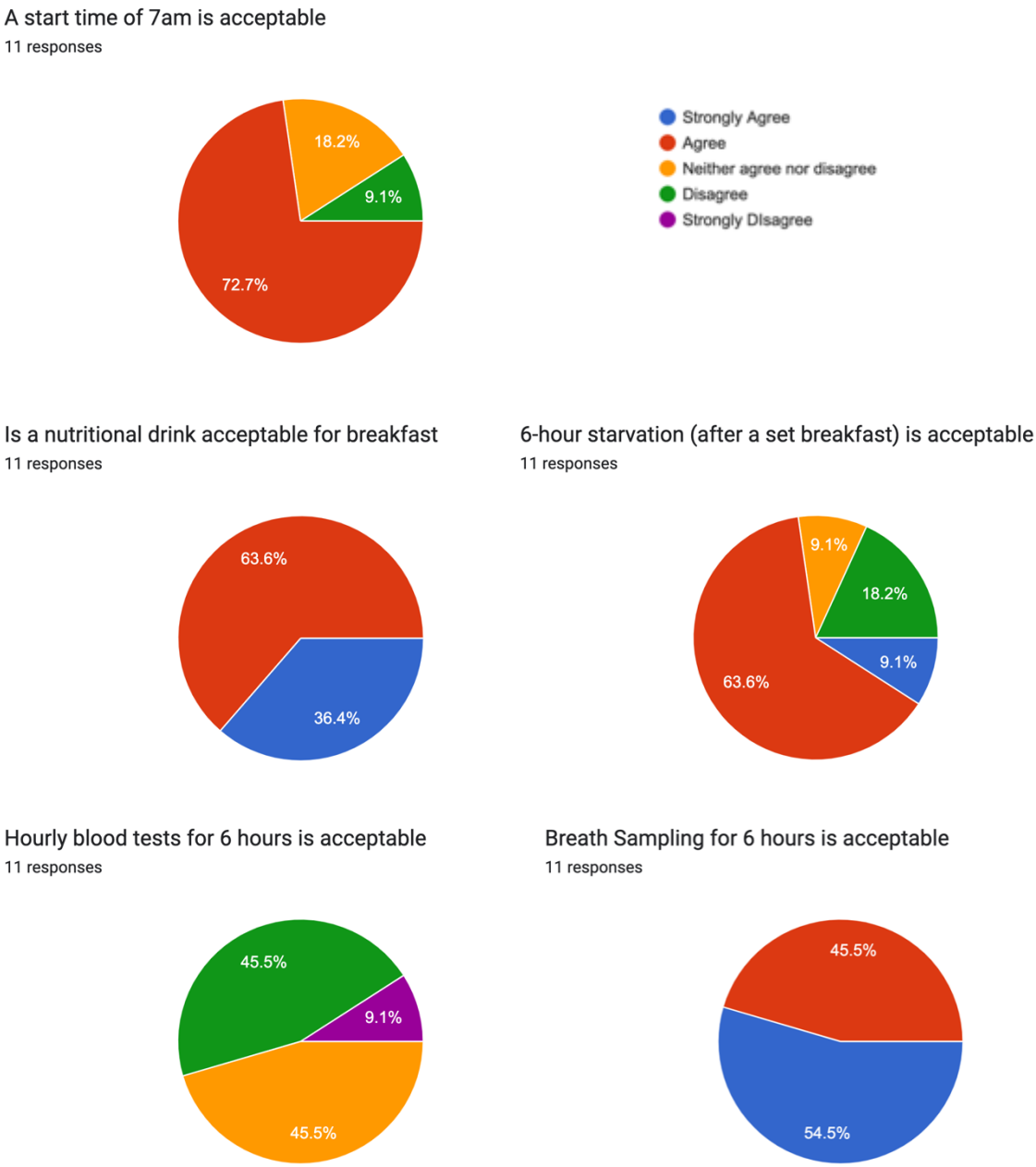
3.4.1 Trial expectations and patient worth

Figure 26 Summary of CRAG group responses regarding trial worth.



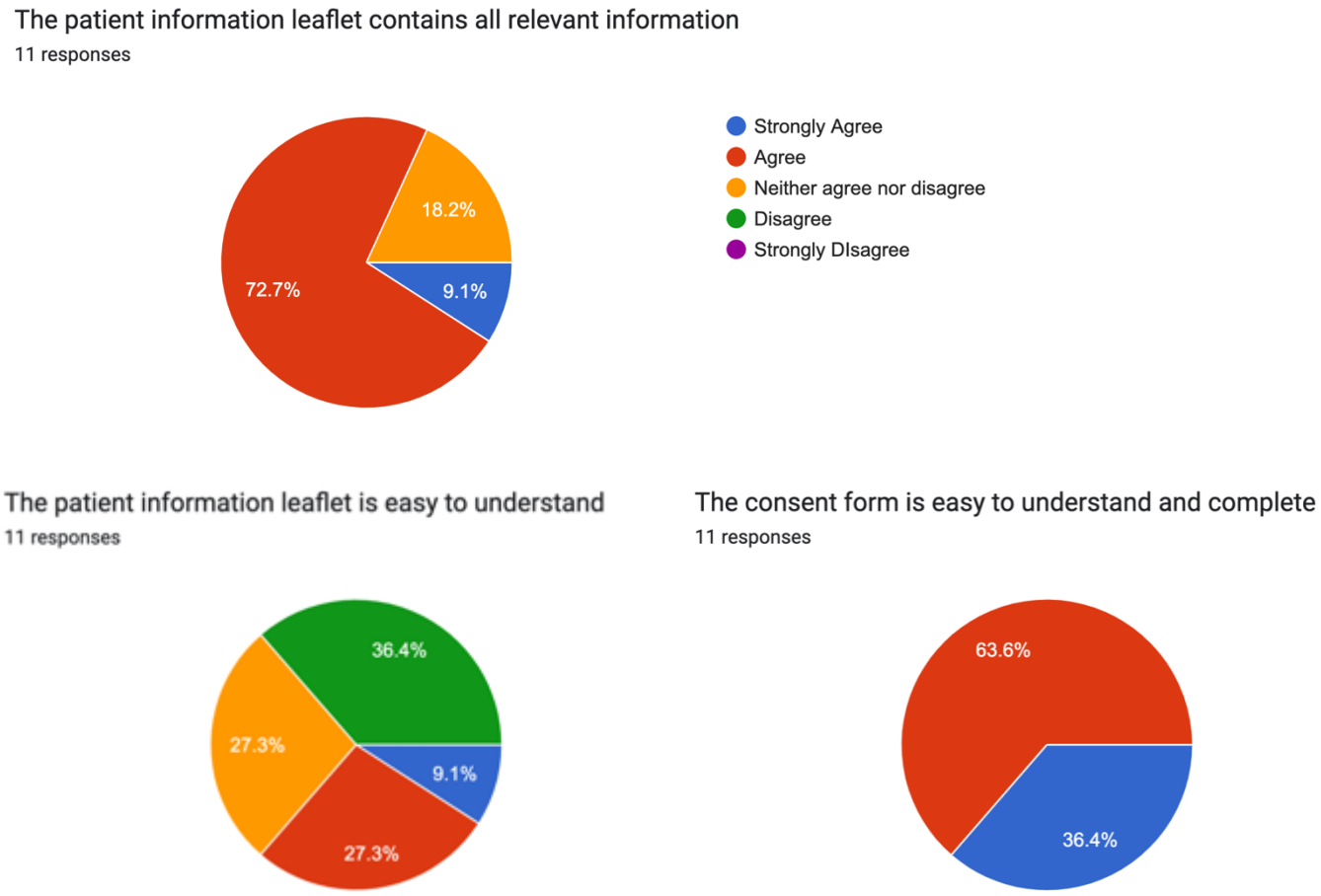
3.4.2 Study day acceptability

Figure 27 Summary of CRAG group responses regarding study day acceptability.



3.4.3 Review of patient facing documents

Figure 28 Summary of CRAG group responses regarding patient facing documents.



3.4.4 Outcomes from PPIE work

In general, CRAG feedback regarding trial worth was excellent with 100% responding agree or strongly agree to the need for a new test, the need for treatment dosing and that this work could be valuable to the patient population. No alterations were made to the study aims from this work. Feedback on the study day itself was very useful, 100% responded neutrally or positively to a 6-hour timeframe for breath sampling and a nutritional drink for breakfast, only 1/11 disagreed with 7am being an acceptable start time and only 2/11 disagreed with a 6-hour period of starvation being acceptable.

Over half (55%) responded negatively to hourly blood sampling for 6 hours and in the free text, 6/11 suggested a limit of 4 hours and several suggested that for the finalized test and ideal time frame would be 2 hours or less. This subject also came up in free text responses to barriers to recruitment with several citing older age, frailty and treatment burden of this patient cohort making a test longer than 4 hours and starvation for this timeframe difficult. After discussion it was agreed that reducing the overall blood sampling timeframe to 4 hours post-prandially would be acceptable to the aim of mapping the post-prandial metabolomic response and more likely to result in effective recruitment.

Around a quarter (27%) responded negatively to a 6-hour starvation period, unfortunately the reference test used to ascertain PEI status (the ^{13}C-MTGT) currently requires this, some work has been done towards shortening this to 4 hours but currently 6 hours is the most widely accepted standard. From a systematic review of ^{13}C breath test methodology (discussed later), the most that could be given during the test would be a small volume of water, some studies gave black tea, but these are in the minority and theoretically caffeine could alter ^{13}C recovery, it was therefore decided to stay with water only. However, when deciding on study day specifics, tea and biscuits on completion was worked into the testing protocol.

Feedback on the patient facing documents resulted in a re-write of the patient information leaflet (PIL) as only 27% responded positively to the statement 'the patient information leaflet is easy to understand'. Input from 2 of the CRAG group members who had expressed a particular interest resulted in a much shorter version with a more succinct summary, less scientific background and less about sample analysis. **See Appendix 3** for finalised patient facing documents.

Two recurring themes in the free comments were firstly that parking and travel issues potentially being a barrier to recruitment and secondly that further PPIE work on test meal acceptability would be ideal. As a result, all grant applications included a set payment value for the day and an additional variable amount to account for parking and travel.

3.5 Discussion – Refinement work to the ^{13}C MTGT, developing a novel challenge meal and the final DETECTION study protocol.

3.5.1 Developing an evidenced, robust protocol for the ^{13}C MTGT

The first hurdle to overcome was the question of how to diagnose PEI in recruits, when the very concept of this work is that there is no ideal test. The ^{13}C MTGT was chosen with FE-1 and PEI-Q used as supporting indicators of disease state. Unfortunately, the accuracy had variable reports, there was no universal protocol, there were huge variations in process and without adjustment to the test meal it would not be suitable to use alongside metabolomic sample acquisition.

To evaluate accuracy, investigate suitability, and develop an evidenced protocol, a systematic review of methodology and meta-analysis of accuracy was performed. Steps with wide variation were examined individually and a suggested protocol based on the available evidence was produced. This work has been published in the peer reviewed, specialist journal *Pancreatology* **(PMID 36805050, Appendix 9).** The key findings were that the ^{13}C MTGT has good sensitivity and specificity for diagnosing PEI but there are several areas of controversy: the ideal test meal, the total testing timeframe, and some smaller protocol points such as the use of metoclopramide, the length of PERT hold and how strict to be regarding limiting smoking and allowing black tea/coffee. This review has been invaluable to my own work and

has also enabled units in other parts of the world to work form an accepted, evidenced protocol. Since publication, a unit in Brazil has set up their own ^{13}C MTGT for the purposes of a national trial, using this protocol and 2 local units have also adopted it within a research setting. **Table 20** shows the key findings as published.

Table 20 Suggested ^{13}C MTGT protocol based on a review of methods.

Summary of Qualitative synthesis	
Areas with good supporting evidence or majority consensus	**Areas of controversy in need of further investigation**
Pre-test control measures	
Limitation of dietary ^{13}C for 48 hours Fasting for 12 hours (except for water) No smoking for 12 hours	PERT cessation duration - authors recommend 48hrs
Test meal and substrate dose	
250mg ^{13}C MTG accurately incorporated into test meal – weigh ^{13}C MTG on a 5-figure balance minimal utensils and transfers to avoid substrate losses. 10g Fat minimum for test meal	Ideal test meal - authors currently recommend 2 slices of toast & butter (to total 16g of fat) + 200ml of water. Metoclopramide - authors do not currently recommend routine use but may be more important if a shortened time frame were used.
In-test control measures	
Remain seated for the duration Remain fasted for duration except for water for comfort	smoking – authors recommend no smoking
Breath sampling and analysis	
At baseline + every 30 minutes for a total of 6 hours Exetainers or breath bags (individual set up) Analyse with IRMS not NDIRS Estimate VCO_2 according to BSA (Appendix 6.)	Potential to shorten to 4 hours if further investigated

Summary of Quantitative synthesis		
Reference test	**Pooled Sensitivity (95%CI)**	**Pooled Specificity**
Duodenal intubation test (S-T, SC-T) 3 papers, 124 subjects	87% (0.65-0.96)	80% (0.64-0.90)
CFA 2 papers, 132 subjects	90% (0.79-0.95)	88% (0.78-0.94)
FE-1 1 paper, 112 subjects	69% (0.49-0.85)	94% (0.86-0.98)
All combined 6 papers, 368 subjects	84% (0.73-0.91)	0.87% (0.79-0.93)

13C = 13 Carbon, PERT = Pancreatic exocrine replacement therapy, ^{13}C MTG = 13 Carbon labelled mixed triglyceride, IRMS = isotope ratio mass spectrometry, NDIRS = non-dispersive infrared spectrometry, VCO_2 = CO2 production, S-T = Secretin test, SC-T = secretin caereulin test, CFA = coefficient of fat analysis, FE-1 = Faecal elastase

A few additional lessons have been learnt along the way. The ^{13}C MTGT protocol as used for this work (described above**)** is necessarily strict and on 3 occasions this was deviated from in some small way, once with a nap and a subsequent strong coffee, once with smoking and once

with more physical activity than 'allowed'. On all three occasions the breath curves did not follow an expected pattern and the end ^{13}C recovery value was suspicious. For 2 of these deviations, repeated measures were available for the same recruit and the difference in ^{13}C recovery was clear and for the other, the supporting measures of PEI quantification (FE-1, PEI-Q and PERT history) did not correlate with the ^{13}C MTGT result. All three of these ^{13}C MTGT results were disregarded, and protocol instructions made clearer to prevent repeat problems. Deviations from protocol such as exertion, sleeping, smoking and caffeine, all have the potential to alter heart rate, temperature, and/or basal metabolic rate which in turn may affect the $\dot{V}CO_2$ (a key component in calculating the cPDR of ^{13}C in exhaled breath). It is possible to measure the $\dot{V}CO_2$ rather than using an estimate (as for this work), however this is impractical for routine use, it is therefore imperative to control the factors that may make the estimate of $\dot{V}CO_2$ inaccurate. For a more extensive understanding of how $\dot{V}CO_2$ relates to ^{13}C recovery and what can affect it, the work by Christine Slater for her thesis has been invaluable and a useful resource for any future work on ^{13}C breath test development.

Of note and previously un-investigated is the potential effect that liver metastasis may have on recovery of ^{13}C during the ^{13}C MTGT. For 2 recruits, both of whom had a significant liver metastasis burden, a breath curve with a more rapid upstroke in ^{13}C recovery was recorded (see **results section 4.3.3.1),** and both recovered more ^{13}C during the 6-hour timeframe than expected. Their Tmax was earlier and their cPDR was higher than anticipated (Clinical history and other supporting tests of PEI indicated at least moderate PEI), however, their decline in ^{13}C recovery was also rapid and extrapolating the curve suggests that should the testing timeframe have continued, their cumulative recovery would indeed have been pathological. There is nothing in the literature previously and this finding is only in 2 patients, but hypothetically, oxidation of ^{13}C octanoate (the product of ^{13}C MTG cleavage by lipase) in the

liver and subsequent exhalation of $^{13}CO^2$ could be more rapid owing to increased vascularisation and delivery secondary to metastases.

As the ^{13}C MTGT is sensitive to even minor deviation on the part of the investigator or the patient, detailed, reproducible step-by-step guides with diagrams were used for both investigators and recruits. This was in addition to the patient information leaflet given in advance. These were developed after several protocol errors in early pilot work and were invaluable to a smooth-running study day.

3.5.2 Designing and validating a novel test meal

Delivering the ^{13}C MTGT requires 6 hours testing time, an overnight fast and strict in test control measures, the acquisition of metabolomic samples requires very similar strictures. When considering the patients under investigation it would have been unreasonable and limiting to recruitment to expect attendance on separate days to obtain the reference ^{13}C MTGT and then the metabolomic samples, therefore a test meal was designed to be used for both. The stages involved were identifying test meal requirements with a systematic review of the literature (both for ^{13}C MTGT methodology, and for existing test meals in use with post-prandial metabolomic testing), confirming adequate tracer incorporation, determining reproducibility with the ^{13}C MTGT, comparing cPDR of ^{13}C with the novel meal in comparison to the standard meal in a 2-way crossover study, and finally confirming reproducibility under study day conditions.

The test meal for the ^{13}C MTGT as highlighted in the previously discussed systematic review is poorly standardised with a wide variety in use. The original work by Vantrappen and Loser used variations on toast and butter, with or without additional chocolate cream.[170, 206] Many

other, some quite complex versions of the test meal, most with a solid-liquid meal and a few with a homogenised or liquid form have come into play since and there have been various attempts to identify the ideal fat content. The only definite features being that it must contain at least 10g of unlabelled fat and physically be able to carry the tracer.

The key requirements and how the novel test meal was chosen is discussed at length in Results **section 3.3,** the result was a Fortisip 2Kcal® and a Nutricia complete crème level 3®. Firstly, as the medium for carrying the tracer had been changed and one of the most important steps is ensuring that the ^{13}C ingested reflects that weighed, a series of experiments were performed to ensure this was accurate. Refinements were made to the process to prevent tracer loss and weights of all instruments and containers pre- and post-weighing and pre-and post- ingestion confirmed adequate tracer consumption using this method. After this, reproducibility under study day conditions was confirmed using repeat measures on a selection of pilot recruits.

To ensure that the novel test meal would deliver equivalent results from the ^{13}C MTGT, a 2-way, crossover trial of the novel meal vs the most widely used meal was performed. This has also been published as a discrete body of work in the peer reviewed, specialist journal 'JHND' (**DOI:10.1111/jhn.13237, Appendix 9)**. To summarise, no difference in the cPDR of ^{13}C using the novel test meal in comparison to toast and butter was seen across repeated measures under similar control conditions. With no difference in cPDR found and similar breath curves in repeat measures, this test meal was then piloted with patients under study day conditions. It was then used without event throughout the trial except for one healthy control who was lactose intolerant.

Although this specific ^{13}C MTGT protocol has not been compared to the previous gold standard of PEI diagnosis (duodenal intubation, which is impractical, expensive and involves potential harm), the incidence of PEI as diagnosed using this ^{13}C MTGT protocol in recruited patients is reflective of that in the wider literature as are the cPDR values in the healthy control cohort. The test meal validation experiment, combined with correlation to the wider literature and complementary methods of PEI assessment (FE-1, PEI-Q and PERT usage) gave confidence that the assessment of PEI status using the ^{13}CMTGT with this protocol was correct.

In addition to enabling the use of the ^{13}C MTGT alongside metabolomic testing for this body of work, creating a test meal that is easy to standardise, doesn't require kitchen equipment and can be used in any setting is an important step in making the ^{13}C MTGT more user friendly and easier to roll out in various hospital and even home settings for routine use. A limitation is that this test meal is not suitable for young children (under 6 years of age) or vegan/lactose intolerant patients. A theoretical limitation is that a significant proportion of the fat content comes from unsaturated fat (via vegetable oil and palm oil), and the changes in lipidomic response may have been more striking and easier to interpret had the fat content come predominantly from saturated fat (SFA). However, there is still a good proportion of saturated fat and there is previous evidence to show a strong post-prandial lipidomic response to a similar test meal with similar fat contributors.

Chapter 4 Results: PEI status and demographics of recruits for the DETECTION trial

4.1 Summary of chapter 4

Recruitment to the DETECTION trial started in 2020, beginning with pilot recruits (results in chapter 3), moving on to the main cohort of participants with pancreatic cancer and PEI, and matched healthy controls. Also planned for recruitment were several sub-groups of patients with PEI for validation of results. At the time of writing this thesis the pancreatic cancer and the NET cohort are fully recruited, the CP cohort is recruiting but with difficulties around patient compliance and the CF cohort is ready to recruit. It is to be noted that the validation studies in non PC cohorts was outside of the remit of the original PhD aims. This chapter covers the demographics of recruits and results of their reference tests used to establish PEI status.

The incidence of PEI following pancreatic resection is under debate owing to the lack of consensus over ideal diagnostic testing. The DETECTION trial used a novel multi-model determination with blinded expert assessment to determine PEI status in 26 post-pancreatic resection patients. This is novel, useful data and is therefore reported independently. Also examined here is the correlation between the diagnostic modalities used to establish PEI status.

4.2: Healthy control recruitment and PEI status

4.3: Main cohort recruitment and PEI status

4.4: NET cohort recruitment and PEI status

4.5: Post-pancreatic resection PEI status

4.6: Correlation between diagnostic modalities for PEI

4.7: Discussion

4.2 Healthy control recruitment

To recruit healthy controls to the main comparative cohort, it was considered essential to minimise the difference in demographics to the diseased cohort. Metabolomic testing is sensitive to multiple factors, both inherent (e.g. age and race) and external such as background diet, exercise, and environmental pollutants. For the pilot and breath test refinement work, healthy controls were identified from friends, family, and colleagues but it was evident that, even with age matching, there would be significant differences in the aforementioned factors between these recruits and my disease population. To try and overcome this, each patient recruited was asked if they would like to bring along their spouse or a friend, this helped recruitment uptake (patients were much happier to spend six hours in a room with someone they knew) and meant that for each diseased recruit a healthy control with similar background diet, environmental exposure and age was also recruited.

All healthy controls underwent the study day as per approved protocol and under the strict conditions described in the methods section. 37 healthy controls (HC) were successfully recruited. A handful were excluded, 1 (HC 43) was unable to complete the breakfast and smoked throughout, their breath curve had several outlying recovery points and cumulative recovery was low, 1 was vegan and unable to eat the test meal and 2 were removed after age and sex matching against the main diseased cohort. The final HC cohort comprised 33 recruits, none had history or symptoms suggestive of pancreatic pathology and none had a pathological ^{13}C MTGT result, the mean age was 64 (IQR 56-67), 14 (42%) were women, and the mean cPDR for the ^{13}C MTGT was 44.60 (IQR 41.32-50.44) full HC demographic data, and ^{13}CMTGT cPDR results are presented below. All recruits provided written, informed consent. See **Table 21** for demographics and cPDR results for main cohort healthy controls.

Table 21 Demographics and cPDR results for main cohort healthy controls for the DETECTION study.

Study ID	Age	Gender	Height (m)	Weight (kg)	BMI	Smoker	cPDR
002 WR	34	M	1.81	78	23.81	N	37.79
004 EC	43	F	1.6	67	26.17	N	37.07
008 EP	64	F	1.61	49.2	18.98	N	49.44
009 MP	70	M	1.76	86	27.76	N	43.5
010 HH	24	M	1.78	85	26.83	N	53.06
011 JT	23	M	1.86	81.3	23.50	N	37.02
014 LP	64	F	1.65	62.6	22.99	N	47.76
016 KK	56	F	1.63	87.2	32.82	Y	45.62
017 TN	66	M	1.87	101.5	29.03	Ex	51.84
019 AP	30	M	1.83	68	20.31	N	36.09
020 GP	51	M	1.72	69.6	23.53	Y	45.9
021 JW	66	M	1.89	86.85	24.31	N	50.58
022 SW	66	F	1.55	74.9	31.18	N	23.52
024 AP	67	M	1.9	108.1	29.94	Ex	41.42
025 CP	63	F	1.65	55.8	20.50	N	50.2
028 MB	61	F	1.71	60.5	20.69	N	41.32
029 JB	61	M	1.91	84.5	23.16	N	52.01
031 AH	68	M	1.91	125	34.26	Y	30.53
032 RN	79	M	1.79	70	21.85	N	50.44
034 LHH	65	F	1.62	63.1	24.04	N	41.87
035 GHH	67	M	1.74	92	30.39	N	38.32
036 RH	57	M	1.75	73.3	23.93	N	36.12
038 DH	47	M	1.93	113	30.34	Y	49.02
040 AW	72	F	1.56	48	19.72	N	50.81
049 DM	45	F	1.73	64.1	21.42	N	43.09
051 MT	79	F	1.71	62	21.20	N	45.82
053 JH	61	F	1.57	53	21.50	N	42.56
055 TM	71	M	1.67	72	25.82	N	46.26
062 SC	67	M	1.72	83.8	28.33	N	48.23
063 SC	65	F	1.66	75.3	27.33	N	57.36
067 JP	80	M	1.62	61.2	23.32	N	45.89
069 BJ	81	M	1.76	90	29.05	N	50.67
076 SY	64	F	1.67	80	28.69	N	50.81

cPDR=Cumulative percent dose recovery, kg=Kilogram, cm=centimetre, M=Male, F=Female

4.3 Main patient cohort (PEI in PDAC) recruitment, investigations, and disease state

4.3.1 Cohort demographics

For the main patient cohort, the target patients were those with PEI, at any stage in their pancreatic cancer journey. Although I ran this trial out of one of the highest throughput units for pancreatic cancer this was a challenging cohort to recruit. As per protocol, patients were identified through HPB clinic listings and approached in a clinic setting (modified to telephone through COVID recovery). All recruited provided written, informed consent. Screening targeted any patient with pancreatic cancer (new diagnosis, pre-operative work up, post-operative follow-up and those attending for chemotherapy either palliative or adjuvant) who had potential PEI. As the study day required the patient to attend early in the morning, stay for 6 hours and remain fasted except for the test meal it was difficult to successfully recruit them in-between their rapid work up for surgery. Those undergoing active chemotherapy were deemed not eligible as the drugs would inevitably alter the metabolomic profile in their own way. Although recruits were successfully recruited from the pre-resection group and metastatic group, the most active recruitment group was those following resection, PEI is recognised as a significant problem in this group (See Introduction Section 1.3.3.2) and many were interested in the trial and in their own results. See **Table 22** for cohort demographics and pancreatic cancer status.

The final patient cohort comprised 43 recruits, mean age 70 (IQR 62-82), 11 (24%) women, mean BMI 23.94 (IQR 21.87-27.92), N=28 (65%) post resection, N=15 (35%) unresected, of the unresected patients 2 were metastatic, 2 were unresectable and 11 were awaiting resection.

Table 22 Demographics of the main patient cohort for the DETECTION study.

Study ID	Age	Sex	Height (m)	Weight (kg)	BMI	Smoker	Pancreatic cancer status	Operation
001 MM	59	M	1.89	105.5	29.53	N	Metastatic	None
003 GB	47	M	1.72	53.4	18.05	Y	Unresectable	None
005 RB	62	M	1.61	58	22.38	N	Metastatic	None
006 DP	77	F	1.52	61.2	26.49	N	Resected	TP
007 TC	59	M	1.66	69.4	25.19	Ex	Pre-operative	None
012 AC	41	M	1.76	67.9	21.92	N	Pre-operative	None
013 RG	61	M	1.7	77	26.64	N	Pre-operative	None
015 DR	56	M	1.8	75.15	23.19	N	Pre-operative	None
018 CM	76	F	1.51	72.4	31.75	N	Pre-operative	None
023 NP	69	M	1.8	67.5	20.83	0	Resected	PPPD
030 PM	66	M	1.73	83.6	27.93	N	Resected	PPPD
033 TN	85	F	1.63	47.5	17.88	N	Resected	PD
037 SH	52	F	1.75	70	22.86	Ex	Resected	PPPD
039 PW	72	M	1.69	54	18.91	N	Resected	PPPD
041 AO	21	M	1.86	82.3	23.79	N	Resected	PPPD
042 PM	73	M	1.66	82	29.76	N	Resected	PPPD
044 JB	50	M	1.72	105	35.49	N	Pre-operative	PPPD
045 MO	58	M	1.74	102	33.69	N	Resected	PPPD
047 SB	68	M	1.77	75	23.94	Y	Resected	PPPD
048 MM	46	M	1.76	113	36.48	N	Resected	PPPD
050 DT	77	M	1.84	85	25.11	N	Resected	PPPD
052 MB	62	M	1.76	84	27.12	N	Resected	PD
054 YM	68	F	1.52	59	25.54	N	Resected	PD
056 TR	70	M	1.65	73.4	26.96	N	Unresectable	None
057 NH	75	M	1.7	77.2	26.71	N	Pre-operative	None
058 PD	65	M	1.75	66.8	21.81	N	Resected	PD
059 TH	59	F	1.65	63.5	23.32	N	Resected	PD
060 RF	70	M	1.76	94	30.35	Y	Pre-operative	None
061 SK	66	M	1.63	79.8	30.04	N	Pre-operative	None
064 JL	82	M	1.72	63	21.30	N	Resected	PPPD
065 LK	87	M	1.7	78.6	27.20	N	Resected	TP
066 SR	75	F	1.48	43.9	20.04	N	Resected	PPPD
068 CJ	78	F	1.74	66.5	21.96	N	Pre-operative	None
070 RB	74	M	1.76	90	29.05	N	Resected	PPPD
071 CB	67	M	1.71	82	28.04	N	Resected	None
072 SD	54	F	1.65	76	27.92	N	Resected	PPPD
073 DL	70	M	1.74	55	19.00	N	Resected	PPPD
074 JA	72	M	1.71	66	22.57	N	Resected	PPPD
075 BY	66	M	1.76	65	20.98	N	Resected	PPPD
077 CE	67	F	1.61	60	23.15	Y	Resected	PPPD
078 AM	73	M	1.63	62	23.34	N	Resected	PPPD
079 KC	74	M	1.73	58	19.38	N	Resected	PD
080 MH	72	F	1.65	44	16.16	Ex	Resected	PPPD

cPDR=Cumulative percent dose recovery, kg=Kilogram, cm=centimetre, TP=Total pancreatectomy, PPPD=Pylorus preserving pancreaticoduodenectomy, PD=Pancreaticoduodenectomy,

4.3.2 Multi-model results, dichotomous decision, and grading of PEI

It was imperative to understand fully the exocrine status of the recruited cohort, the ^{13}C MTGT whilst being the best available test has potential problems in its methodology and although used extensively in some countries with several publications using it to determine PEI in pancreatic cancer patients, has not been specifically validated in our cohort. Therefore, when finalising the dichotomous yes/no decision regarding PEI and then further grading PEI from mild through to severe, a multi-model approach was used with 4 assessors blinded to the other's decisions. A consultant HPB surgeon with a specialist interest in PEI, an academic dietician specialising in PEI, an intercalating student undertaking their degree in exocrine insufficiency and myself reviewed each recruit's faecal elastase, PEI-Q, PERT responsiveness and ^{13}C MTGT result. Spurious results from any investigative modality were discussed between the group and were excluded if appropriate. Results, assessor decisions (0= No PEI, 1=Mild, 2=Moderate, 3=Severe) and final PEI status decisions for the recruited patient cohort are shown in **Table 23**. Missing data from faecal elastase was due to the following reasons, 4 could not produce a sample, 6 were too liquid for processing and 3 were not analysed owing to lack of facilities during the COVID pandemic. There was 1 missing ^{13}C MTGT result from damage to the vials during transport and 1 ^{13}C MTGT result that could have been spurious (discussed below). 2 recruits did not complete their PEI-Q. Results felt to be spurious by all 4 assessors are highlighted in **Table 23**, reasoning to follow. There were 2 patients that did not have a unanimous yes/no decision on their PEI status after blinded review (045 MO and 047 SB). These 2 were discussed as a group, their investigations and CRF reviewed for response to PERT and PERT dosing requirements, after which a final PEI status decision was made, for both the group ultimately reached agreement. The final decision identified 34 patients with PEI and 9 without.

Table 23 Results of multi-modal PEI assessment for main patient cohort recruits.

Study ID	FE-1	Duct (mm)	PEI-Q (MSS)	^{13}CMTG cPDR:	Blinded assessor decisions 0,1,2,3				PEI status	Grade average
001 MM	124	8	2.20	39.94	2	2	3	2	Y	2.25
003 GB	90	6.9	1.72	27.51	3	3	3	2	Y	2.75
005 RB		8	1.32	28.93	3	3	3	2	Y	2.75
006 DP		9	2.20	11.38	3	3	3	3	Y	3
007 TC		9	0.25	22.02	2	2	2	3	Y	2.25
012 AC		11	3.07	10.51	3	3	3	3	Y	3
013 RG	86	10	1.68		3	3	3	2	Y	2.75
015 DR	218	5	1.63	42.39	0	0	0	0	N	0
018 CM	37	10	1.92	30.04	3	3	3	2	Y	2.75
023 NP	15	9	1.49	25.99	3	3	3	3	Y	3
030 PM	91	7	1.8	19.62	3	3	3	3	Y	3
033 TN	122	7	0.95	25.67	2	2	3	2	Y	2.25
037 SH	98	5.5	1.55	27.29	3	3	3	2	Y	2.75
039 PW	143	5	1.49	29.44	2	2	2	1	Y	1.75
041 AO		1	1.57	26.1	2	2	2	2	Y	2
042 PM	225	4	0.8	58.01	0	0	0	0	N	0
044 JB	257	14	1.49	28.12	2	2	2	2	Y	2
045 MO	238	1		41.98	0	0	1	0	N	0.25
047 SB		6	1.82	38.2	0	0	1	2	Y	0.75
048 MM	500	4	1.13	47.6	0	0	0	0	N	0
050 DT	54	8	2.18	24.97	3	3	3	3	Y	3
052 MB	77	5.5	1.68	27.54	3	3	3	2	Y	2.75
054 YM	15	8	0.56	29.3	3	3	3	3	Y	3
056 TR	84	6.5	1.2	28.55	3	3	2	2	Y	2.5
057 NH		13	0.65	45.27	0	0	0	0	N	0
058 PD	93	5	1.86	27.83	2	3	3	2	Y	2.5
059 TH		7	1.72	16.31	3	3	3	3	Y	3
060 RF		12	1.22	8.58	3	3	3	3	Y	3
061 SK	23	13	1.51	20.26	3	3	3	3	Y	3
064 JL	125	3	1.55	31.06	1	1	1	1	Y	1
065 LK		7	2.2	13.5	3	3	3	3	Y	3
066 SR	46	6	1.24	28.91	3	3	3	2	Y	2.75
068 CJ	385	10	1.24	52.46	0	0	0	0	N	0
070 RB	500	4		56.26	0	0	0	0	N	0
071 CB	264	4.5	0.96	45.52	0	0	0	0	N	0
072 SD	200	1	0.25	45.91	0	0	0	0	N	0
073 DL	15	8	1.76	28.49	3	3	3	3	Y	3
074 JA	21		0.61	29.47	2	2	3	2	Y	2.25
075 BY	15	6	2.09	16.6	3	3	3	3	Y	3
077 CE	62	2	1.26	43.06	2	1	3	2	Y	2
078 AM		9	1.84	6.16	3	3	3	3	Y	3
079 KC	15	8	3.07	19.36	3	3	3	3	Y	3
080 MH	127	3	2.20	32.7	2	2	3	2	Y	2.25

FE-1=faecal elastase, PEI-Q=PEI questionnaire, 13C MTGT = 13C mixed triglyceride test, cPDR=cumulative percent dose recovery, PEI=Pancreatic exocrine insufficiency, MSS=mean symptom score

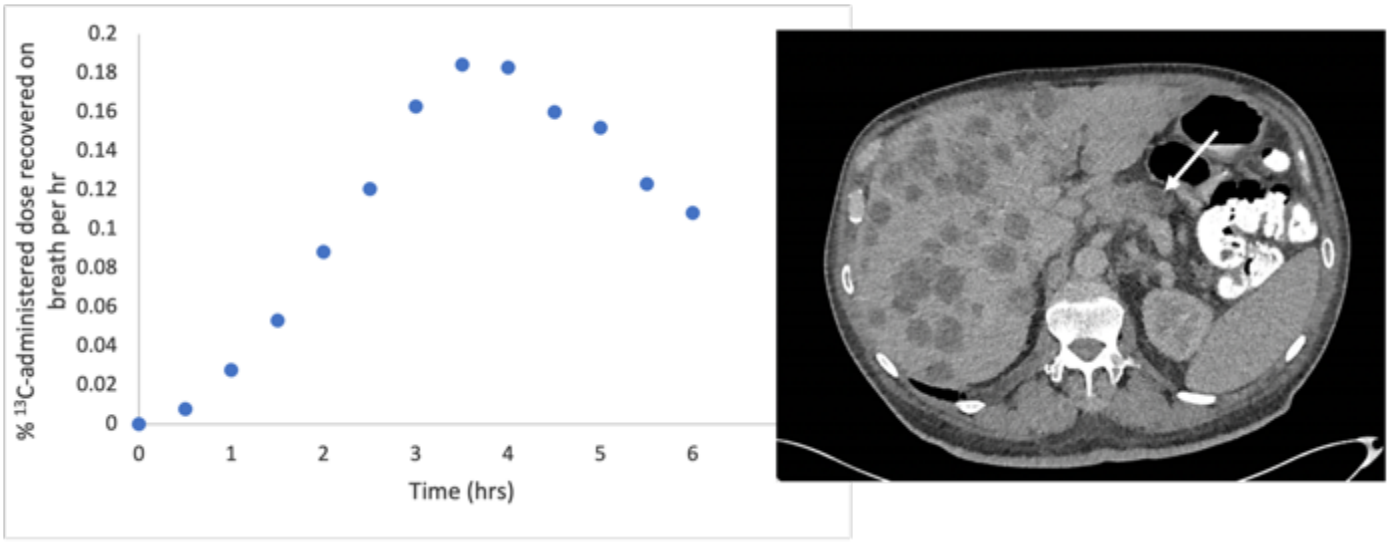
4.3.3 Issues potentially affecting the accuracy of diagnosis of PEI.

4.3.3.1 Liver metastasis

Two patients had normal results on their ^{13}C MTGT, 001 MM and 077 CE but had an overall clinical picture indicative of PEI with possible spurious cPDR results owing to liver metastases. They are discussed below.

001 MM had a heavy liver metastasis burden, and his breath curve showed a much more rapid cPDR spike than expected with an earlier tMax (3.5 hrs) and a much faster drop after tMax than usual (**Figure 29**). With a diagnostic FE-1 (125), clear symptoms of PEI, an atrophic pancreas and wide pancreatic duct (8mm) on CT (**Figure 29**) this recruit was still deemed to have PEI. Theoretically the high liver metastasis burden could have increased the blood flow to the liver where ^{13}C is metabolised to $^{13}CO_2$ prior to exhalation with earlier recover of ^{13}C in breath and had I run a longer ^{13}C MTGT, the cPDR would potentially be pathological (looking at the fast downstroke in ^{13}C recovery).

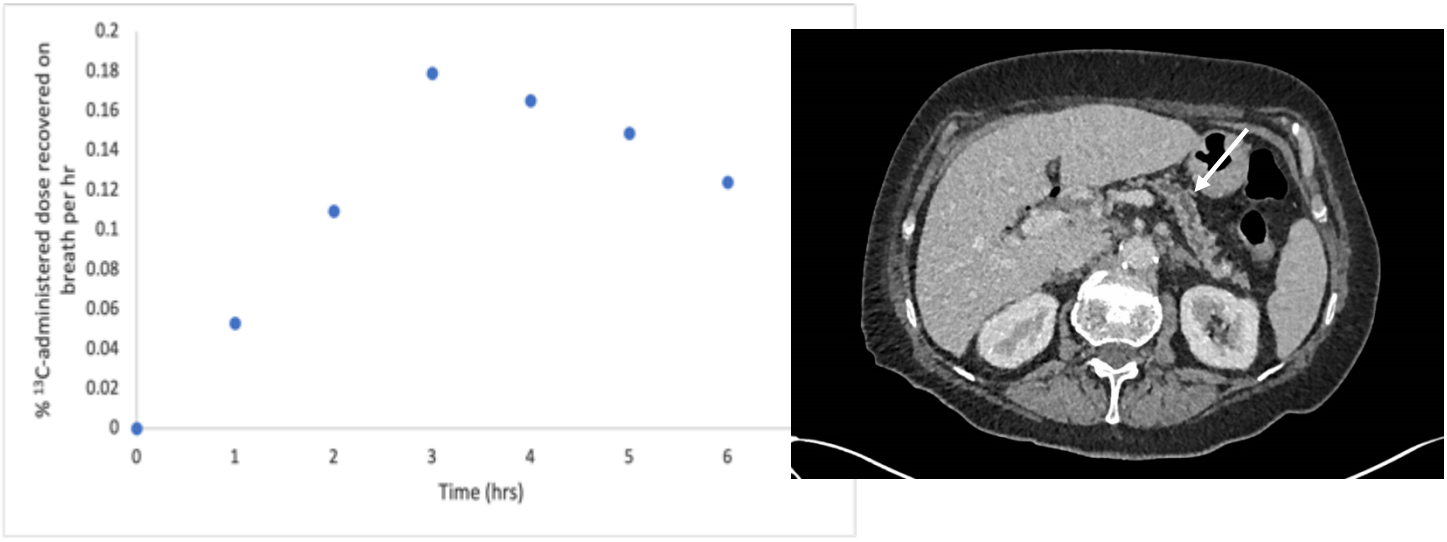
Figure 29 cPDR curve and snapshot of CT scan for 001MM.



077 CE also had metastasis (although with a lesser burden than 001 MM and post resection), their curve also showed a more rapid upstroke, an earlier tMax (3 hours) and a rapid

downstroke. Their cPDR was 43 (normal) but with a FE-1 of 62, an atrophic pancreas on CT and symptoms suggestive of PEI improved by PERT, this recruit was also deemed to have PEI and included in the pathological cohort. Reasoning behind their discordant cPDR could be either metastases or study day issues (it was noted that she was unable to go the 6-hour duration without smoking and took several trips outside), it was therefore decided that her cPDR should be disregarded. See **Figure 30,** white arrow to atrophic pancreas with dilated duct.

Figure 30 cPDR curve and snapshot of CT scan for 077CE.



4.3.4 Final included recruits for the main cohort of the DETECTION trial

The main comparative cohort is patients with PEI vs healthy controls, comparing these two groups there is no significant difference between age, gender, and BMI but there is a significant difference in cPDR from the ^{13}C MTGT (**p=0.0000**), See **Table 24**. The group of patients with no PEI are to be used as a secondary control group to ensure differences identified are from PEI status not down to underlying disease status (pancreatic cancer). There is no significant difference seen in age or gender, but patients with PEI have a significantly lower BMI, lower

FE-1, lower cPDR, wider pre-operative pancreatic duct and worse PEI-Q scores, see **Table 24** for a summary of these results. **Figure 31** Shows the difference in cPDR results from the ^{13}C MTGT between the 3 groups and **Figure 32** shows the other markers of PEI compared between the PEI and non-PEI patient group.

Table 24 Demographics and markers of PEI compared between patients with PEI and patients without PEI and between patients with PEI and healthy controls.

	Patient cohort			**Healthy controls (N=33)**	
	PEI – No (N=9)	***PEI – Yes (N=34)***	***P***		***p* (vs PEI)**
Age (Years)	67 (56-74)	68 (61-73)	0.77	64 (56-67)	0.06
Gender (F) N (%)	2 (22%)	9 (26.5%)	0.80	14 (42.4%)	0.17
BMI (kg/m^2)	28 (26.7-29.8)	23.3 (21-27)	**0.01**	24 (21.9-28.8)	0.19
FE-1	251 (222-443)	81 (22-110)	**<0.01**	N/A	
cPDR (%)	45.9 (45.3-52.5)	27.5 (19.6-29.3)	**<0.01**	45.9 (41.4-50.4)	**<0.01**
Duct width (mm)	4 (4-5)	7 (6-9)	**0.04**	N/A	
PEI-Q (MSS)	0.96 (0.65-1.24)	1.68 (1.32-1.92)	**<0.01**	N/A	

FE-1=faecal elastase, PEI-Q=PEI questionnaire, 13C MTGT = 13C mixed triglyceride test, cPDR=cumulative percent dose recovery. Data are reported as N (%), with p-values from Chi^2/Fisher's exact test, or as median (interquartile range), with p-values from Mann-Whitney U tests, unless stated otherwise. Bold p-values are significant at p<0.05.

Figure 31 cumulative percent dose recovery of 13C in the recruited cohorts.

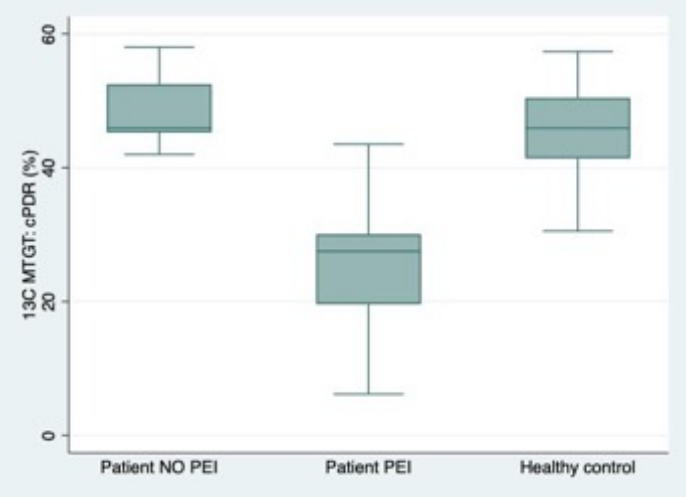
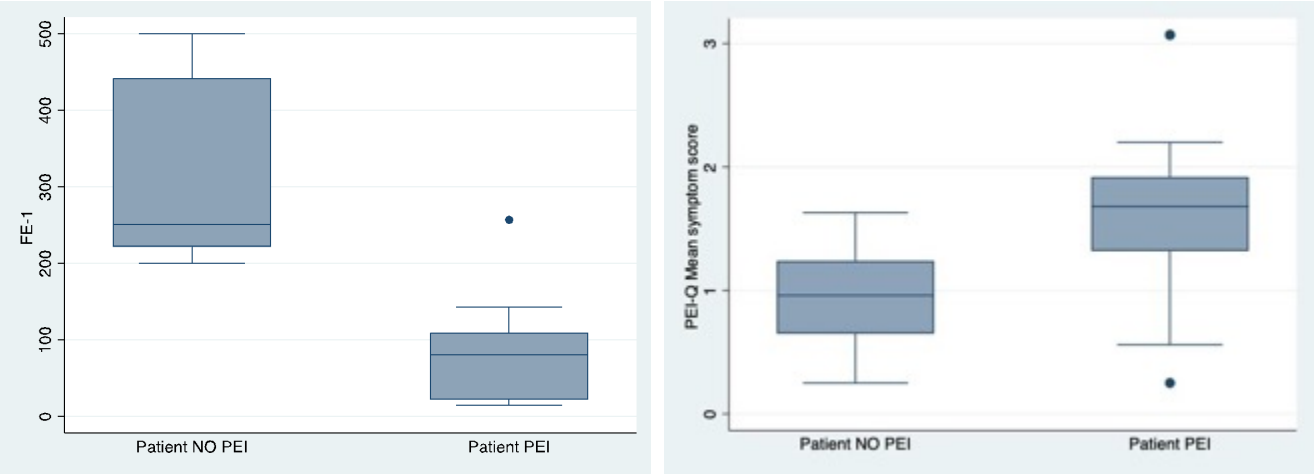


Figure 32 PEIQ and FE-1 in the PEI patient cohort and the no PEI patient cohort.



In summary, healthy controls are demographically well matched to the PEI cohort except for PEI status. Final recruitment for the main cohort after exclusions was 34 patients with pancreatic cancer and PEI (mean cPDR 27.5 (19.6-30)) to be compared against 33 healthy controls (mean cPDR 45.9 (41.4-50.4)). 9 patients with pancreatic cancer and no PEI have also been recruited, when comparing this group to the patients with PEI and pancreatic cancer they are well matched for demographics and have significantly higher cPDR, narrower duct width, better PEI-Q scores, and lower faecal elastase.

4.4 NET cohort recruitment, investigations, and disease state .

This section covers the NET cohort recruitment and their PEI status. PEI status in this cohort is poorly investigated and using the ^{13}C MTGT to try and establish the drop in exocrine function after initiating SSAs is wholly novel. For this section of my trial, I worked with an iBSc student (LH) who carried out most of the patient recruitment (following training from myself and initially under my supervision), I analysed the ^{13}C MTGT data, LH completed the comparative statistics and wrote this work up for his iBSc dissertation, I will ensure that any work done by

him is attributed correctly. Part of this work has been published in a peer reviewed journal: ***Somatostatin Analogues among Patients with Neuroendocrine Tumours? Cancers (Basel). 2023 Mar 23;15(7):1933. PMID: 37046594.***

11 eligible patients with NETs were recruited prior to initiating SSA therapy (written, informed consent given). 10 successfully completed pre and post therapy investigations and were included in analysis. Baseline characteristics shown in **Table 25**. All have plasma samples frozen at -80 awaiting metabolomic analysis, their demographics, and investigations of PEI status pre and post SSA therapy are reported here. 2 patients had pancreatic NETS (without pancreatic duct obstruction or any suspicion of pre-SSA PEI) and 8 had extra-pancreatic NETs.

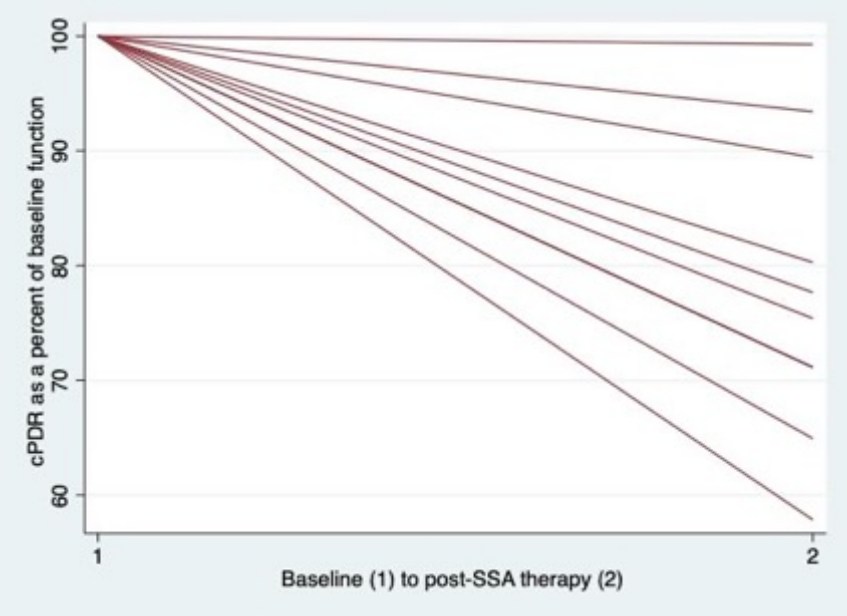
Table 25 Patient demographics, tumour details and cPDR results pre- vs post- SSA initiation for NET patient cohort.

ID	Age	Sex M/F	BMI (kg/m^2)	Tumour			cPDR from the ^{13}C MTGT		SSA type
				Location	Grade	Status	Before SSA therapy	After SSA therapy	
N1	63	F	36.6	Pancreas	2	Fu	43.9	33.1	L
N3	58	M	28.5	Pancreas	3	Fu	65.0	37.6	L
N4	73	M	24	Small bowel	3	Fu	59.3	55.4	L
N5	72	M	34.5	Small bowel	3	Fu	57.8	46.4	O
N6	78	M	23.5	Small bowel	2	Fu	57.7	51.6	O
N7	64	M	27.1	Lung	3	Fu	47.0	36.5	L
N8	84	M	26.9	Small bowel	3	Fu	38.8	27.6	L
N9	62	M	28.3	Small bowel	0	nFu	41.2	40.9	L
N10	65	F	31.7	Small bowel	3	Fu	61.3	39.8	L
N11	76	M	24.7	Small bowel	3	Fu	48.5	34.5	L

*Functional status of tumour determined by baseline symptoms + 24 h urinary 5-hydroxyindoleacetic acid (5-HIAA) levels. *F, female; M, male; BMI, body mass index; SSA, somatostatin analogue. L=Lanreotide, O=octreotide. Fu=functional, nFu=Non functional*

All 10 performed the ^{13}C MTGT pre and post commencement of SSA therapy with no deviation from protocol. All patients had a drop in their cPDR, the median drop form baseline was 23.4% (range 0.5-42.1%, p=0.005), **Figure 33** displays change from baseline in cPDR for each recruit.

Figure 33 ^{13}C MTGT cPDR represented as % of baseline in NET patients commencing SSAs. Produced using STATA 16®.



Faecal elastase was performed before and after SSA commencement in 7 of the 10 patients (3 samples were too liquid to assay) and there was no significant change between the paired measures. The PEI-Q was completed by all 10 at baseline and post SSA therapy, there was a statistically significant decrease in mean symptom score (indicating improvement in bowel symptoms) of −31.5% (range: −56.8–1.9%, p = 0.005).

Although the drop in cPDR for all patients suggests that SSAs do indeed affect exocrine function, few met the diagnostic threshold for PEI, therefore this cohort are stored for future metabolomic analysis but will not be used as a validation cohort for metabolomic markers indicative of PEI. A body of work is underway to robustly investigate the impact of this fall in exocrine function in NET patients starting on SSAs and whether they warrant treatment with PERT.

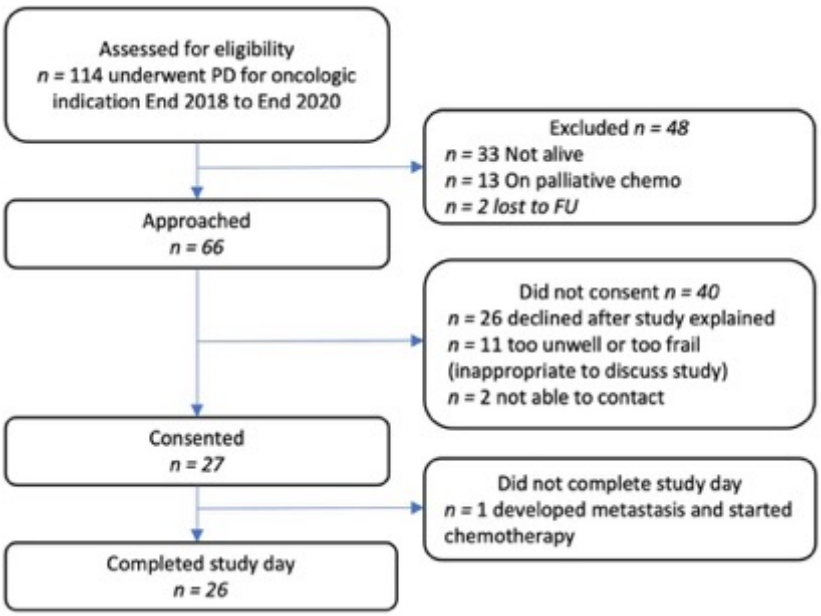
4.5 The long-term incidence of PEI following PD or PPPD

As part of the DETECTION trial patients post PD were recruited, as the incidence of PEI in the long-term following PD is still poorly understood, the results of their multi-model PEI work up were examined separately. This body of work shows the incidence of PEI in the long term after pancreatic resection (very poorly investigated previously) and examines consensus between the different available diagnostic modalities. This has been published as an independent piece of work; *Comprehensive, long-term evaluation of pancreatic exocrine insufficiency after pancreatoduodenectomy. Pancreatology. 2023 Dec doi.org/10.1016/j.pan.2023.11.016 . (1st Author). (214)*

4.5.1 Cohort characteristics

A total of N=114 patients underwent PD/PPPD for an oncologic indication between September 2018 and December 2020, and were assessed for study eligibility. Ultimately N=26 were successfully recruited and completed the study day (as part of the DETECTION trial), see **Figure 34** for a flow chart of exclusions.

Figure 34 Post resection inclusion flowchart



These patients had a median age at the time of surgery of 69 years (IQR: 62-73), with the majority having PDAC (69%) and undergoing PPPD (77%); the study day was a median of 15 months (IQR: 12-21; range: 8-27) post-resection. Full cohort details are reported in **Table 26**

Table 26 Demographics of post PD or PPPD patients recruited to the DETECTION trial.

	Whole Cohort		Consensus PEI Diagnosis		
	N	***Statistic***	***No***	***Yes***	***p-Value***
At Time of Surgery					
Age at Surgery (Years)	26	69 (62, 73)	58 (54, 73)	69 (66, 73)	0.313
Sex (% Male)	26	18 (69%)	4 (80%)	14 (67%)	1.000
Histology	26				**0.029**
PDAC		18 (69%)	1 (20%)	17 (81%)	
Ampullary		4 (15%)	2 (40%)	2 (10%)	
Cholangio		2 (8%)	1 (20%)	1 (5%)	
Others		2 (8%)	1 (20%)	1 (5%)	
Pancreatic Duct Width*					
Width (mm)	25	5 (2, 7)	2 (1, 3)	6 (3, 8)	**0.026**
Dilated (>3mm)	26	16 (62%)	1 (20%)	15 (71%)	0.055
Type of Surgery	26				0.298
PD		6 (23%)	0 (0%)	6 (29%)	
PPPD		20 (77%)	5 (100%)	15 (71%)	
On Study Day					
Days from Resection to Study Day	26	452 (375, 650)	579 (542, 650)	422 (375, 586)	0.536
Charlson Comorbidity Index	26	3 (2, 3)	1 (1, 3)	3 (2, 3)	0.318
Smoker	26				1.000
Non-		22 (85%)	5 (100%)	17 (81%)	
Ex-		2 (8%)	0 (0%)	2 (10%)	
Current		2 (8%)	0 (0%)	2 (10%)	
Adjuvant Chemotherapy	26	22 (85%)	4 (80%)	18 (86%)	1.000
Weight (kg)					
Pre-op	26	77.2 (69.5, 83.4)	83.4 (81.7, 104.1)	75.1 (69.4, 78.0)	**0.010**
Study Day	26	66.4 (59.0, 82.3)	90.0 (82.0, 102.0)	63.5 (58.0, 70.0)	**0.004**
*Change***	26	-6.4 (-10.7, -4.0)	-1.4 (-2.4, 8.9)	-8.1 (-11.6, -6.0)	**0.002**
BMI (kg/m^2)					
Pre-op	26	25.9 (23.9, 28.9)	30.3 (30.0, 33.6)	25.5 (23.6, 26.7)	**0.005**
Study Day	26	23.2 (20.8, 27.1)	29.8 (29.1, 33.7)	22.6 (20.0, 23.8)	**0.001**
*Change***	26	-2.2 (-3.6, -1.3)	-0.5 (-0.8, 2.9)	-2.6 (-4.0, -1.9)	**0.004**

4.5.2 PEI Prevalence

All N=26 patients completed the ^{13}CMTGT-cPDR; however, one patient was unable to refrain from smoking for the duration and their ^{13}CMTGT-cPDR was excluded from analysis, leaving N=25. N=22 patients had FE-1 levels reported, N=4 were unavailable; two samples were not processed because of COVID-19 pandemic related laboratory service problems, one patient

was unable to produce a sample, and one sample was too liquid. The PEI-Q was completed by N=25; with one patient not returning their questionnaire.

After evaluating the PEI assessments, the four reviewers gave a concordant assessment of PEI status for 24 of26 patients (92%). Of the two patients where consensus was not achieved, the first was ascribed mild PEI by one assessor and no PEI by the other 3. This patient had a ^{13}CMTGT-cPDR of 42.0%, and a FE-1 of 238µg/g; PEI-Q data were not available. Further review found that they were receiving low dose PERT and had no definitive symptoms of PEI after a PERT hold for the study day, and after discussion they were classified having no PEI. The second patient had a mild and moderate PEI classification from 2 assessors and no PEI by the other 2. This patient had a ^{13}CMTGT-cPDR of 38.2%, a PEI-Q of 1.82, and no available FE-1. On review of PERT hold, this patient experienced significant symptoms and clinical steathorrhoea after a the study day hold and was therefore diagnosed with PEI after discussion. Accordingly, the prevalence of PEI in this post pancreatic resection cohort was 81% (95% CI: 61-93%; 21/26).

Reviewers additionally quantified the severity of PEI on a four-point scale. High levels of consistency were observed, with an ICC of 0.90 (95% CI: 0.82-0.95), and 76% (118/156) pairs of assessments having concordant PEI severities. Where there were discrepancies, these were generally differences of one severity grade, most commonly between moderate vs. severe PEI (*Table 2*). Averaging the grades across reviewers found that 50% (N=13) of the cohort were classified as having severe PEI, with 23% (N=6) moderate, and 8% (N=2) mild. Patients with a consensus PEI diagnosis had significantly greater PD width on pre-operative CT, with a median of 6mm (IQR: 3-8) compared to 2mm (IQR: 1-3) in those without PEI (p=0.026, *Table 1*). PEI was also significantly more common in patients with PDAC, compared to other

histologies (94% [17/18] vs. 50% [4/8], p=0.029). Patients with PEI had significantly lower BMI at the pre-operative assessment (median: 25.5 vs. 30.3kg/m^2, p=0.005), which was followed by a significant greater reduction in BMI between the pre-operative assessment and the study day (median reduction: 2.6 vs. 0.5kg/m^2, p=0.004).

4.5.3 Comparisons between PEI assessments

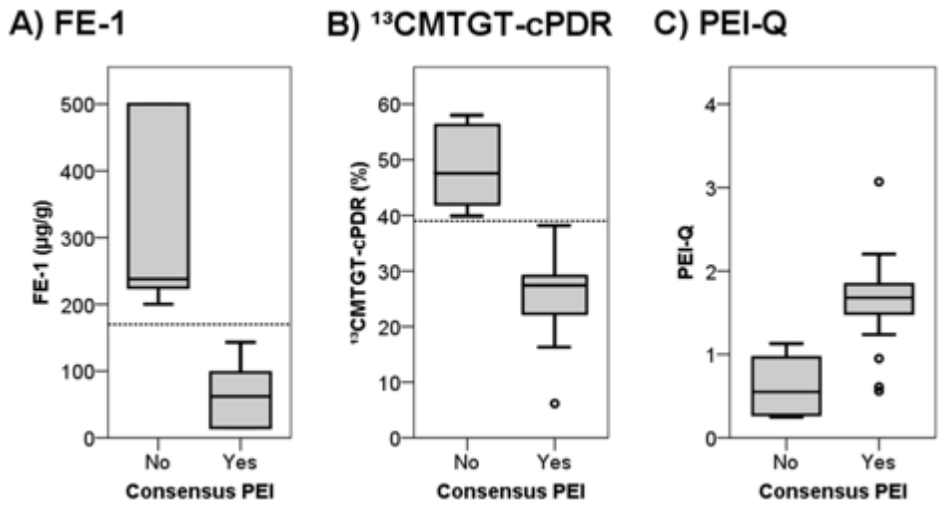
The distributions of each of PEI assessment for the cohort as a whole, as well as by PEI status are reported in **Table 27**, and **Figure 35**. For FE-1, the pre-specified threshold of ≤200µg/g yielded a PEI rate of 82% (18/22). This threshold was consistent with the consensus diagnosis for all but one patient, who was deemed not to have PEI, despite an FE-1 of 200µg/g. The highest observed FE-1 in a patient with PEI was 143µg/g; hence, a threshold within the range 143-200µg/g (e.g. 170µg/g) would result in concordance with the consensus diagnosis of PEI for all patients in this cohort.

Table 27 PEI assessments.

	Whole Cohort		**Consensus PEI Diagnosis**	
	N	***Statistic***	***No***	***Yes***
FE-1 (µg/g)	22	92 (21, 143)	238 (225, 500)	62 (15, 98)
<100 (Severe PEI)		13 (59%)	0 (0%)	13 (76%)
100-200 (Mild-Moderate PEI)		5 (23%)	1 (20%)	4 (24%)
>200 (Normal)		4 (18%)	4 (80%)	0 (0%)
^{13}CMTGT-cPDR (%)	25	27.8 (25.7, 31.1)	47.6 (42.0, 56.3)	27.4 (22.3, 29.1)
≤29% (PEI)		15 (60%)	0 (0%)	15 (75%)
>29% (Normal)		10 (40%)	5 (100%)	5 (25%)
PEI-Q	25	1.55 (1.13, 1.82)	0.55 (0.28, 0.97)	1.68 (1.49, 1.84)
<0.60 (Normal)		3 (12%)	2 (50%)	1 (5%)
0.60-1.39 (Mild PEI)		6 (24%)	2 (50%)	4 (19%)
1.40-1.79 (Moderate PEI)		8 (32%)	0 (0%)	8 (38%)
≥1.80 (Severe PEI)		8 (32%)	0 (0%)	8 (38%)

Average values are reported as median (interquartile range). Each assessment is also divided into categories based on the predetermined diagnostic thresholds, with the proportion of patients in each category reported.

Figure 35 Association between PEI assessments and consensus PEI diagnosis.



Points indicate outliers as defined by Tukey's Fences method, namely cases that were above the upper quartile or below the lower quartile by >1.5 times the interquartile range. Broken lines are plotted at the thresholds that perfectly divide the consensus PEI and non-PEI groups in this cohort, namely FE-1 = 170µg/g and ^{13}CMTGT-cPDR = 39%; this was not possible for PEI-Q.

For ^{13}CMTGT-cPDR, the pre-specified threshold of ≤29% yielded a PEI rate of only 60% (15/25). Whilst all N=15 patients below this threshold had a consensus diagnosis of PEI, 50% (5/10) of those with "normal" ^{13}CMTGT-cPDR also had a consensus diagnosis of PEI, implying that the threshold was too low. The highest observed ^{13}CMTGT-cPDR in the consensus PEI group was 38.2%, with 39.9% being the lowest in the non-PEI group. As such, moving the threshold to be within this range (e.g. ≤39%) would result in concordance with the consensus diagnosis of PEI for all patients in the cohort.

Finally, for PEI-Q, the pre-specified threshold score of ≥0.6 resulted in a PEI rate of 88% (22/25). This threshold was inconsistent with the consensus PEI diagnosis in N=3 patients, with one patient having a consensus diagnosis of PEI despite a score of 0.56, and two patients being in the non-PEI group despite scores of 0.80 and 1.13, respectively. Unlike the other two assessments, it was not possible to define a diagnostic threshold for PEI-Q that would be concordant with the consensus diagnosis of PEI in all cases.

4.5.4 Summary of results: examining PEI in the longer term after PD or PPPD

The aim of examining PEI reference test in this sub-cohort was to determine the incidence of PEI in the years following PD for oncologic indication. The key finding is that patients after oncologic PD have a long term (median 15 months) incidence of PEI of 80% Additional findings are that both pre-operative pancreatic duct width and pre-operative BMI are associated with post-operative PEI, the ^{13}C MTGT an FE-1 results correlate well but the PEI MSS does not, and those with PEI show a much greater weight loss than those without, despite all being treated with PERT. For full discussion of these results see discussion chapter, **section 4.2.1.**

4.6 Correlation between diagnostic modalities (whole cohort)

Using a multi-modal approach to defining PEI status in recruits was at the centre of the DETECTION trial as none of the 3 modalities currently available (PEI-Q, ^{13}C MTGT and FE-1) are robust enough to rely wholly on. Using an expert multi-disciplinary panel reviewing both the results of the ^{13}C MTGT, FE-1 And PEI-Q, and the more subjective picture of response to PERT hold and CT findings, recruits have a robust, accurate assessment of their PEI status. 43 patients with pancreatic cancer were recruited to the DETETION trial giving a unique opportunity to examine the correlation between diagnostic modalities. For examination of correlation, the 2 ^{13}C MTGT cPDR results from patients with liver metastasis and suspected spurious ^{13}C MTGT results have been removed, as has the single FE-1 outlier results that was also suspected to be spurious .

Correlation between individual test and overall PEI status was examined. Both the ^{13}C MTGT cPDR and FE-1 yielded excellent negative correlation (Spearman's test) with overall PEI status (rho: -0.72 and -0.76 respectively), and although there were a handful of cPDR results from the PEI group that were above the accepted threshold for PEI, there was no cross over between

the PEI and no PEI groups, and no patients without PEI fell below the diagnostic threshold. The PEI-Q MSS (mean symptom score) however showed only a moderate correlation with overall PEI-Q status and there was significant cross over between the 2 groups with N= 4 (44%) of those without PEI had a score indicative of at least mild PEI. Correlation between diagnostic modalities found a very strong, statistically significant, positive correlation between ^{13}C MTGT-cPDR and FE-1 (rho: 0.70, p=0.000) but only a moderate correlation between PEI-Q and either FE-1 or ^{13}C MTGT-cPDR (rho 0.39 and 0.33 respectively) See **Fig 36, 37 and 38** .

Figure 38 Association between FE-1 and 13C MTGT-cPDR, very strong correlation, Rho: 0.70.

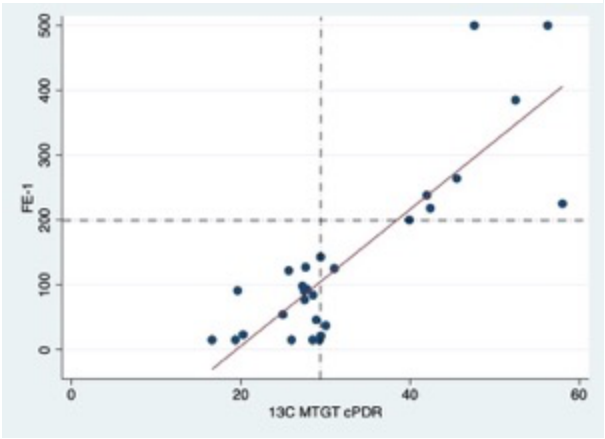


Figure 37 Association between FE-1 and PEI-Q MSS, moderate correlation, Rho: 0.39.

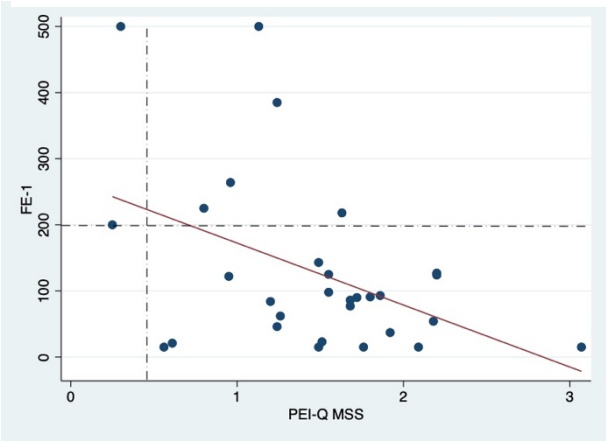
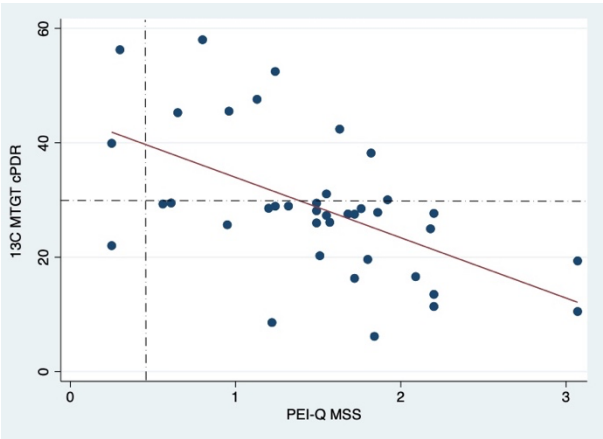


Figure 36 Association between 13C MTGT-cPDR and PEI-Q MSS, moderate, Rho: 0.33.



4.7 Discussion of results chapter: PEI status and demographics of recruited cohorts

4.7.1 Patient recruitment

The concept for the DETECTION trial was originally born out the problems around PEI diagnostics in pancreatic cancer. Therefore, recruitment for the main body of work was aimed at pancreatic cancer patients at any point in their cancer journey.

The protocol was carefully thought through to enable smooth delivery but along the course of the trial several barriers had to be overcome. It became apparent from the pilot work that healthy controls would have to be better matched as the metabolome is sensitive to both inherent, lifestyle and environmental factors. Traditional age, sex and race matching can be done with little difficulty, but tackling lifestyle and environmental factors is far more difficult. To overcome this as far as possible, the recruitment of patient partners, immediate family, or close friends to run as healthy controls alongside them was performed. This worked well and meant factors such as long-term dietary intake, exposure to environmental pollutants etc that are impossible to account for with standard matching would be as closely accounted for as possible.

Patient recruitment was challenging owing to age, frailty, and geographical factors: As a tertiary referral unit, many patients are not local, owing to the disease itself and the wider impact of its treatment, most patients were older and potentially frail. With the protocol requiring an overnight fast, an early morning attendance to UHB followed by a 6-hour starvation period and serial blood tests there were a significant number of patients who declined. This could potentially bias included patients towards those that were younger, fitter

and with a less aggressive pathology and thus likely to have less severe PEI. This should not lessen the significance of the results, but it is worth considering that the true incidence and severity of PEI is probably underestimated.

The COVID-19 Pandemic started a little way into recruitment and initially shut down the trial completely, on re-starting there were strictures in place for social distancing that limited recruitment speed and affected the analysis of faecal elastase samples. With slower than expected recruitment, although fully recruited for the main cohort of pancreatic cancer, the sub-study cohorts of chronic pancreatitis and cystic fibrosis are still incomplete. The advantage to running these cohorts after full examination of the main study cohort is that metabolomics can be run in a more targeted fashion, targeting a refined metabolomic panel which is both cheaper and results in a more accurate analysis.

Overall, recruitment of the main cohort, although slower and having faced a few problems along the way was satisfactory. A good spread of patients across mild to severe PEI, unresected and resected, and a reasonable number of patients with pancreatic cancer but no PEI have been successfully recruited. As discussed previously the rapid work up for pancreatic cancer and the intense nature of chemotherapy meant that it was more difficult than expected difficult to recruit pre-operatively and not appropriate to recruit during adjuvant chemotherapy.

4.7.2 Ongoing recruitment

Current active recruitment is aimed at cystic fibrosis patients, these are proving an easier, more consistent cohort to recruit with a generally younger, fitter population that are interested in their pancreatic function and more willing to engage in research. The aim is to recruit 15 patients with CF to act as a validation cohort but with additional funding this could potentially

be increased. The diagnosis of PEI is less difficult in this cohort as they have serial FE-1 measurements taken as part of their routine care and, unlike in pancreatic cancer this is likely to be reflective of their underlying disease state. With existing results as a template and the end goal of a short test for clinical use the blood sampling time frame can be reduced to 4 hours. Ultimately this cohort should be quicker and easier to recruit. Unfortunately, having identified the problems with healthy control matching, ideally a new set of healthy controls selected from their partners, friends and family is required. Discussions are underway as to whether a small validation cohort with existing ethics and funding is enough, or whether the ethics should be amended, and further funding sought to enable larger scale recruitment alongside matched healthy controls. A question for future research and of great interest would be whether this work could be adapted to run a similar trial in infants, there is increasing evidence to show that early treatment with CREON and disease modifying drugs has the potential to significantly improve outcomes. The main hurdle would be to modify the test meal, from previous work done on the ^{13}C MTGT work that breast milk and formula produce an adequate pancreatic challenge to evaluate enzyme response, however it is unlikely that the metabolomic findings resultant from the complex nutritional supplement (as used in work to date) would be reflected in the response to just milk. This body of work would require careful planning, new ethics and a dedicated researcher and is outside the scope of my current work.

Recruitment of patients with chronic pancreatitis to perform the study day on the days on 3 different doses of PERT (as per protocol – dosing sub-study) to evaluate whether the metabolome could be returned to 'normal' with adequate treatment has proven unsuccessful so far with patients showing initial interest but then becoming uncontactable, unwilling to return for sequential days or unwilling to participate at all. There are several potential factors underlying this, identification of chronic pancreatitis patients has been via the inpatient lists

which has meant most have an active problem and although showing inpatient interest on the ward, once discharged they rarely show continued interest and often do not attend. Several patients have been put off by the need for smoking cessation for 24 hours and many said that they would not be prepared to starve for the test duration. I am uncertain why the responses are so different from the pancreatic cancer, NET, and cystic fibrosis cohorts but recruitment for this group would potentially benefit from a targeted PPIE group review and from a different approach to patient identification. Approach via gastroenterology team outpatient clinic lists could be potentially more fruitful as these patients are likely to be more stable, and less likely to not attend as they are already engaging in the outpatient system.

4.7.3 Incidence of PEI in patient having undergone PD

The aim of examining this cohort independently was to determine the incidence of PEI in the years following PD for oncologic indication. The key findings of this work are an 80% incidence of PEI long-term following oncologic PD, that pre-operative pancreatic duct width and lower BMI correlate to post-operative PEI, and, despite supposedly 'adequate' PERT treatment, those with PEI have a significantly lower BMI in the long-term. Examining the correlation between diagnostic modalities; the ^{13}C MTGT and FE-1 results correlate well with each other, but the PEI MSS does not correlate well with either.

The two existing reviews of PEI incidence after PD show differing results with Tseng showing an incidence of 74% and Moore showing an incidence of just 43%. Both looked at post resection PEI in those undergoing PD, but Moore included those with no diagnostic test and just symptomatic assessment. Both reviews are limited by length of follow up, heterogeneity of included patients and problems with diagnostic modalities used.[51, 207] The median follow up time of the included studies is just 4 months. More recently, Hartman et al evaluated PEI 6

weeks following PD in 78 patients using the ^{13}C MTGT and found an incidence of 64.1%.[208] The timing of assessment is important with a large proportion of patient developing PEI at more than 90 days following PD, therefore a longer follow up period is essential to fully realise incidence.[209]

This cohort of DETECTION trial patients post resection have a median follow up of 15 months, using a multi-modal approach with expert review helped to ameliorate the limitations of individual diagnostic tests and this data and identifies a long-term PEI incidence of over 80%. In the absence of other appropriate tests, by using the ^{13}C MTGT breath test in this study, (supported by FE-1 and PEI-Q and assessed by a panel of experts), we believe that we have given the most practical and accurate assessment of PEI in long-term survivors after PD currently possible.

Unlike with bowel resection where the remaining bowel adapts to recover digestive function, these results indicate that PEI persists for years after pancreatic resection.[210] As previously discussed, there is a worrying disconnect between the incidence of PEI and PERT treatment. Despite campaigns highlighting the broad benefits of PERT and new NICE guidelines arising from the 2018 U.K national audit RICOCHET (which identified less than 75% of resectable pancreatic cancer patients had PERT prescribed), a 2023 OpenSAFELY research platform publication still found under 50% PERT prescribing in pancreatic cancer.[5, 211] This problem is reflected worldwide.[7, 8, 212]

The key limitation of this work was small sample size, especially for the non-PEI cohort. Therefore, there were wide confidence intervals for the PEI incidence estimates and comparisons of patient characteristics between the PEI and non-PEI cohort had potential for a

higher false-negative rate. Also, due to the nature of study requirement, recruits are non-frail, alive, and not on chemotherapy approximately 1-2 years post-resection, which likely represents a younger, fitter cohort with less aggressive pathology, thus potentially underestimating PEI incidence.

To summarise, existing literature shows that PEI is a significant problem after PD, especially for malignancy, but longer-term incidence has previously been poorly investigated with reviews limited by short follow up and heterogenous diagnostic techniques. This sub-study of the DETECTION trial employs a multi-modal approach to diagnosis and shows an incidence of over 80% at a median of 15 months post pancreatic head resection. The take home from this is that PEI is prevalent and sustained after pancreatic head resection, patients should remain on PERT lifelong, and all healthcare professionals involved in any stage of care must remain attentive to ensure correct PERT prescribing and compliance.

4.7.4 Incidence of PEI in NET patients starting Somatostatin analogues

Iatrogenic exocrine inhibition in the form of somatostatin analogues (SSAs) is a poorly investigated area, partly because NETs are uncommon, and partly because PEI diagnosis and treatment is especially challenging in these patients as the systemic symptoms of NET disease can overlap with those of PEI. University Hospitals Birmingham is a tertiary referral centre for NET patients, performing multiple points of clinical contact both before and after starting SSAs. Therefore, an opportunity arose to investigate PEI before and after SSA initiation. The key finding in this sub-group was that 8 weeks after commencing SSAs, all patients showed a reduction in pancreatic exocrine function. However, despite a clear drop in exocrine function, the PEI-Q demonstrated an improvement in mean symptom score after patients commenced SSAs, showing that symptom assessment alone is not a valuable diagnostic tool in this cohort.

This is likely due to the symptom overlap between PEI and systemic NET disease, with the NET symptom burden prevailing. On the ground recruitment of this cohort was run, in part by an iBSc student LH, under my supervision, whilst breath analysis and interpretation were done by myself. This work has been published in a peer reviewed journal (**See Appendix 9, PMID: 37046594**).

The literature around SSA-induced PEI incidence in NETs is poor with only 4 studies prior to this body of work, these studies are less than ideal with questionable diagnostic techniques and timepoints, however they suggest that PEI occurs between 2.9 and 6.5 months after SSA initiation, at a rate of about 20% (range 12 to 40%).[63-66] With existing PEI diagnostic modalities being less than ideal, many clinicians rely on symptom assessment alone (bloating, urgency, diarrhoea and steathorrhoea), unfortunately, owing to symptom overlap with systemic NET disease, a more objective measure is required for NET patients.[213] The next most common routine diagnostic strategy is FE-1, however, this requires solid stool for assay and with diarrhoea being prevalent in this cohort, FE-1 is often not possible, this is reflected in our cohort with 25% of samples not being solid enough to assay. The use of the ^{13}C MTGT for assessing PEI in this cohort has not been previously described, there are no theoretical reasons why it should not reflect the true exocrine state in these patients, and it has the advantage of not relying on stool or symptomatic assessment. The ^{13}C-MTGT allowed sequential quantification of exocrine function rather than a simple dichotomous Yes/No. All 10 recruits demonstrated a drop in exocrine function from before, to 8 weeks after initiation of SSA therapy, with a median drop in function of −23.4% (−42.1–0.5) ($p = 0.005$). These results indicate that not only does SSA therapy have a consistent impact on exocrine function, but that it occurs much earlier in the disease process than previously thought. The relevance of this is difficult to interpret and although patients acted as their own controls, the ^{13}C MTGT has never

been used in this group previously. The incidence of patients below the recognised diagnostic threshold for PEI was so much lower than in pancreatic cancer that the same 'treat all' policy for PERT would be difficult to apply (despite it being highly likely that all will experience a drop in their exocrine function, a reasonable proportion may not meet the criteria for PEI). The wider literature on PERT treatment for PEI has proven repeatedly that PERT improves quality of life, clinical outcomes and even survival in other groups. Other work from our own research group has also suggested that PERT may convey a survival advantage specifically in NET patients starting SSAs.[67] However there is very little literature around which patients on SSAs should receive PERT, what dose they should receive and what the benefits are.

To conclude, SSA initiation in NET patients consistently induces an early decline in exocrine function, but the diagnostic and treatment strategy remains unclear. This work has further shown the limited use of FE-1 and symptom assessment in this cohort, and the ^{13}C-MTGT, although accurate is too cumbersome and time-consuming to roll out routinely following SSA initiation. More work is needed to investigate how and when PERT should be prescribed for this cohort. As follow on from this work, our research group is designing a trial of PERT vs placebo, The PrEPARe Trial: anticipatory PErt prescription in Patients commencing ssA theRapy: A placebo-controlled, randomised, two-arm crossover trial that will hopefully answer some of these questions.

Regarding metabolomics in this cohort, all 10 NET patients have frozen plasma samples prepped for metabolomic assessment, however with the metabolomic profile for PEI in its infancy, the numbers would be too low for a meaningful comparison therefore they will be stored until the profile has been validated in a larger cohort of patients that are all exocrine insufficient (likely to be the cystic fibrosis cohort, recruitment underway).

4.7.5 Value of a multi-modal approach, correlation between diagnostic tests and limitations.

Early in the development of The DETECTION trial it became apparent that using a multi-modal approach to defining PEI status in recruits would be beneficial. None of the 3 modalities currently available (PEI-Q, ^{13}C MTGT and FE-1) are robust enough to rely wholly on (one of the reasons this body of work is being undertaken). The PEI-Q is subjective, has poor specificity, is not well validated outside of chronic pancreatitis, and many of our recruits may have other pathology that could confound the results (diarrhoea, SIBO, Bile salt malabsorbtion e.t.c). The ^{13}C MTGT is extremely sensitive to external factors, does not have a universally agreed protocol, and again is not well validated in our main cohort (pancreatic cancer). FE-1 has been shown to be of low sensitivity following pancreatic resection and there are problems with assaying loose samples. However, by combining all three of these, reviewing patients CT scans, assessing their response to stopping PERT, and having experts in the field to discuss borderline cases, I believe we have been able to give an accurate assessment of PEI status. It has also resulted in some interesting data on correlation between diagnostic tests. 43 patients with pancreatic cancer were recruited to the DETECTION trial and all underwent the above-described panel to determine PEI status, this gave a unique opportunity to examine the correlation between diagnostic modalities.

Correlation between individual tests and overall, PEI status showed excellent negative correlation for both FE-1 and ^{13}C MTGT-cPDR, and overall PEI status. For the ^{13}C MTGT there were a handful of patients that were above the accepted threshold for PEI but still classed as PEI, however there was no cross over between the PEI and no PEI groups, and no patients without PEI fell below the diagnostic threshold. From the other results assessing response to PERT hold and CT review there is little doubt that despite not meeting the standard diagnostic threshold that these patients had PEI. Either there is something about these individuals or their

test that has created spuriously high results, or the threshold for PEI in this cohort should be raised. The former is more likely, with the ^{13}C MTGT being extremely sensitive to external factors it is possible that these patients either had an inherent factor in their physiology that increased *V*CO2 or something on the day artificially raised their *V*CO2 resulting in an earlier recovery of ^{13}C despite the strict control measures employed. FE-1 data was clearer cut, again, there was excellent correlation between FE-1 results and overall decision on PEI-status and the accepted diagnostic threshold reflected status for all but one patient who had a normal FE-1 but was exocrine insufficient on all other assessments. Correlation between FE-1 and ^{13}C MTGT-cPDR was also very strong.

The results of the PEI-Q in this cohort were inconsistent with nearly half of those without PEI meeting the diagnostic threshold for at least mild PEI on their MSS, only moderate correlation between PEI-Q MSS and overall PEI status and only moderate correlation between the PEI-Q and both ^{13}C MTGT-cPDR and FE-1. To summarize, in this cohort, the ^{13}C MTGT and FE-1 results correlate well with overall PEI status as well as with each other, but the PEI-Q MSS does not. This work suggests that employing the 13C MTGT and FE-1 will give an accurate reflection of PEI status but that using the PEI-Q in this cohort is not useful in determining status.

Chapter 5 : Biological interpretation of metabolomic analysis.

5.1 Summary of chapter 5

The DETECTION trial compared participants with clearly defined PEI and matched healthy controls for metabolomic analysis using Ultra High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS).

This chapter addresses the biological results of metabolomic analysis, using UHPLC-MS. A summary of key pilot data is followed by results from the main cohort analysis (pancreatic cancer with PEI *vs.* controls with no PEI). Three types of samples are referred to: biological samples (BS), quality control samples (QC) and process blank samples (PB). Samples have been analysed in two batches using two complimentary UHPLC-MS assays: a HILIC assay (to study water soluble metabolites) and a lipidomics assay (to study lipids). After reducing and cleaning the data into a filtered data matrix, more traditional statistical techniques have been applied.

The following sections are described below:

- 5.2 UHPLC-MS of pilot samples
- 5.3 Creating a quality filtered data matrix for main cohort analysis (PEI vs No PEI)
- 5.4 Comparing baseline samples (main cohort)
- 5.5 Examining time related changes independent of phenotype (main cohort)
- 5.6 Discussion of results chapter 5, biological interpretation of LC-MC main cohort results

5.2 UHPLC-MS analysis of pilot samples

As described in methods section 2.3.1, five patients with clinically likely PEI (no reference tests, but all with clinical symptoms indicative of significant PEI, and scans consistent with a head of pancreas cancer) and five healthy controls were recruited. All pilot recruits had baseline blood taken, consumed the same fatty test meal and then had hourly bloods collected up to 4 hours post-meal. The plasma samples underwent UHPLC-MS untargeted HILIC and lipid assays in a single batch. Raw data was processed using XCMS, and putative metabolite annotation was performed using the software BEAMS. (see methods section 2.4) A two-way ANOVA applying time and phenotype (PEI vs Control) as the two factors was performed. Box and whisker plots have been generated for the most biologically relevant metabolites. The description of the results has been kept to a minimum as these recruits were not well controlled.

5.2.1 Changes with time, independent of phenotype

35 unique metabolite features demonstrated a statistically significant difference across time with correction for multiple testing, ($p<0.05$). The main metabolite type perturbed postprandially were water soluble (as detected in the HILILC assay). See **Figure 39,** these changes were independent of phenotype and confirm that the challenge meal is having a significant effect on the course of post-prandial metabolites, regardless of phenotype.

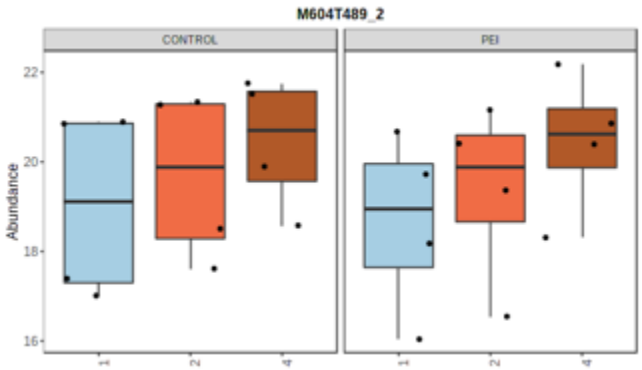


Figure 39 Changes in abundance over time, HILIC assay metabolite, pilot cohort, shown for both PEI and control.

5.2.2 Changes with phenotype, independent of time

Independent of statistically significant changes in time, 3 unique metabolite features demonstrated a statistically significant difference between PEI and control classes with correction for multiple testing ($p<0.05$). **See Figure 40 and 41** for visualization of examples from both HILIC and lipidomic assays with box and whisker plots.

Figure 41 Changes in abundance over time (Lipids positive assay) for a triacylglyceride, PEI vs Control

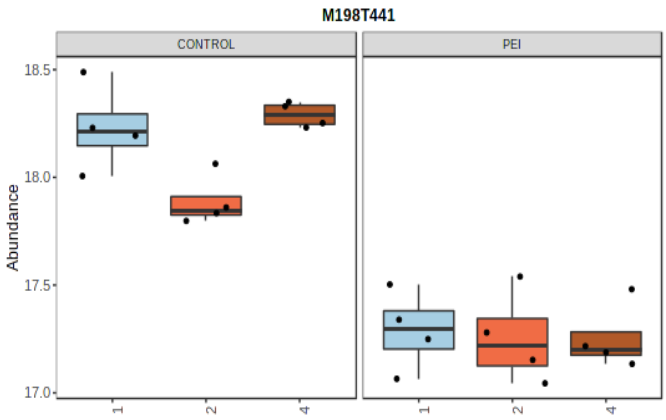
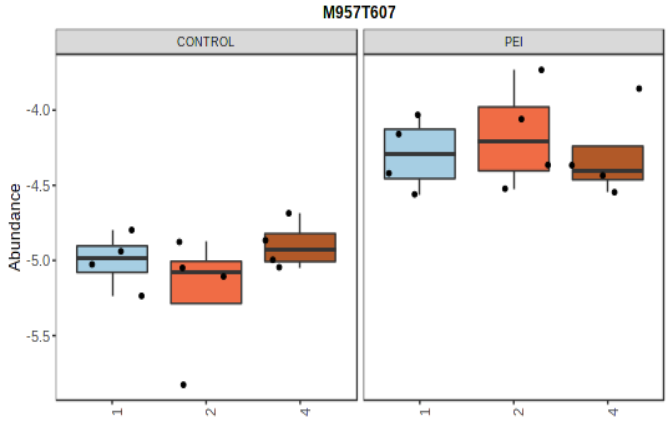


Figure 40 Changes in abundance over time (HILIC negative assay), pilot cohort, shown PEI vs Control.



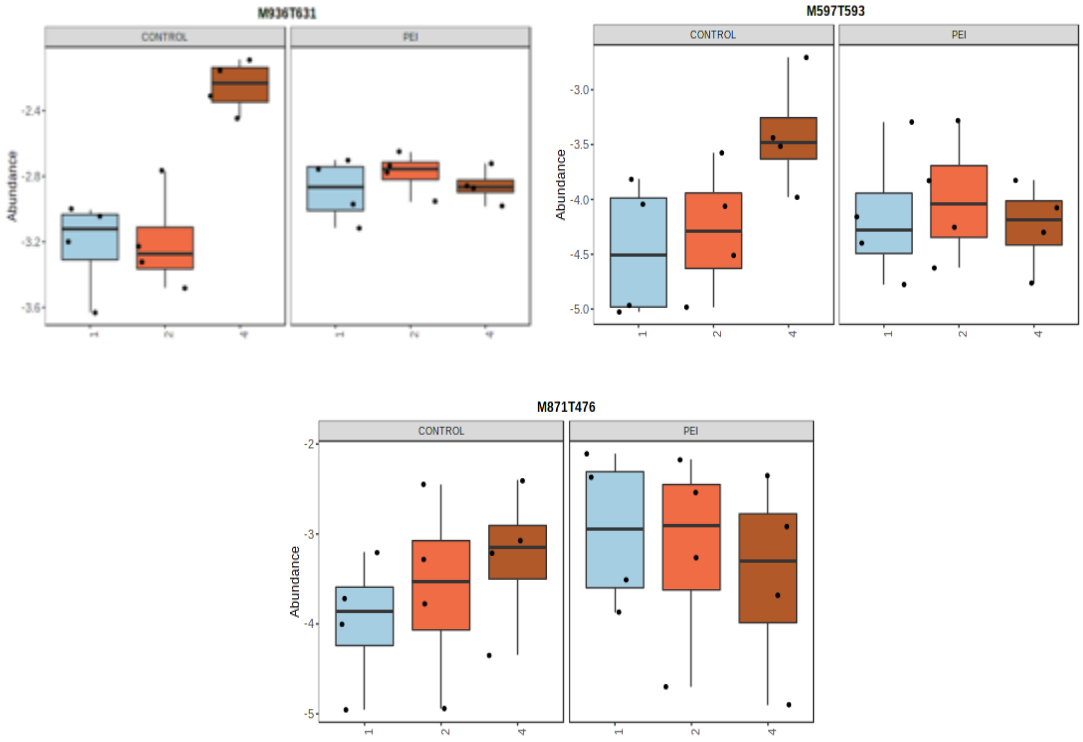
These changes are independent of time and the major type of metabolite perturbed are lipids especially triacylglycerides and diacylglycerides. As they are independent of time, these changes are difficult to interpret, that they are present at baseline shows there is a significant difference in these between our patient group and our recruited cohort. They could therefore suggest that those with PEI have a baseline difference but confounding factors with recruitment of this pilot data are possible .

5.2.3 Interaction of phenotype and time

This evaluates whether the post-prandial course of metabolites in the disease cohort is different to that of the control cohort. 5 unique metabolite features demonstrated a statistically

significant difference between PEI and control classes with correction for multiple testing ($p<0.001$), these are visualized in the below box and whisker plots, **Figure 42 (lipid assay metabolites).**

Figure 42 Changes in abundance over time (Lipids positive assay) for a triacylglyceride, a diacylglyceride and a glycerophospholipid, PEI vs Control.



The main limitations of this control cohort were that they were young, fit individuals not well matched to the disease cohort. Fasting time and time of meal administration were also not well controlled.

To summarise, this pilot data supported the hypothesis that the post prandial metabolme would differ between PEI and no PEI and enabled further funding to be obtained. A larger, better controlled, cohort would be examined in more detail within the main cohort samples.

5.3 UHPLC-MS analysis of main cohort : Creating a quality filtered data matrix.

As described in **section 2.2.4,** control recruitment for the main cohort was age matched, and sought to overcome differences in external factors by recruiting the partners or close friends and family of disease recruits. The final cohort constituted 34 patients with pancreatic cancer and PEI to be compared against 33 healthy controls and 9 patients with pancreatic cancer and no PEI. For cohort characteristics see **results section 4.3.1**. Each recruit had baseline and 4 post-prandial samples taken hourly, giving a total of 380 samples for analysis, these were stored at -80°C until recruitment was complete and samples were ready for analysis. Each biological sample, quality control sample and process blank sample were analysed using a HILIC assay to study water-soluble metabolites and a lipidomics assay to study lipids. Changes at baseline, changes between phenotype alone and the interaction between time and phenotype will be discussed in turn.

After XCMS raw data processing, quality control techniques were employed (described in **Methods section 2.3.4**) to construct a quality-filtered data matrix for each assay. The resultant data matrix is essentially a list of metabolite features with their relative concentrations, for each sample, from each assay, that has been filtered for outliers and adjusted for analytical problems (such as drift of signal intensity over time). PCA scores plots were used after initial data reduction and cleaning to examine clustering of variables, and remove outlying or erroneous samples, these are presented below for each assay. As the QC samples are the same sample analysed multiple times they should be more tightly clustered in the PCA scores plots when compared to the biological samples.

PCA scores plots showed good tight clustering of QC samples (green) compared to BS samples (pink) for batch 1 but for the batch 2 lipids negative assay there was a small outlying QC and BS cluster, these samples were removed from the dataset prior to statistical analysis.

5.3.1 Lipidomics assay – negative and positive ion mode, removing outliers

Figure 46 Lipids negative assay; QC and BS batch 1, Pre-signal correction. BS 50% filter, QC 70% filter.

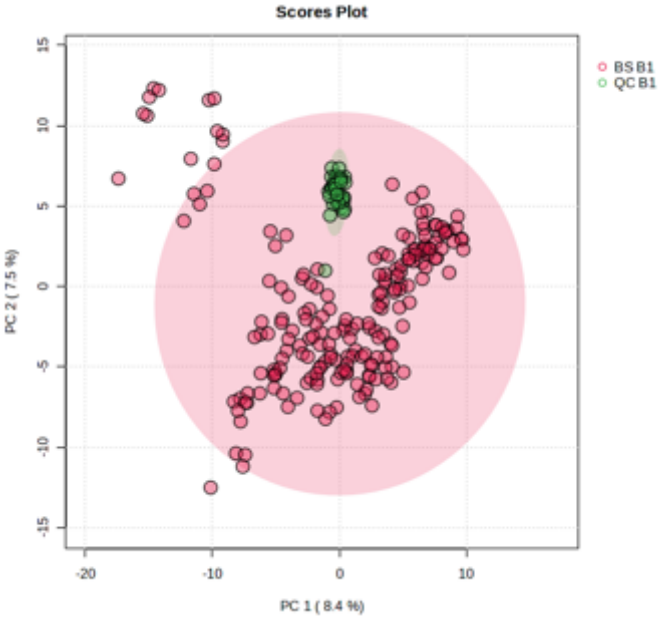


Figure 46 Lipids negative assay; QC and BS Batch 2, Pre-signal correction, BS 50% filter, QC 70% filter. first samples of batch removed batch 2 reduced.

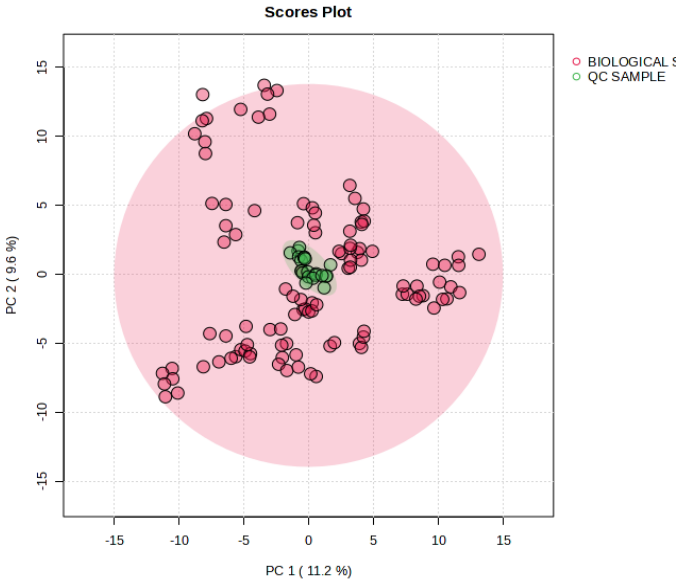


Figure 46 Lipids positive assay; QC and BS batch 1, pre-signal correction. BS 50% filter, QC 70% filter.

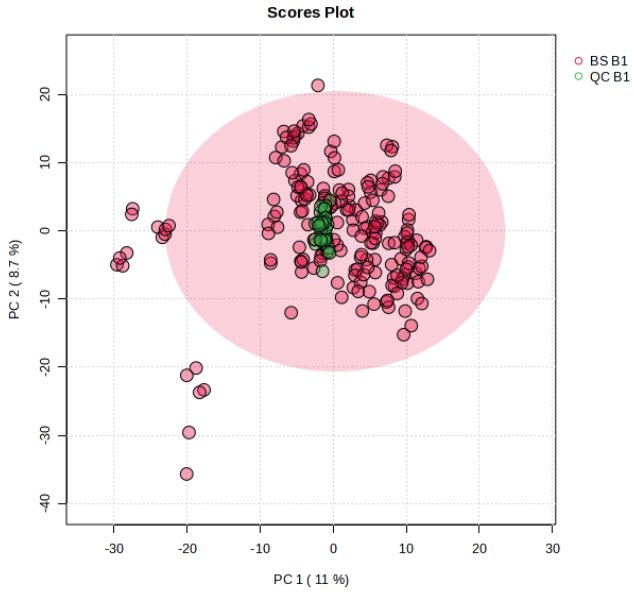
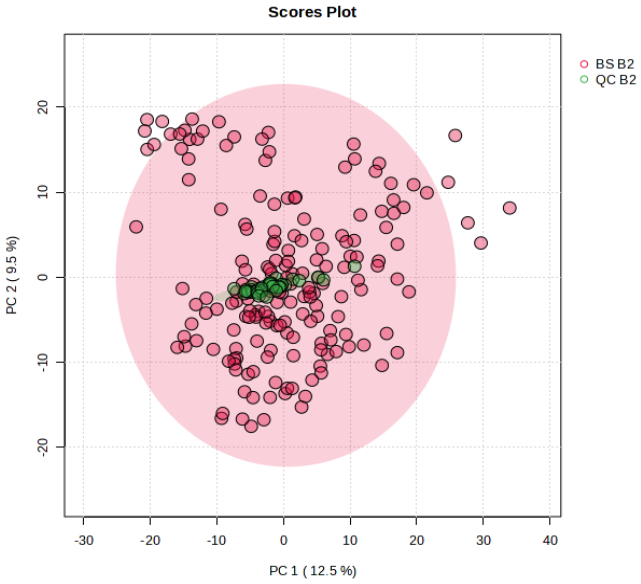


Figure 46 Lipid positive assay; QC and BS batch 2, pre-signal correction. BS 50% filter, QC 70% filter.



5.3.2 HILIC assay – negative positive ion mode, removing outliers

Figure 50 HILIC assay, negative ion mode, batch 1, pre-signal correction, after filtering.

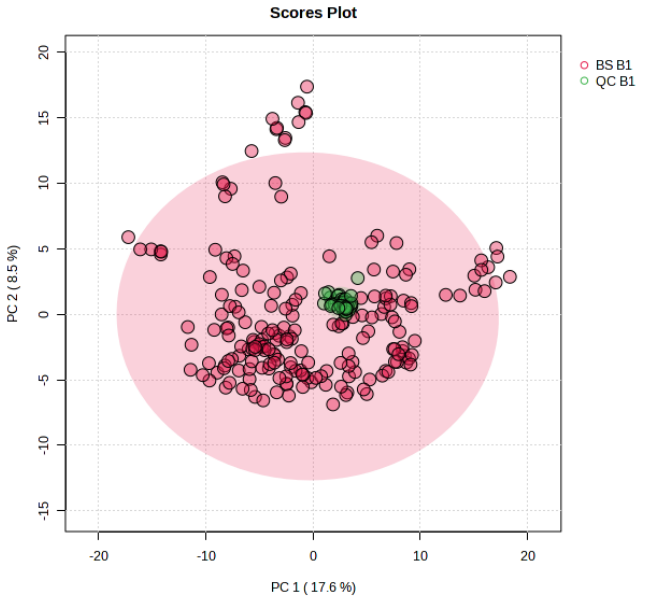


Figure 50 HILIC assay, negative ion mode, batch 2, pre-signal correction, after filtering.

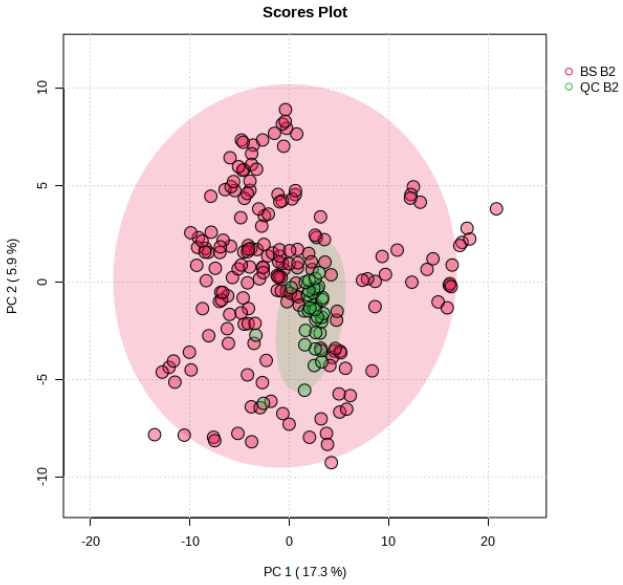


Figure 50 HILIC assay, positive ion mode, batch 1, pre-signal correction, after filtering.

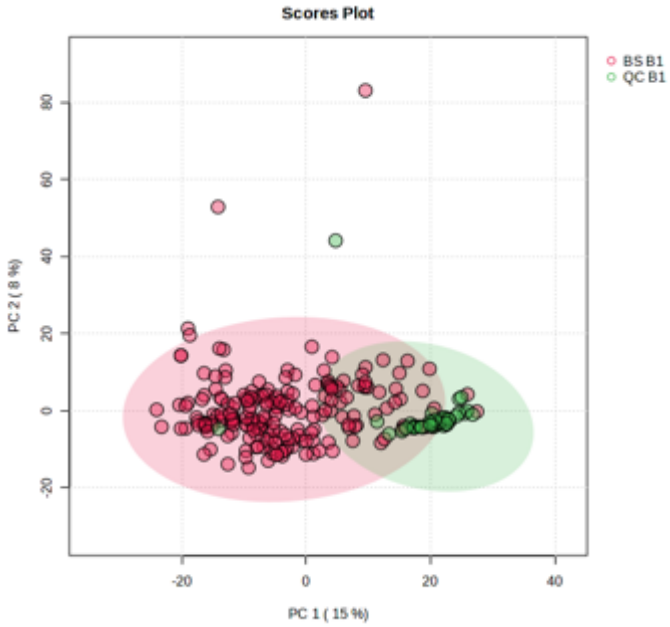
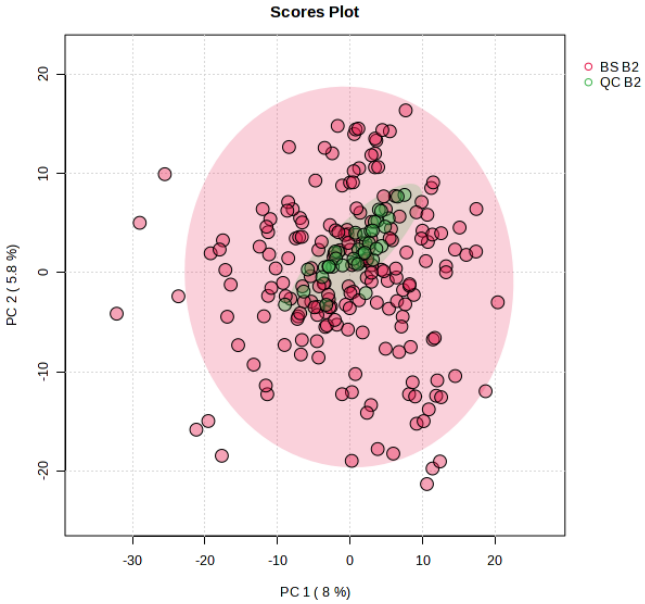


Figure 50 HILIC assay, positive ion mode, batch 2, pre-signal correction, after filtering.



5.3.3 Final dataset

Table 28 defines the number of metabolite features pre- and post- filtering/quality control in the data matrices for each assay (HILIC positive/negative, Lipids positive/negative) for each batch (one and two). A significant number had to be removed from the HILIC negative batch 2. See section **2.3.4** for data filtering and reduction methods.

Table 28 Number of metabolite features after quality control and data filtering for each assay and batch.

Assay	Before filtering	After filtering
HILIC negative		
Batch 1	5938	1469
Batch 2	5938	80
HILIC positive		
Batch 1	11274	3795
Batch 2	11274	4048
LIPIDS negative		
Batch 1	7921	1607
Batch 2	7921	1651
LIPIDS positive		
Batch 1	9694	2446
Batch 2	9694	2964

* *HILIC*= Hydrophilic interaction liquid chromatography

5.4 Significant differences at baseline between phenotypes

A one-way ANOVA of baseline (pre-test meal, T=0hrs) data for all three phenotype classes (Pancreatic cancer and PEI, healthy controls, and pancreatic cancer no PEI) was performed. Results were reported after correction for multiple testing (Benjami-Hochberg) with $p<0.05$ being considered statistically significant. 36 statistically significant metabolites were observed with the most statistically significant metabolite holding a p-value of 0.0012. All of these are from the HILIC positive assay, with no consistent class of metabolite represented.

To conclude, there are minimal metabolic differences at baseline between the disease and control population, suggesting that firstly our populations are well matched and secondly that

significant class differences represented post-prandially are more likely to be resultant from exocrine function rather than pancreatic cancer itself. See **Appendix 7** for the 15 most significant metabolites from this analysis.

5.5 Changes with time, independent of phenotype.

Independent of statistically significant changes in time, 659 unique metabolite features demonstrated a statistically significant difference across time, independent of phenotype (PEI *vs.* No PEI). All results are reported after correction for multiple testing (Benjami-Hochberg) with significance set at $p<0.05$. 659 metabolites reach significance at $p<0.05$, 517 metabolites at $p<0.01$, 396 at $p< 0.001$ and 316 at $p<0.0001$. The top 65 most significant metabolites are all at $p<0.00000000001$.

This is a vast number of changes and is encouraging as it shows that that regardless of phenotype, the challenge meal used is creating very significant changes in the measurable blood metabolome.

5.6 Interaction between phenotype and time.

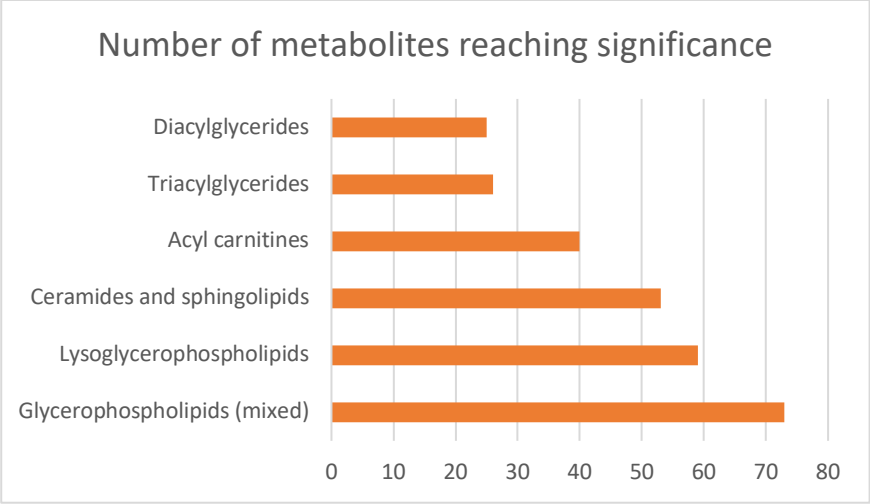
A two-way ANOVA analysis of all data for timepoints 0-5 (baseline to 4 hours) with two factors (phenotype (no PEI, PEI) and time) was performed. All results are reported after correction for multiple testing (Benjami-Hochberg) with significance set at $p<0.05$.

Looking at the interaction between time and phenotype, (i.e. those that show a statistically significant change in abundance over time between the 2 classes), there are 213 metabolites that reach significance at $p<0.05$, 139 metabolites at $p<0.01$, 77 at $p< 0.001$ and 43 at $p<0.0001$. The top 15 most significant metabolites are all at $p<0.00001$. This level of significance

indicates that over the course of 4 hours, those with PEI have a significantly different post-prandial response in a small number of metabolites than those without PEI. See **Appendix 8** for a list of the top 15 most significant metabolites from this analysis.

To identify which classes of metabolites overall are responsible for these differences, a pathway enrichment analysis (PEA) was performed for all metabolites with $p<0.05$. Firstly, a manual classification of lipid metabolites followed by grouping in to lipid classes was performed and any class with more than 20 metabolites present is likely to represent an important metabolic difference between the two phenotypes. **See Figure 51.**

Figure 51 Most relevant metabolite classes and number of metabolites within that reached significance



Secondly, pathway enrichment analysis was performed in the software MetaboAnalyst with the results shown in **Table 29.** Pyrimidine metabolism, cysteine/methionine metabolism and aromatic amino acid metabolic pathways were observed to be the most significant (match status defined as X/Y where X = number of statistically significant metabolites and Y = number of metabolites in metabolic pathway).

Table 29 Significant pathways and the number of metabolites within those pathway metabolite lists that reached significance

Pathway Name	**Match Status**	**p-value (FDR corrected)**
Pyrimidine metabolism	12/39	0.00018945
Cysteine and methionine metabolism	10/33	0.0005958
Phenylalanine metabolism	5/10	0.0033096
Tryptophan metabolism	10/41	0.0033096
Glycine, serine and threonine metabolism	8/33	0.010165
Pantothenate and CoA biosynthesis	6/19	0.010165
Phenylalanine, tyrosine and tryptophan biosynthesis	3/4	0.0104480000000
Alanine, aspartate and glutamate metabolism	7/28	0.0135380000000
Nicotinate and nicotinamide metabolism	5/15	0.0151900000000
Histidine metabolism	5/16	0.0190680000000

5.1 Discussion of chapter 5, biological interpretation of LC-MC main cohort analysis

To summarise, a one-way ANOVA performed at baseline showed minimal significant differences between recruited phenotypes and no significant differences in overall metabolite classes. From this it can be inferred that our disease and healthy control recruits are well matched. Minimal baseline difference also gives confidence that the post-prandial changes that follow are likely to be related to exocrine status rather than background health and disease of the recruits.

A two-way ANOVA looking at the interaction between phenotype and time (i.e. how the post-prandial changes in those without PEI compare to those with PEI) show that there are significant class differences, the most important of which are glycerophospholipids (in particular, lysoglycerophospholipids), ceramides and sphingolipids, and acyl carnitines.

5.6.1 Lipid class significance

Glycerophospholipids were the largest class represented as significantly different between those with and without PEI. These represent glycerol-based phospholipids and are the main structural component of biological membranes. Their metabolism involves the addition/transference of fatty acid (FA) chains to a glycerol resulting in lysophosphatidic acid (LPA) which is then acylated to become phosphatidic acid (PA). PA can then be dephosphorylated to diacylglycerol which is core to the creation of phosphatidylcholines (PC). PC can then be converted to other glycerophospholipids like phosphatidylserines (PS).

The most significant glycerophospholipids were lysoglycerophospholipids (e.g. LysoPG(O-18:0) which was one of the top ten most significant metabolites from the 2-way ANOVA comparing interaction of phenotype and time.) Elevated levels of glycerophospholipids have been identified in other disease states such as acute coronary syndrome and gastric cancer; however, the associated mechanism is poorly understood.[215]

The post prandial lipidome profile reflects the capacity to process a fatty meal, this is a complex balance of intra-luminal enzymatic activity (under scrutiny) to enable absorption then secretion and clearance of lipid-carrying compounds in the circulation. PEI, whether primary or secondary, results in defective lipolysis. This process is essential to the initial steps of lipid metabolism, generating FAs and glycerol for absorption and subsequent metabolism (as described above, FAs and glycerol are the basis of the glycerophospholipid class). The downstream metabolic pathways reliant on the absorption of these building blocks will inevitably be perturbed. Of note, there is good evidence of altered glycerophospholipid and FA (especially essential FAs) metabolism in cystic fibrosis with PEI being an independent risk

factor, this has been theorised to be due to the altered fat absorption increasing the ratio of bioavailable carbohydrate to fat leading to de novo synthesis of SFAs.[216, 217]

The changes seen here represent the early metabolic response to the given challenge meal, the significant differences in lipid classes could potentially form part of a diagnostic panel for PEI.

5.6.2 PEA – sub-pathway disturbance

Using pathway enrichment analysis (PEA), overarching metabolic pathways can be studied for significance, the results are presented as a level of significance (p-value) and 'match status' showing the proportion of metabolites reaching significance within that pathway. The top three most significant pathways and their potential links to this study cohort are discussed below.

5.6.2.1 Pyrimidine metabolism, match status 12/29, p=0.0001

Pyrimidines can be produced from amino acids *de novo,* or by salvaging circulating purines and pyrimidines. Glutamine (the key amino acid for de-novo pyrimidine synthesis), orotic acid (also part of de-novo synthesis) as well as two of the core extra-cellular pyrimidines uridine and cytidine were observed to be statistically significant. Glutamine is an abundant amino acid found in many food products such as seafood, dairy and nuts and it has previously been shown to be significantly reduced in patients with chronic pancreatitis.[218]

To know where in the metabolic process this difference has arisen is not possible. The challenge meal is dairy based and thus a potential source of glutamine; however, the consumption and synthesis of glutamine is a complex web of interactions involving various organs that can also influenced by a multitude of exogenous factors such as cancer, sepsis,

infections, and exercise. Glutamine is also an important precursor for gluconeogenesis, PEI affects glucose and lipid homeostasis in many ways; reduced capacity to break down carbohydrates for absorption, altered levels of circulating FFAs leading to insulin resistance and impairment of the incretin hormone response (which facilitates insulin secretion).[219] Ultimately, it would be impossible to tell exactly where in the pyrimidine metabolism the underlying pathology is having its effect, it could be directly related to reduced proteolysis and lipolysis from poor exocrine function, a more deep-rooted disturbance to glucose and lipid homeostasis, or a combination of all of these. As these metabolites came out as significant across time following a challenge meal and not at baseline, it is likely that they are related to post-prandial perturbance, however there is evidence in the literature for various oncologic pathways influencing pyrimidine metabolism.

5.6.2.2 Cysteine and methionine metabolism, match status 10/33, p=0.0005

Cysteine and methionine are essential amino acids that must be ingested and cannot be synthesised *de-novo.* Methionine metabolism is involved in a multitude of cellular functions including polyamine synthesis, folate metabolism and methylation reactions. Methionine is an intermediate in the biosynthesis of cysteine (AA), taurine (AA), carnitine (a transporter of FFAs), lecithin, phosphatidylcholine, and other phospholipids. Cysteine plays a crucial role in protein synthesis and enzyme catalysis.[220]

The level of methionine in the diet has been proven to have a big effect on methionine metabolism at a cellular level and a link has been established between nutrition, methionine metabolism and the resultant effect on health. The methionine pathway feeds into purine and pyrimidine synthesis (discussed above) and is crucial to glutathione synthesis and methylation

reactions. Methylation is one of the most prominent biological functions of this pathway, modulating things such as phospholipid integrity, signalling pathway activity and polyamine synthesis. Direct links between methionine metabolism and lipid metabolism have been shown; Methionine restriction increases de-novo lipogenesis, lipolysis, fatty acid oxidation and decreases triglycerides, insulin resistance and leptin levels, whereas hypermethioninemia provokes a reduction in serum LDL cholesterol.[221, 222]

The complex, multisystem interactions of this metabolic pathway are beyond the scope of this thesis, yet it is clearly significant in this cohort with a third of this pathway showing significant differences between those with PEI and those without in the post prandial metabolic response.

5.6.2.3 Phenylalanine metabolism, match status 5/10, p=0.003

Phenylalanine is an essential amino acid (cannot be made *de novo),* it is found in milk, eggs, chicken, beef, soyabeans and artificial sweetener. It is a precursor of tyrosine and monoamine neurotransmitters. 5 of the 10 compounds in this pathway were significantly different between those with PEI and without PEI. As the ingested challenge meal contained cow's milk protein this could be a potential source, with the differences between the cohorts theoretically arising from alteration in proteolytic activity in the intestinal lumen. Importantly, other amino acid pathways were also perturbed.

Interestingly, there has been only one other untargeted metabolomic study investigating exocrine insufficiency, this was a study in dogs with PEI, and found a significant difference in the phenylalanine sub pathway metabolites when comparing dogs with PEI to healthy controls.[223] Essential amino acid levels have also been investigated in patients with chronic

pancreatitis and a significant decrease in the serum concentration of essential and aromatic (phenylalanine, tyrosine, tryptophan and histidine) amino acids has been shown. The authors theorise that this is most likely due to the reduction in the pancreatic protease chymotrypsin (which catalyses peptide bonds contributed to by aromatic amino acids).[218]

5.7 Conclusions from biological interpretation of main cohort metabolomic analysis

This trial has recruited a cohort of patients with clear PEI and a well-matched control group. UHPLC-MS has delivered a vast quantity of data on the metabolome that must be interpreted with caution owing to the complexity of the underlying metabolic pathways.

With similar baseline findings it is likely that significant findings from analysing the interaction of time and phenotype relate to the post-prandial response of test subjects. Key findings on biological interpretation are that the overarching class of lipids differing between the cohorts is that of glycerophospholids and the metabolic pathways that are most different are pyrimidine metabolism, methionine and cysteine metabolism and phenylalanine metabolism. Significant differences in glycerophospholipid metabolism potentially results from reduction in intraluminal lipolysis reducing free fatty acids and glycerol available for the numerous lipid pathways but could also be contributed to by the more complex interaction of glucose homeostasis. The differences in pathway analysis are likely to relate to the reduction in intra-luminal enzymatic activity affecting availability of particular amino acids that feed into these pathways.

Chapter 6 Results: Identifying a potential biomarker panel for PEI

6.1 Summary of chapter 6

This analysis moves away from biological interpretation of metabolite pathways and classes, and focuses on identifying the most useful potential metabolites in identifying PEI from a meal challenge test. Chapter 5 shows minimal changes at baseline between PEI and no PEI subjects and a significantly different pattern of post-prandial metabolites in those with PEI compared to those without PEI. However, a test lasting 5 hours and requiring multiple sampling points is time consuming, costly, and likely unacceptable to patients. This chapter first looks at which time points independently show the most biological differences between those with PEI and those without and then explore the most significant metabolites at those timepoints to identify a single metabolite or panel of metabolites which show most promise for diagnosing PEI. Finally, the sensitivity and specificity of combinations of the most promising metabolites/metabolite panel will be evaluated.

- 6.2 Analysis of biological differences at 2 and 3 hours post-prandial
- 6.3 Sensitivity and specificity of a metabolite panel for identifying PEI
- 6.4 discussion of chapter 6

6.2 Analysis of biological differences at T2 and T3

The maximum number of statistically significant biological differences were shown at the 2- and 3-hour time point following a challenge meal, for ease these will be referred to as T2 and T3. For appreciating the magnitude of difference in abundance between the classes, fold change (FC) will be examined in addition to p-values (fold change (FC) is the ratio of the mean metabolite abundance in the disease cohort over that of the healthy cohort, a FC of <1 shows an increase in concentration in the healthy cohort and >1 an increase in the diseased cohort. A FC of <0.5 or >2 is considered substantial).

6.2.1 Metabolites with significant Differences at T2 and T3

A one-way ANOVA comparing PEI to No PEI at 2 hours (T2) and at 3 hours (T3) was performed. **Table 30** presents the number of metabolites at T2 and T3 that were significantly different. All results are reported after correction for multiple testing (Benjami-Hochberg) with p<0.05. Also presented in **Table 30** are the number of metabolites that had a fold change of either <0.5 or >2.

Table 30 Number of metabolites reaching significance for the 2 hour and 3 hour time points, in batch 1 and batch 2. Reduced by whether the have fold change of either <0.5 or >2.

Assay	Number of metabolites p <0.05	Number of metabolites with a fold change of <0.5 OR >2		
		Total	<0.5	>2
Time point 2				
Batch 1	246	116	59	57
Batch 2	176	92	54	38
Time point 3				
Batch 1	314	141	71	70
Batch 2	121	71	44	27

To identify those metabolites most consistently different between those with and without PEI and to narrow down the list of metabolites for further investigation, only those that reached

significance across **both** batch 1 and batch 2 and for **both** T2 and T3 were taken forward. This reduced the list of potential metabolites to 17.

Table 31 P value and Fold change for each metabolite with significance at the 2-hour time point and the 3 hour time point.

Metabolite	Time point 2				Time point 3				Take further
	Batch 1		Batch 2		Batch 1		Batch 2		
	p	FC	p	FC	p	FC	p	FC	
1771	0.021	0.29	0.016	0.19	0.018	0.28	0.044	0.16	**YES**
4150/4171	0.033	0.25	0.006	0.15	0.028	0.23	0.005	0.16	**YES**
3470/3109	0.011	0.27	0.009	0.23	0.006	0.23	0.017	0.23	**YES**
3227/3206	0.009	3.35	0.044	3.18	0.006	4.82	0.014	2.89	**YES**
6059	0.026	0.20	0.006	0.37	0.045	0.17	0.008	0.30	YES
4586	0.002	3.71	0.002	4.82	0.001	3.55	0.007	6.12	**YES**
3385	0.001	7.54	0.004	4.88	0.005	4.62	0.001	5.42	**YES**
4835	0.047	0.29	0.009	0.23	0.044	0.28	0.005	0.28	YES
5989	0.046	2.83	0.042	2.37	0.009	2.29	0.039	2.13	**YES**
7061/7048	0.036	11.95	0.0023	4.88	0.039	2.14	0.003	5.05	**YES**
11174	0.032	2.96	0.005	4.75	0.027	2.99	0.003	3.81	YES
8925	0.005	0.23	0.003	0.20	0.004	0.22	0.005	0.18	YES
5665/10462	0.022	1.77	0.034	3.22	0.004	1.79	0.003	1.76	**YES**
5610/5607	0.004	2.23	0.047	1.41	0.039	1.70	0.011	1.49	Maybe
3598	0.022	0.7	0.008	0.6	0.009	0.64	0.14	0.68	NO
3740/3763	0.05	0.8	0.025	0.79	0.034	0.78	0.015	0.76	NO
4504	0.012	0.53	0.013	0.54	0.004	0.50	0.025	0.58	NO

P values from one way ANOVA comparing those with Pei to those without PEI at specific time points. FC=Fold change

Of these 17 metabolites that were significantly different at both T2 and T3 and across both batch 1 and batch 2, the fold changes were examined and only those with a FC of with <0.5 or >1.5 were examined further, this amounted to 13 metabolites (see **Table 31**).

6.2.2 Feature annotation and visual representation of the most relevant metabolites for a potential biomarker of PEI.

Those metabolites maintaining significance across T2 and T3, B1 and B2 that also had a supportive FC are tabled below with their tentative annotation based on mass spectral libraries.

Table 32 Annotation and class of metabolites with significance at both 2 hours and 3 hours following a challenge meal and with a fold change of <0.5 or >1.5.

Identifier	Compound name	Class
5989	PI(O-38:6)||PI(P-38:5)	Glycerophospholipid
5665/10462	PC(40:8)	Glycerophospholipid
7061/7048	PS(44:0)	Glycerophospholipid
5610/5607	PG(34:1)||PC(33:2)||PE(36:2)	Glycerophospholipid
11174	TG(61:14)	Triacylglyceride
3227/3206	Ergothioneine||Cysteinyl-Glutamate||Glutamylcysteine	Dipeptide - Amino acid
3470/3109	Asparaginylhydroxyproline	Dipeptide - Amino acid
6059	Hexadecanedioic acid mono-L-carnitine ester|| 1-eicosanylglycerol	Acyl carnitine
4835	O-sebacoylcarnitine	Acyl carnitine
4586	Homodeoxycholic acid||(22E)-3alpha,6beta,7beta-Trihydroxy-5beta-chol-22-en-24-oic Acid||(22E)-3alpha,7alpha,12alpha-Trihydroxy-5beta-chol-22-en-24-oic Acid	Other class
3385	N-Phenylacetyl pyroglutamic acid|| Phenylalanylproline||tetradecadienoic acid	Mixed class
1771	4,4'-Thiobis-2-butanone	Other class
8925	3'-Sialyllactose||6'-Sialyllactose	Oligosacccharide
4150/4171	Acetyl-N-formyl-5-methoxykynurenamine||Formyl-N-acetyl-5-methoxykynurenamine||N(2)-phenylacetyl-L-glutaminate ||Phenylacetylglutamine ||2-Hydroxydecanedioic acid||Thiamine acetic acid	Mixed class

These metabolites are shown in box and whisker plots below (4 box and whisker plots are shown for each metabolite, from left to right, these represent T2 Batch 1, T2 Batch 2, T3 Batch 1 and T3 Batch 2).

Figure 52 Metabolite 5989, box and whisker plots comparing concentration in No PEI (0) vs. PEI (1)

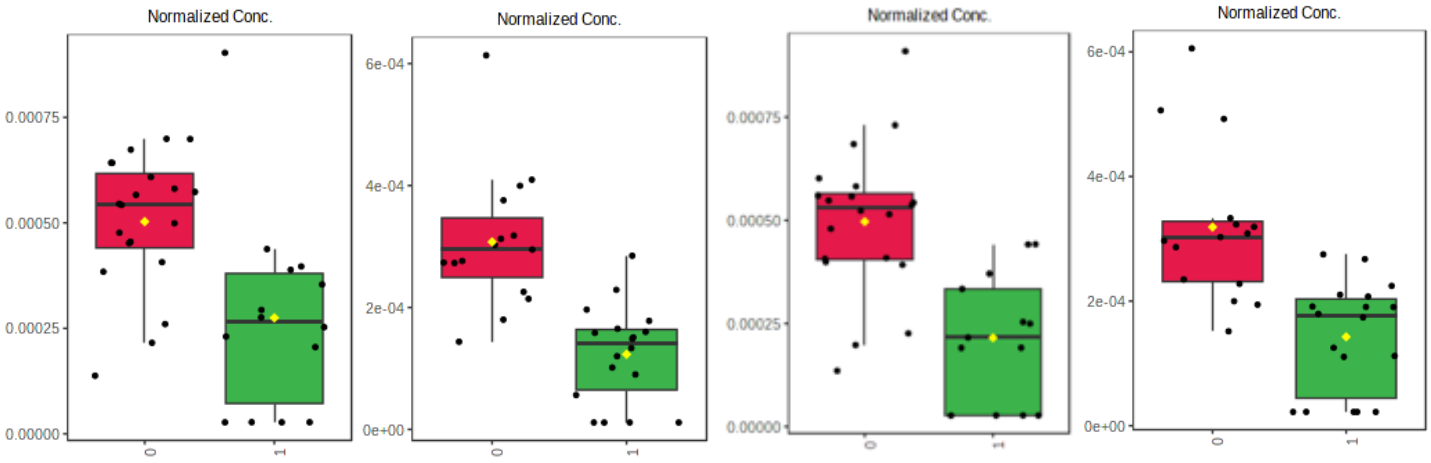


Figure 53 Metabolite 5665, box and whisker plots comparing concentration in No PEI (0) vs. PEI (1)

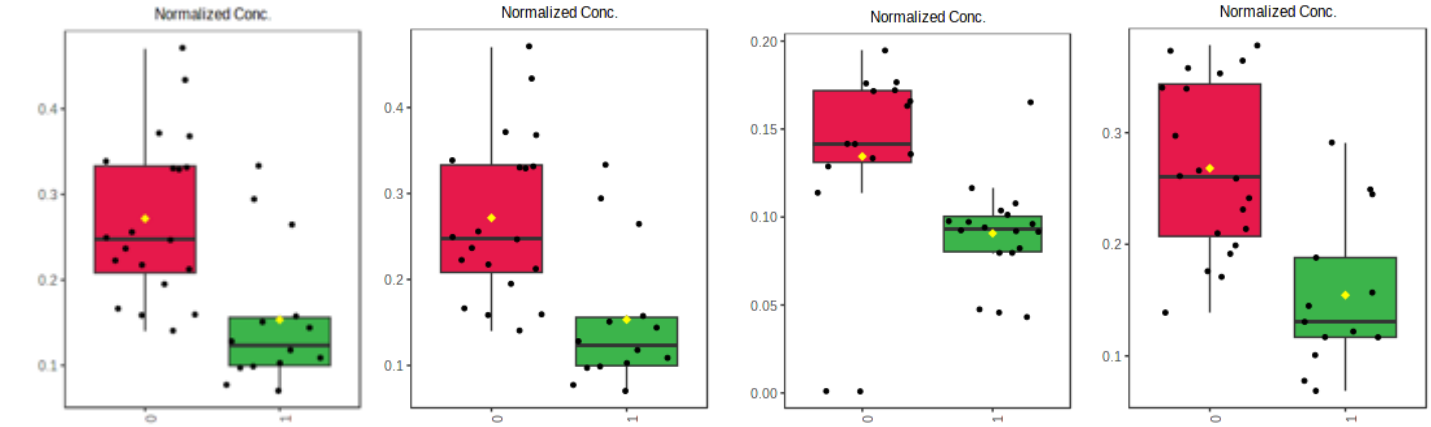


Figure 54 Metabolite 7061/7048, box and whisker plots comparing concentration in No PEI (0) vs. PEI (1)

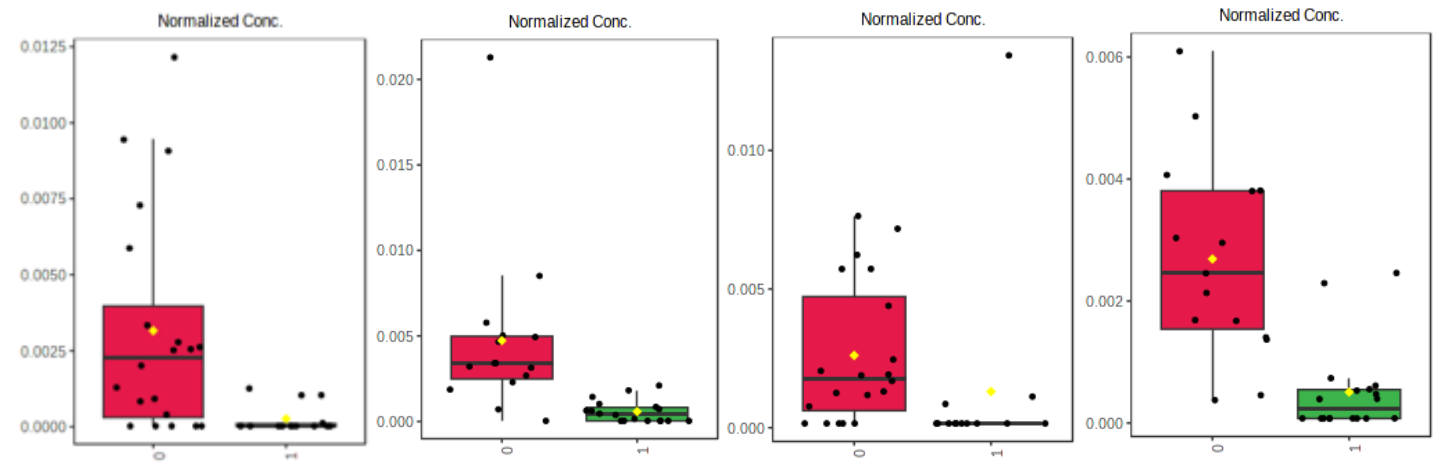


Figure 56 metabolite 3206/3227 Box and whisker plots comparing concentration in No PEI (0) vs PEI (1)

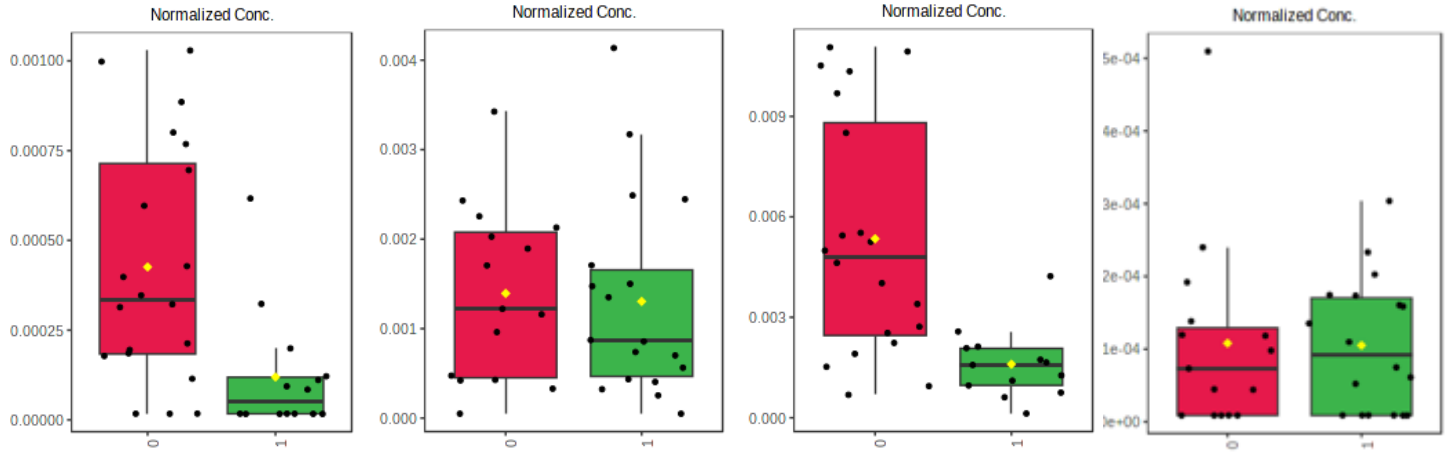


Figure 55 Metabolite 5607/5610 box and whisker plots comparing concentration in No PEI (0) vs. PEI (1)

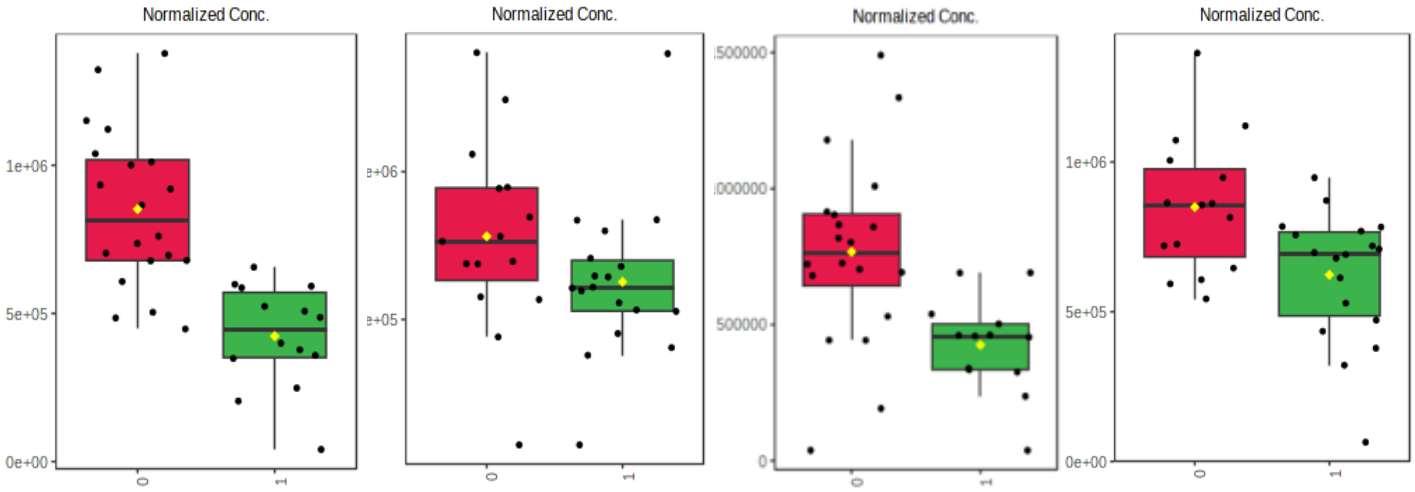


Figure 57 Metabolite 3109 Box and whisker plots comparing concentration in No PEI (0) vs PEI (1)

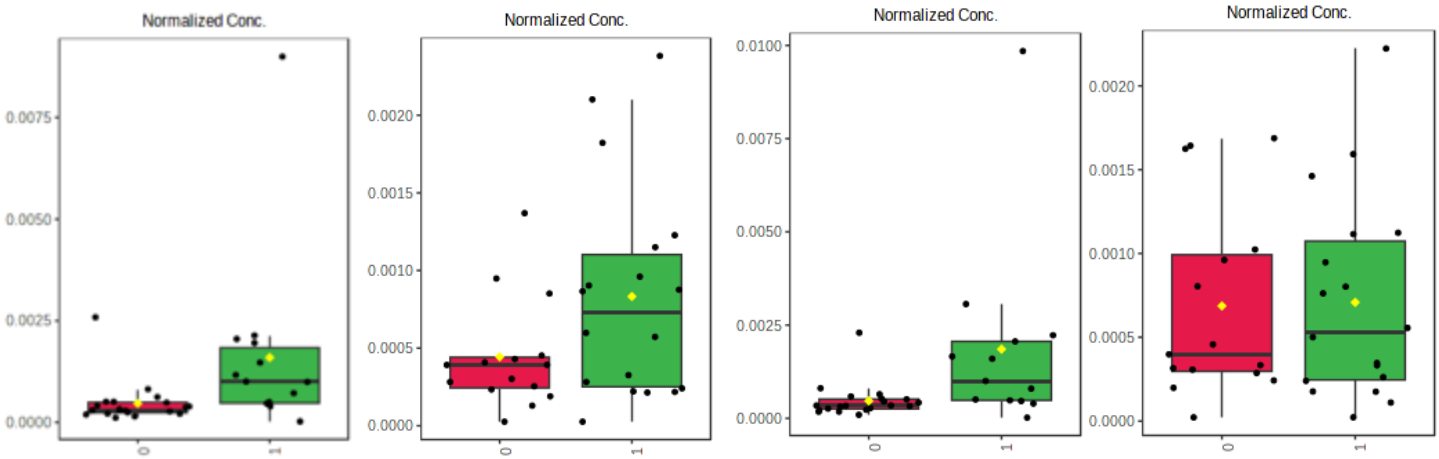


Figure 58 Metabolite 4586 Box and whisker plots comparing concentrations in No PEI (0) vs. PEI (1)

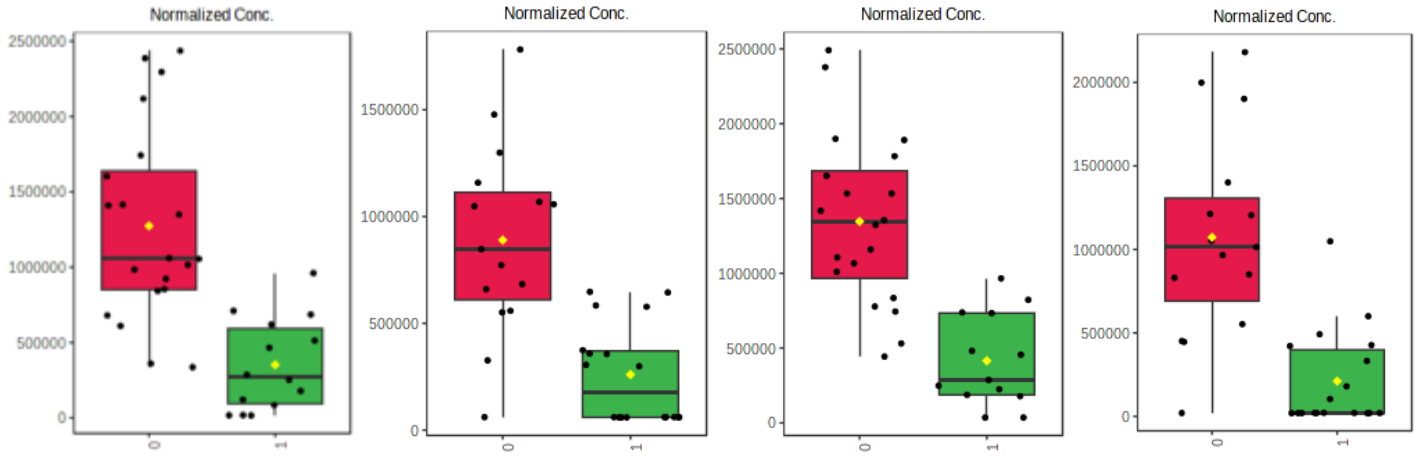


Figure 59 Metabolite 3385 Box and whisker plots comparing concentrations in No PEI (0) vs. PEI (1)

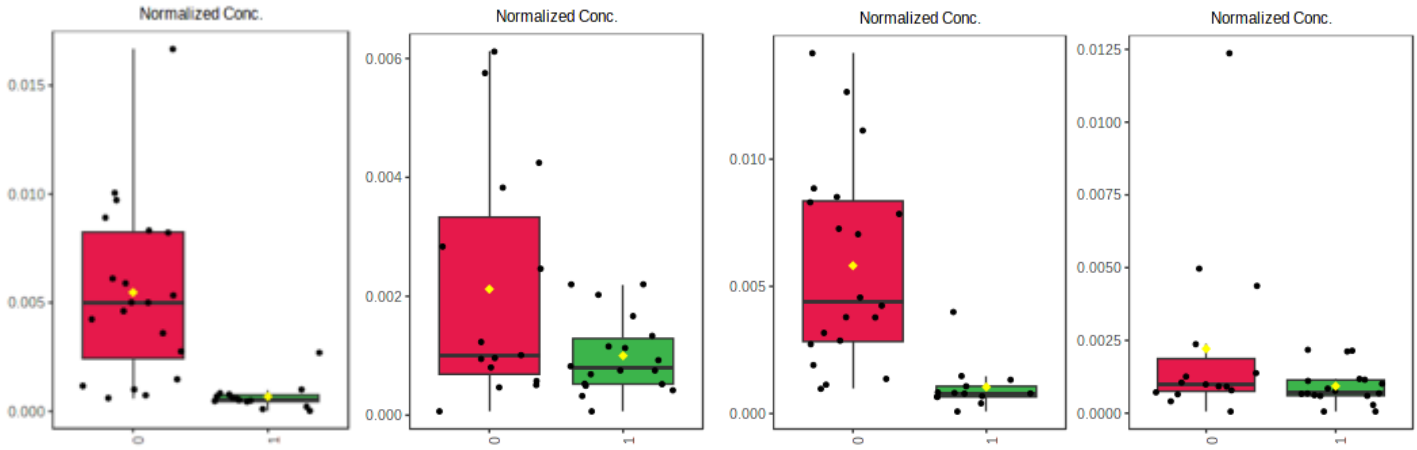


Figure 60 Metabolite 1771 Box and whisker plots comparing concentrations in No PEI (0) vs. PEI (1)

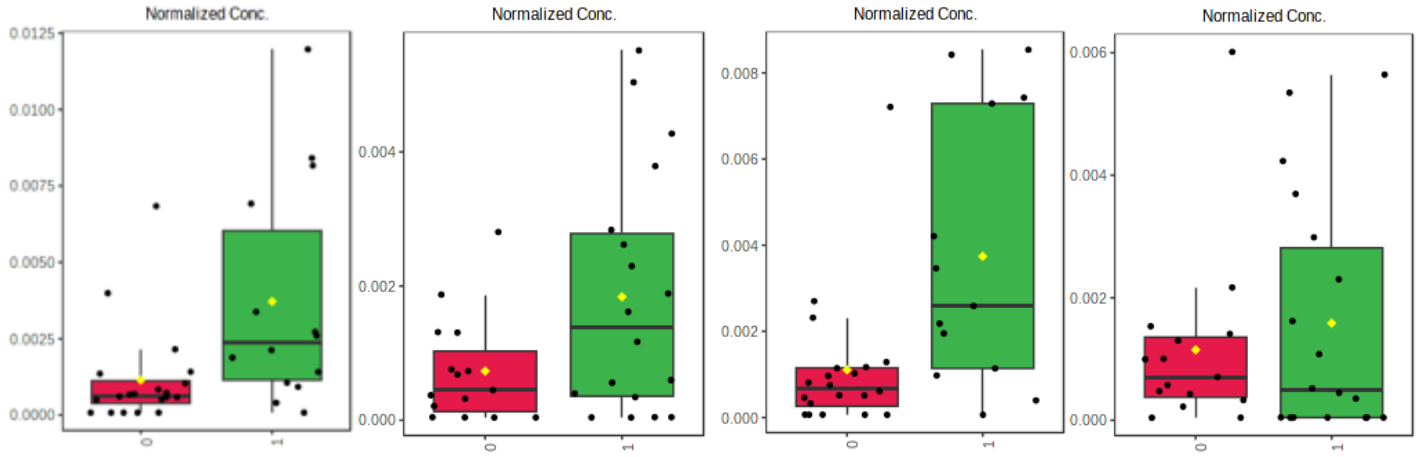


Figure 62Metabolite 4150 Box and whisker plots comparing concentration in No PEI (0) vs. PEI (1)

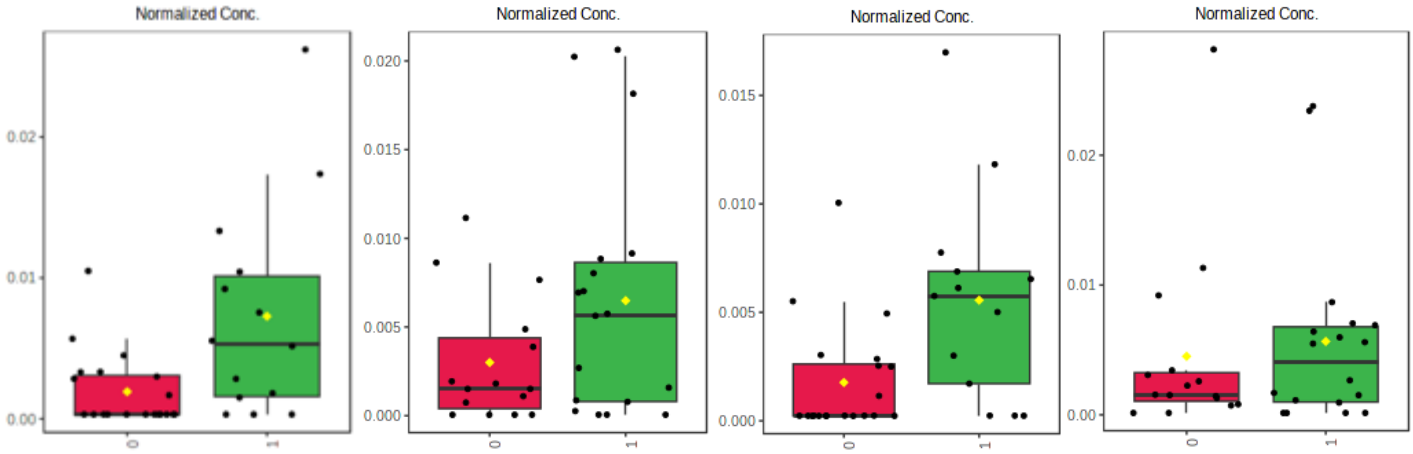
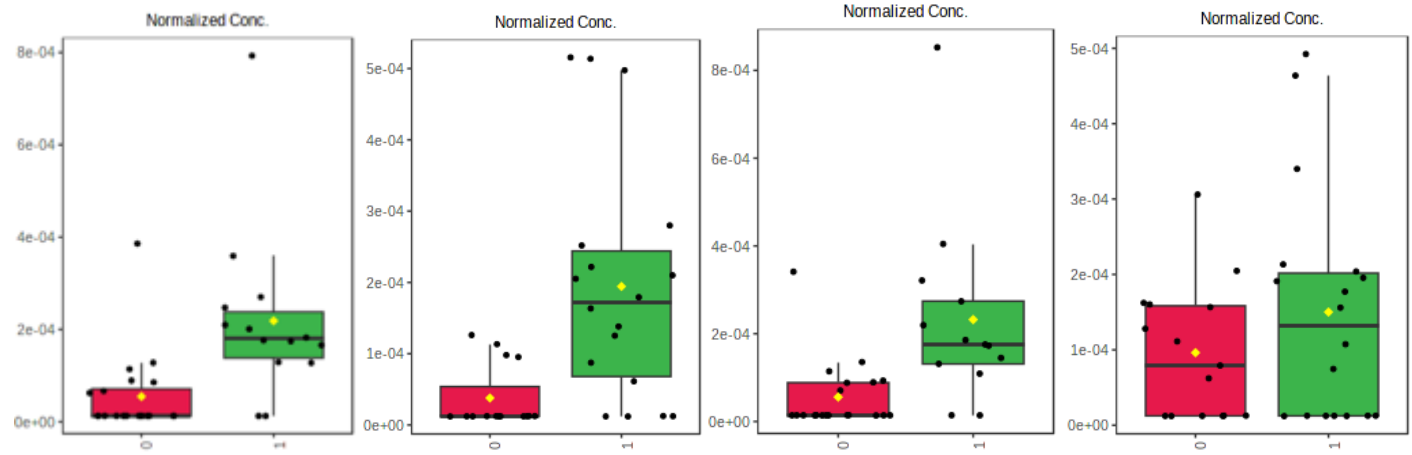


Figure 61 Metabolite 8925 Box and whisker plots comparing concentrations in No PEI (0) vs. PEI (1)



The above plots make it easier to visualise those metabolites lacking in consistency between the time points and batches, e.g metabolite 3227/3206, metabolite 1771 and metabolite 3109 and remove them from further investigation.

6.3 Sensitivity and specificity of metabolite panels for identifying PEI

Having identified a list of potentially significant metabolites, this section moves away from understanding the underlying biology, and towards the creation of a diagnostic panel of metabolites. Using a multivariate regression model, combinations of the potential biomarkers

above have been examined. The 2-hour time point and the 3-hour time point have been examined separately for each batch creating AUROC and CI for combinations of the most significant metabolites. Presented in **Table 33** are the top performing metabolites on univariate analysis for each time point and batch alongside AUROC curves produced using a random forests multivariate regression modelling. Each is presented alongside the confusion matrix (effectively presenting sensitivity and specificity) and selection frequency (showing how heavily weighted the model is on each metabolite).

6.3.1 2 hour time point batch 1 and 2, univariate and multivariate analysis

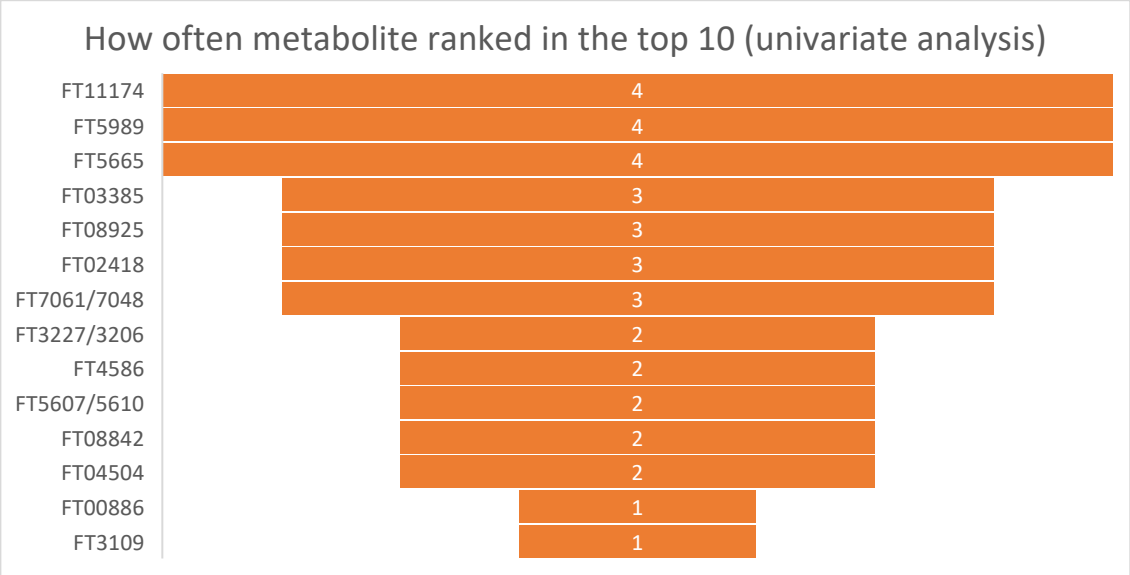
Table 33 Univariate analysis of most significant metabolites (top 10 for each time point and batch)

Metabolite	**Univariate analysis**		**Metabolite**	**Univariate analysis**	
Time point 2 Batch 1	AUC	T-test	**Time point 2 Batch 2**	AUC	T-test
FT03385	0.95	0.0001	FT4586	0.98	0.0000
FT08925	0.86	0.0017	FT5989	0.94	0.0000
FT5665	0.85	0.0007	FT7048	0.91	0.0026
FT5989	0.83	0.0018	FT08925	0.86	0.0011
FT11174	0.83	0.0006	FT5607	0.85	0.0014
FT02418	0.83	0.0010	FT5610	0.85	0.0012
FT03206	0.81	0.0041	FT5665	0.83	0.0170
FT03109	0.81	0.0358	FT08842	0.78	0.0224
FT7061	0.81	0.0061	FT11174	0.78	0.0047
FT03227	0.81	0.0034	FT04504	0.71	0.1099
Time point 3 Batch 1	AUC	T-test	**Time point 3 Batch 2**	AUC	T-test
FT03385	0.94	0.0002	FT5665	0.96	0.0000
FT5989	0.89	0.0001	FT4586	0.92	0.0000
FT03227	0.87	0.0012	FT5989	0.91	0.0001
FT5665	0.87	0.0002	FT5607	0.90	0.0001
FT04504	0.86	0.0043	FT7048	090	0.0000
FT02418	0.85	0.0007	FT5610	0.84	0.0002
FT03206	0.85	0.0009	FT11174	0.68	0.0998
FT08925	0.85	0.0021	FT08842	0.67	0.3990
FT11174	0.82	0.0009	FT02418	0.65	0.1039
FT00886	0.82	0.0006	FT03385	0.64	0.1266

A univariate analysis was used to investigate how well individual metabolites identify PEI at both the 2 hour mark and the 3 hour mark. There is good consistency across the rankings with 3 metabolites consistently ranking in the top 10 across all 4 analyses (Across both time points

and both batches): 5665, 5989 and 11174. A further 3 metabolites make the top 10 in 3/4 : 3385, 8925, 2418 and 7061/7048. There are some differences across the 2 batches with 3 metabolites ranking well in batch 2 but not within the top 10 for batch 1. The 2 most promising metabolites from univariate analysis are metabolite 5665 and metabolite 5989, these have and AUC of greater than 0.8 and a *p* value of 0.01 or less across both time points and both batches. Although metabolite 11174 ranked consistently in the top 10, the AUC fell beneath 0.8 in batch 2 and the *p* value was not significant at the 3 hour mark in batch 2.

Figure 63 Frequency of being ranked in the top ten on univariate analysis.



Next, we calculated the AUC with CI for combinations of metabolites from multivariate analysis using random forests multivariate regression model, these are presented below (**Figures 64-67)** with their selection frequencies.

Figure 65 T2 B1 AUC from multivariate random forests model, alongside selection frequency of metabolites

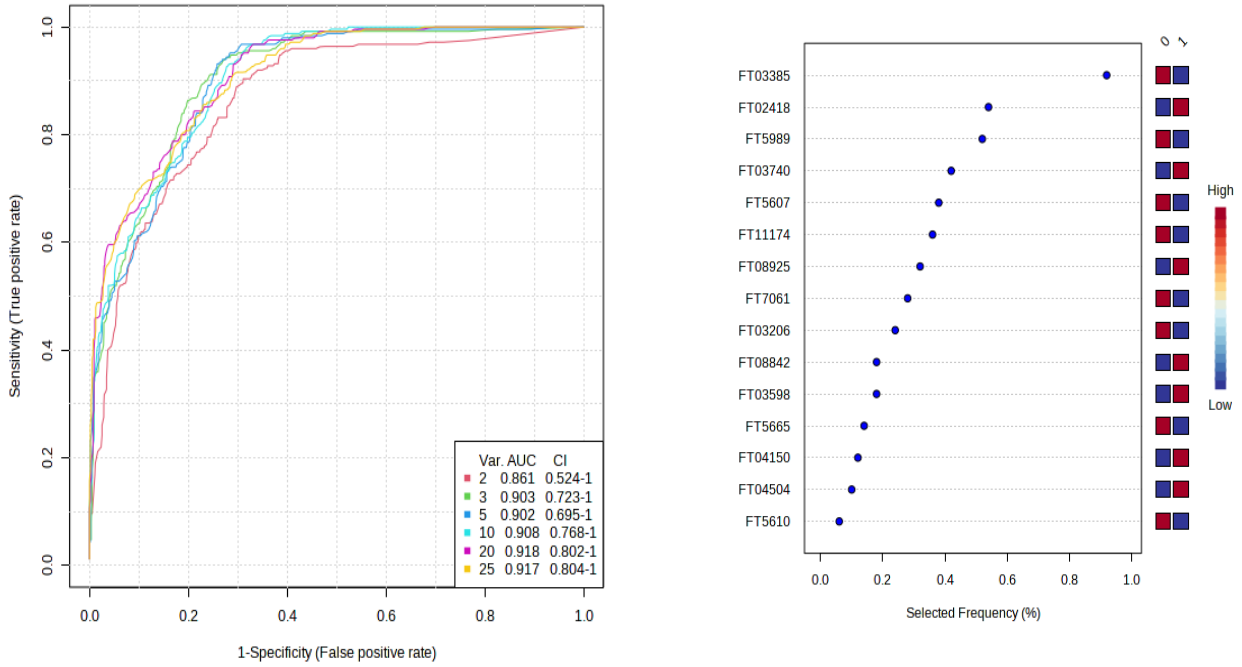


Figure 65 T2 B2 AUC from multivariate random forests model, alongside selection frequency of metabolites

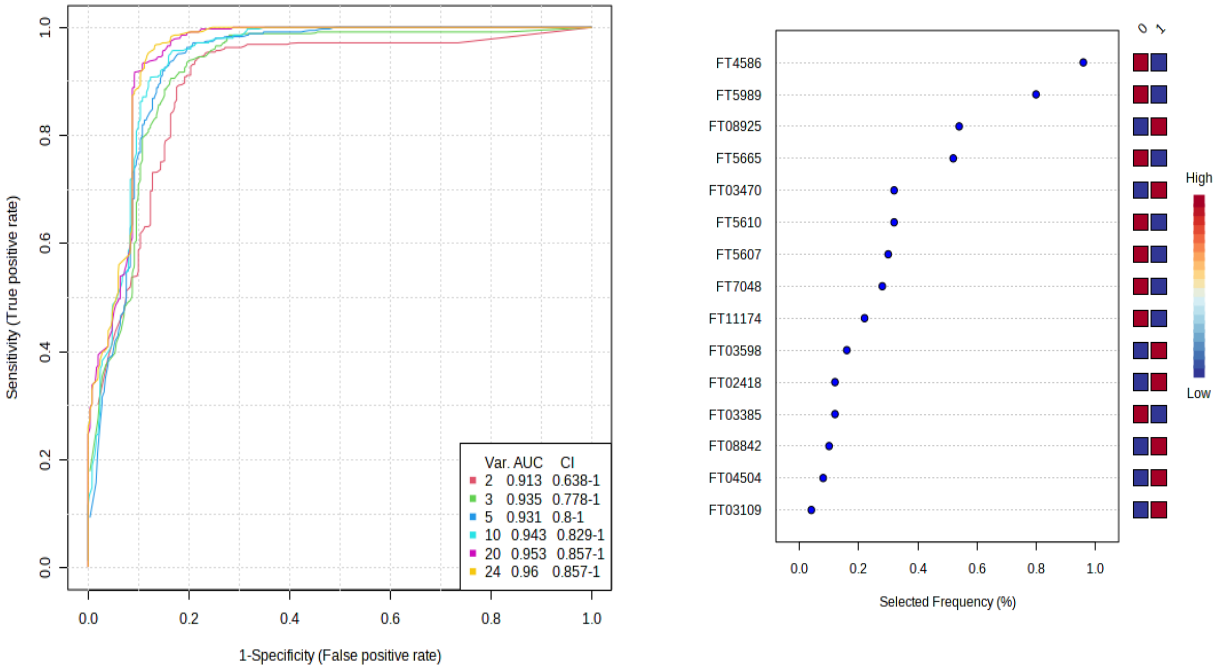


Figure 67 T3 B1 AUC from multivariate random forests model, alongside selection frequency of metabolites

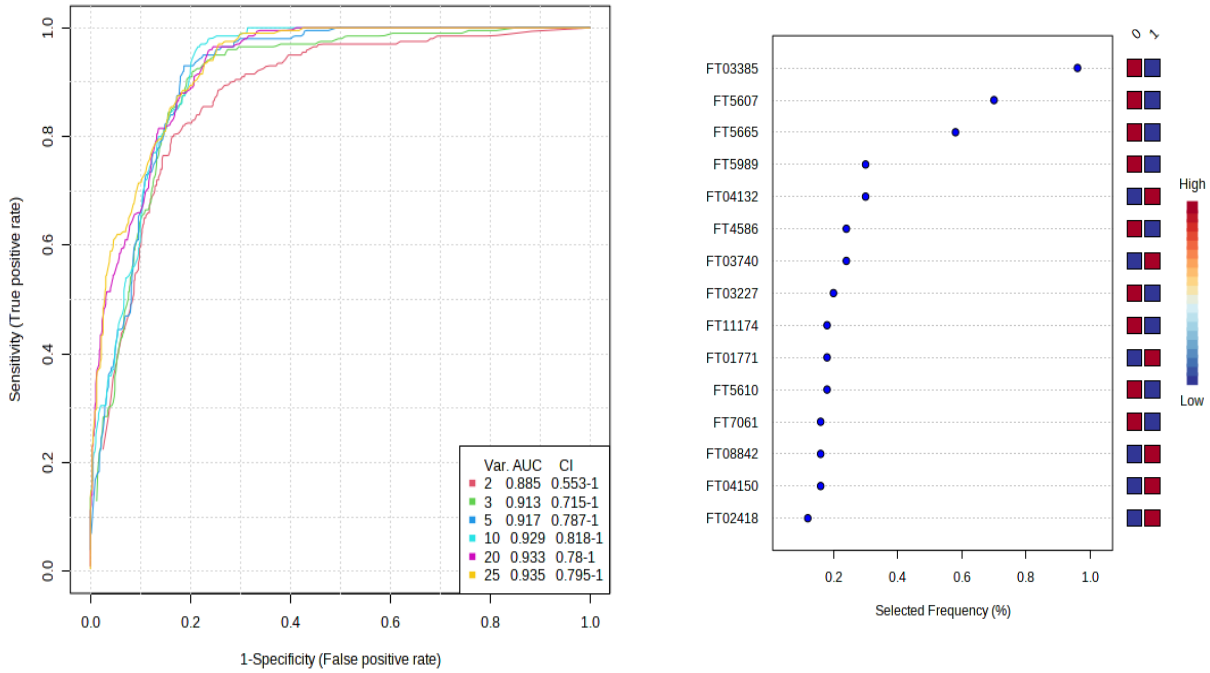
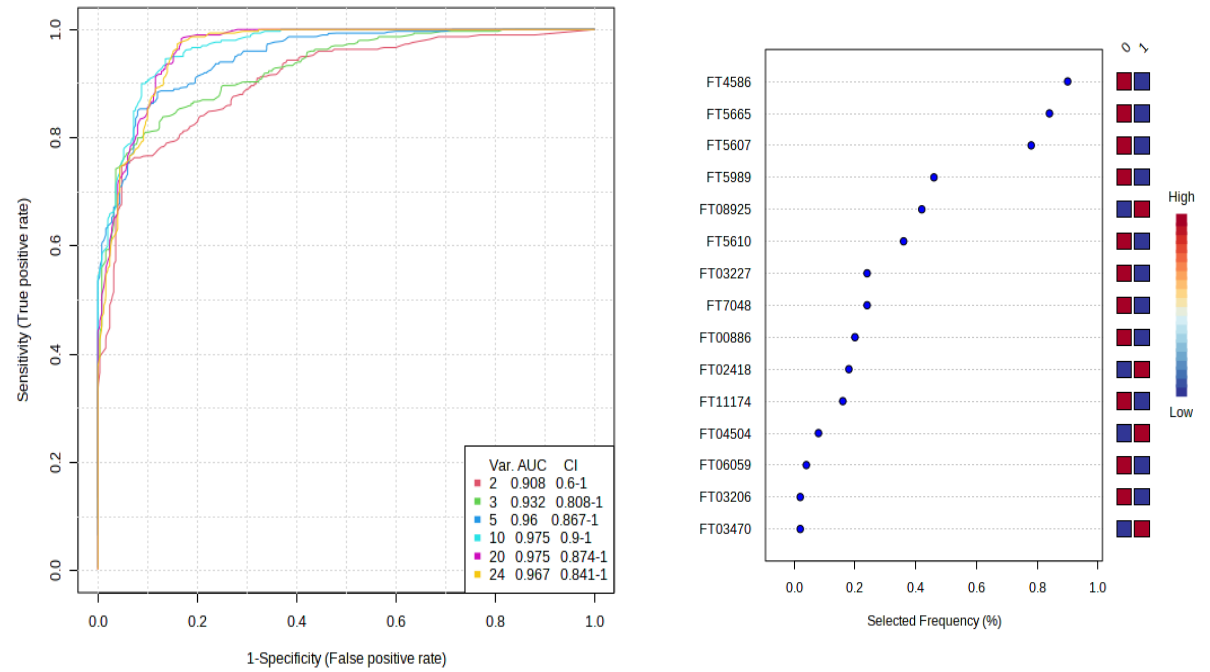


Figure 67 T3 B2 AUC from multivariate random forests model, alongside selection frequency of metabolites



Using multivariate random forests model, results in an excellent (>0.9-1) AUC at both time points and across both batches when using 3 or more metabolites and a good (0.8-0.9) AUC using just 2 metabolites. The frequencies of selection and how they compare to univariate analysis are described below.

6.3.2 Targeting metabolites constant across MV time points and batches.

The above UV and MV analysis represent AUC from models based on separate time points and batches. Collated below in **Figure 68** are the highest represented metabolites in order of rank from each MV. This information enables the selection of the most consistently represented metabolites from which a refined MV analysis can be done on a narrower selection of metabolites. Of note, two types of modelling have been performed, random forest (RF) and linear SVM (LSVM) (via Metabolanalyst ®).

Figure 68 Top 10 most represented metabolites from multivariate analysis at 2 hours and 3 hours, for batch 1 and batch 2.

	T2, B1		T2, B2		T3, B1		T3, B2	
Rank	LSVM	RF	LSVM	RF	LSVM	RF	LSVM	RF
1	3385	3385	4586	4586	3385	3385	4586	4586
2	2418	2418	5989	5989	5607	5607	5665	5665
3	5989	5989	5665	8925	5665	5665	5607	5607
4	3740	3740	5610	5665	5989	5989	5989	5989
5	5607	5607	8925	3470	4586	4132	5610	8925
6	1174	1174	5607	5610	2418	4586	7048	5610
7	8925	8925	7048	5607	8842	3740	8925	3227
8	7061	7061	3470	7048	4132	3227	0886	7048
9	3206	3206	2418	11174	11174	11174	3227	0886
10	8842	8842	8842	2598	7061	1771	111174	2418

LSVM = linear SVM, RF = Random forests, T2=2-hour time point, T3=3-hour time points, B1=Batch 1, B2=Batch 2

Reviewing the top 5 most highly ranked metabolites in the above figure (**figure 68**), the most consistently represented are 5989 (represented across all 4 and in every MV model), 5607 (also known as 5610, represented across all 4), and 5665 (represented in 3 of the 4, not in the top 5 ranked for T2 B1 still in the MV selection model but ranked lower in the selection frequency (12^{th}), see **Figure 68.**). Although MV analysis in the context of biomarker identification does not focus on biological interpretation, for interest, the biological annotations of these three metabolites are shown below (**Figure 69)**.

Figure 69 Univariate AUC for the most consistently performing, highly ranked metabolites, along with their annotation and class.

	AUC (from UV analysis)				Compound	Class
	T2 B1	T2 B2	T3 B1	T3 B2		
5607/5610	0.853	0.9	0.853	0.842	PG(34:1)||PC(33:2)||PE(36:2)	Glycerophospholipid
5665	0.85	0.826	0.865	0.962	PC(40:8)	Glycerophospholipid
5989	0.832	0.942	0.885	0.913	PI(O-38:6)||PI(P-38:5)	Glycerophospholipid

AUC=Area under the curve, UV=univariate T2=2-hour time point, T3=3-hour time points, B1=Batch 1, B2=Batch 2

Finally, these three most consistent, highly ranked metabolites have been used to perform a random forest multivariate analysis, creating an AUC and a CI for a combination of either two (red line) or three (green line) metabolites (Shown below in **Figures 70 and 71)**. The results are an AUC of more than 0.9 and a CI of 0.8 for the combination of all three (maintained across T2, T3, B1 and B2) and an AUC of more than 0.9 and a CI of more than 0.7 for a combination of two (maintained across T2, T3, B1 and B2).

Figure 71 AUC and CI from MV analysis of the most consistently highly ranked metabolites (T2 B1 and T2 B2).

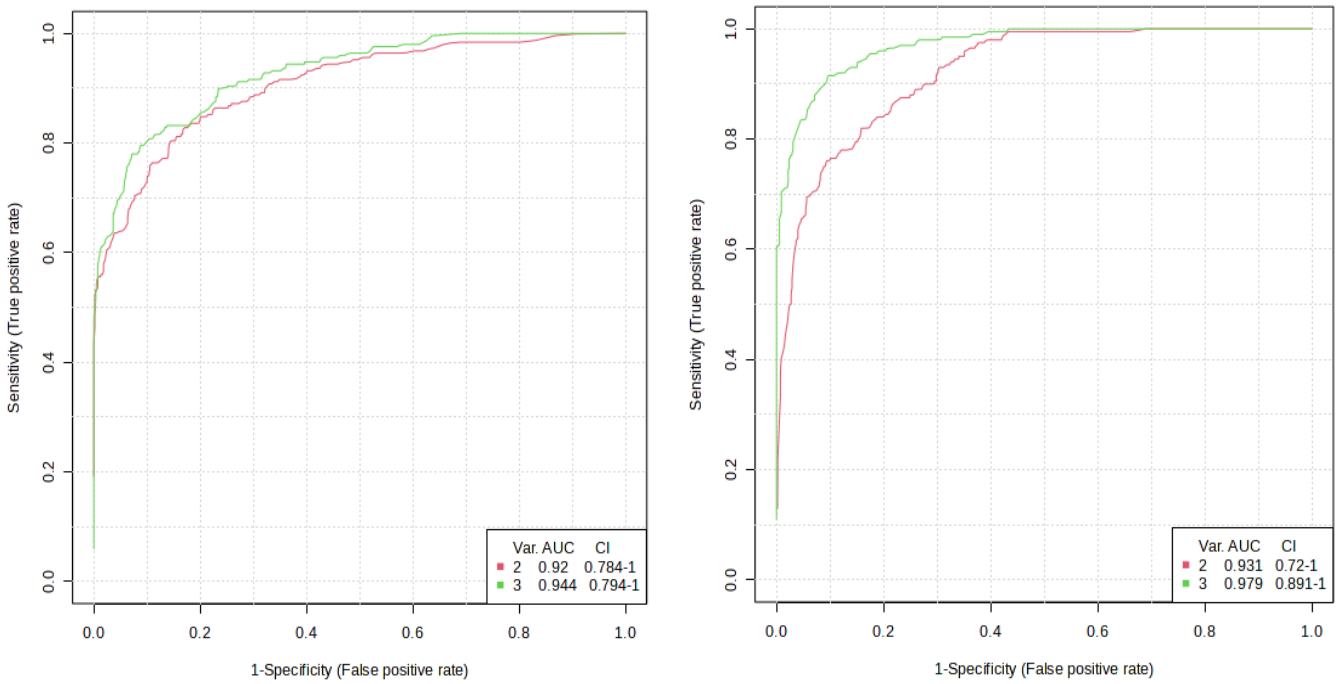
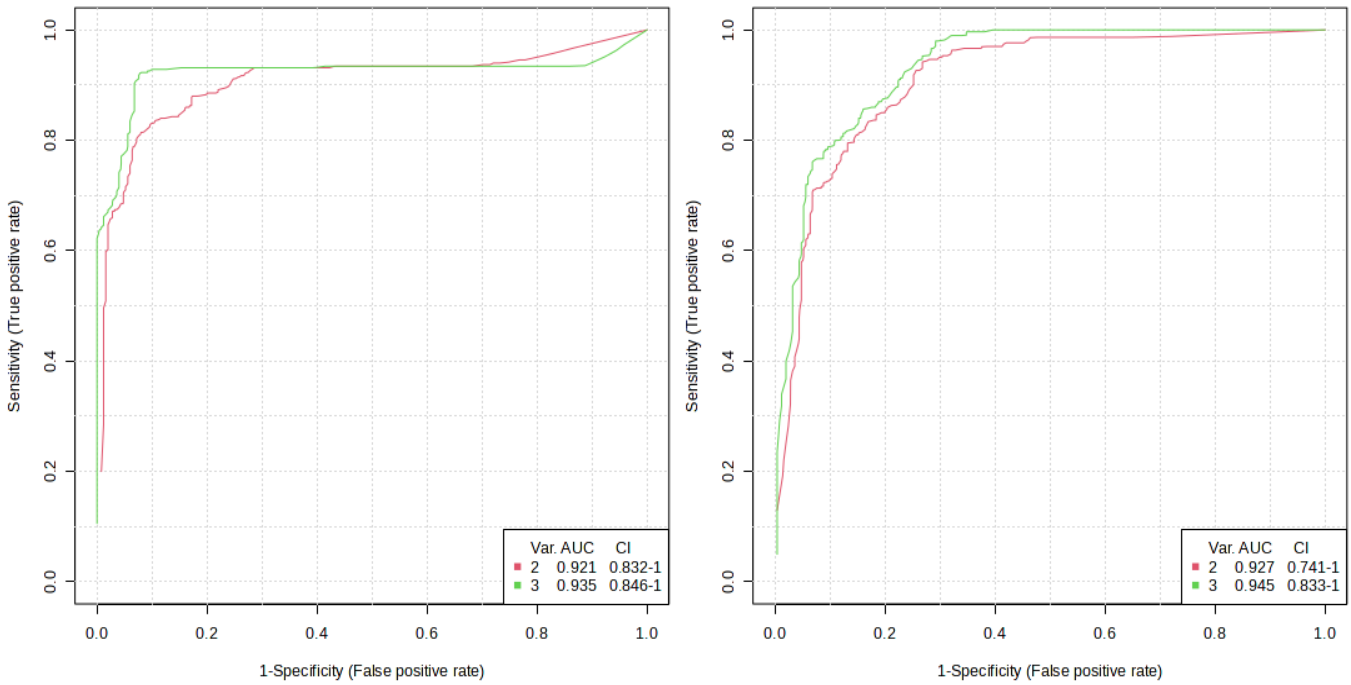


Figure 71 AUC and CI from MV analysis of the most consistently highly ranked metabolites (T3 B1 and T3 B2).



From the above results, using three metabolites produces an AUC that is closer to the ideal value of 1.0 than using two metabolites and it is the recommended [method]. As a test lasting two hours is more acceptable to patients than a test lasting three hours, the former is recommended.

6.4 Discussion of chapter 6

This chapter culminates in a discussion about the selection of those metabolites which are most different between those with and without PEI and that are most consistently significant across two separate time points and across the two batches. Even after significant reduction and selection there are ample metabolites to choose from in the creation of a biomarker panel. Therefore, those that have ranked most highly across the time points and batches have been selected. The best combination used three metabolites at the two-hour time point and produces an AUC of 0.94 in batch 1 and an AUC of 0.98 in batch 2. This is a very promising finding and although it requires validation in other groups of patients with PEI, it has the potential to be the basis of a novel diagnostic test for PEI.

The validation of this panel would be a much more refined process than the original DETECTION trial protocol; an overnight fast and the same challenge meal would still be required, but just a single blood test two hours post-prandially on which targeted UHPLC-MS would be used. With fewer sampling points, a shorter test, and a targeted approach to metabolomic processing this would be cheaper and easier to recruit for than the DETECTION trial. The next steps are already in motion with a potential cohort of cystic fibrosis patients identified, ethics in place for recruitment and preliminary funding from PCUK secured.

Chapter 7 Conclusions and future work

My postgraduate research sought to design and deliver a trial to investigate the post-prandial metabolome of those with and without PEI and identify potential biomarkers for the diagnosis of PEI. This work culminated in the selection of a panel of 3 metabolites most likely to identify those with PEI at 2 hours post prandially (AUC 0.98). This is a very promising finding that has the potential to be a novel diagnostic test for PEI.

During trial design, much work had to be done on the refinement of the ^{13}C MTGT, a robust framework for a set test protocol had to be formed and areas lacking in evidence and regulation had to be identified. A novel, evidence based, acceptable, challenge meal was created, and a sub-study was conducted to validate this against the most used existing challenge meal. This work is an important contribution to improving the accessibility and standardisation of the ^{13}C MTGT, a useful aid to PEI diagnosis. Both the systematic review of ^{13}C MTGT methodology and the novel test meal validation study have been published and several other research groups have started using the ^{13}C MTGT based on this protocol.[224, 225]

The main recruitment cohort for the DETECTION trial comprised patients with pancreatic cancer at various points in their treatment journey. To establish PEI status, a battery of existing diagnostic measures was used. Establishing the true incidence of PEI in the longer term following pancreatic resection was challenging, as the literature is sparse and limited by studies adopting short follow up times and heterogenous diagnostic techniques. To try and answer this question the reference tests done on those post pancreatic resection recruited to the DETECTION trial were examined independently, the key findings being:

- There was an 80% incidence of PEI long-term following PD for pancreatic cancer .

- Pre-operative pancreatic duct width and lower BMI correlated with post-operative PEI.
- Despite supposedly ‘adequate’ PERT treatment, those with PEI had a significantly lower BMI in the long-term.
- The ^{13}C MTGT and FE-1 results correlated well with each other, but the PEIQ (symptom score) did not correlate well with either.

This work has been published with the take-home message that PEI is prevalent and sustained after pancreatic head resection, and patients should remain on PERT for the rest of their lives, with adequate monitoring to ensure PERT prescribing and compliance.[214]

Another cohort of the DETECTION study that warranted independent examination of PEI incidence was the NET cohort. This was a group of patients in whom the use of somatostatin analogues inhibited pancreatic secretion, however, prior to the DETECTION study little had been done to investigate PEI or standardise treatment with PERT.

- The key finding was that all patients had a drop in their cPDR of ^{13}C on repeated measures of the ^{13}C MTGT pre and post SSA therapy. This confirmed that SSAs affect exocrine function. As few patients met the diagnostic threshold for PEI, this cohort will not be used for validation. However, the samples taken are stored for future metabolomic analysis.

This work has been published and a trial is currently in development to robustly investigate the impact of this fall in exocrine function in those initiating SSAs and how to determine need for PERT treatment.[69]

The main cohort for the DETECTION study comprised 33 healthy controls, 34 patients with pancreatic cancer and PEI and 9 patients with pancreatic cancer and no PEI. Recruits were matched for baseline characteristics. Bloods taken at baseline and 1, 2, 3 ,4, and 5 hours post-prandially were processed using UHPLC-MS and analysed using metabolanalyst.

- Key findings on biological interpretation were that glycerophospholids were the main metabolite class differing between those with and without PEI. The metabolic pathways that were most different were pyrimidine metabolism, methionine and cysteine metabolism and phenylalanine metabolism.
- A 2-way ANOVA was used to investigate the overall interaction of phenotype and time. It found a vast number of significantly different metabolites, with 213 metabolites that reached significance at $p<0.05$. The top 15 metabolites were all significant at $p<0.00001$. This strongly supports the DETECTION trial hypothesis that those with PEI have a significantly different post-prandial response to those without PEI.
- Examining the differences between phenotypes at individual timepoints found minimal differences at baseline with no consistent class represented. This suggested that the cohorts were reasonably matched at baseline and the subsequent post-prandial changes related to differences in their ability to metabolise the challenge meal. Some changes were seen at 1 hour, but maximum biological differences were found at 2 and 3 hours.
- A one-way ANOVA comparing phenotypes at the 2-hour mark and the 3-hour mark was performed. Only metabolites that were significantly different (after corrected for multiple testing) and had an appropriate fold change across both time points and in both analysis batches were included in further analysis. This amounted to 13 metabolites.
- Univariate and multivariate analysis was then performed on promising metabolites, those that ranked most highly (and consistently across time points and batches) on univariate analysis and those that had the most promising selection frequency were taken further.
- The best results came from a combination of three metabolites at the 2-hour time point, these produced an excellent AUC of 0.94 (CI 0.8) in batch 1 and 0.98 (CI 0.9) in batch 2. This is an extremely promising finding and has the potential to be a future biomarker panel for the diagnosis of PEI.

There are limitations to this work, both in trial delivery and in sample processing:

- Main cohort recruitment was guided by circumstance, the study (and researcher) being based at a very high throughput unit for pancreatic cancer and embedded in a pancreatic cancer research team meant that pancreatic cancer was the obvious choice. However, pancreatic cancer is a rapidly progressing disease, which presents challenges to recruitment. For patients who can undergo resection the treatment journey is swift , which limits pre-surgery recruitment. For those who cannot undergo resection the short survival time and the intensity of chemotherapy often makes time-consuming research inappropriate. Although I am confident that my main recruitment cohort all had PEI, it was also a heterogenous cohort, with patients recruited from all points along their cancer journey. To try and mitigate the effect of this heterogeneity, patients across all stages, but without PEI were also included in the control cohort However, there are very few patients with pancreatic cancer but without PEI, thus using this group alone as a control was not feasible.
- There are inherent limitations in high throughput analytical techniques, with difficulties in annotating large datasets and obtaining chemical standards for the definitive identification of metabolites. Quality control was imperative for UHPLC-MS, and a significant number of samples had to be excluded from analysis from batch 2, as there was evidence of a processing issue with them.
- For some very specific cohorts of patients, such as those with active pancreatic cancer, there is a question as to whether a novel diagnostic marker is required given that current NICE guidance advises that all be treated with PERT regardless. This may limit the applicability of a novel test. However, should a novel test be able to guide dosing and help identify severity of PEI it would still be of significant benefit.

- Finally, time was a major limitation. Developing a trial, obtaining ethical approval, delivering the trial during the COVID-19 pandemic, and analysing the resultant vast quantity of data proved to be challenging within a PhD term.

A very promising panel of metabolites has been identified as a potential novel biomarker of PEI, and the next step is to validate this biomarker panel in other patient cohorts. The cystic fibrosis cohort is ideal: with a centre of excellence located within the same NHS trust, there is a ready stream of patients with confirmed PEI who are younger, fitter and already acclimatised to research. A more streamlined, less costly, and easier to run trial could be delivered with blood sampling just at the 2- and 3-hour mark post-prandially. Another potential cohort is that of chronic pancreatitis, however this cohort has proven more difficult to recruit with a higher incidence of smoking (patients unwilling to comply with non-smoking requirements for the ^{13}CMTGT) and lesser engagement with health services. PPIE work targeted specifically at CP patients should be done before further attempts at recruitment of this cohort.

Finally, should validation cohorts support these metabolites as a biomarker panel, a dosing study to evaluate whether PERT can effectively return the biomarker panel to (or towards) normal should be instigated.

To conclude, this work supports the hypothesis that the post-prandial metabolome in patients with PEI is significantly different to those without. A promising metabolomic panel for identifying PEI with an excellent AUC has been developed, with further work this has the potential to greatly improve PEI diagnosis and treatment.

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Appendices

Appendix 1 – Research Ethics Committee and UHB approval documents
- HRA/REC approval of original IRAS application 271410
- UHB authorisation of original IRAS application 271410
- SA01, SA02

Appendix 2 – PPIE feedback questionnaire for the CRAG group.

Appendix 3 – Patient facing documents (latest versions)
- Patient information leaflet V1.3
- Consent form V1.3
- Step by step guide to the 13C MTGT
- PEI-Q

Appendix 4 – Full search terms for SR/MA on 13C MTGT accuracy and methodology

Appendix 5 – Auto calculate Excel with worked example from P1 for PDR, cPDR and breath curves.

Appendix 6 – Summary of formula.

Appendix 7 – Statistically significant metabolites at baseline (one way ANOVA, 3 class analysis)

Appendix 8 – Statistically significant metabolites over time

Appendix 9 - Publications and presentations relating to this work

Appendix 1 – The DETECTION study approvals (from the West Midlands Research Ethics committee and University Hospital Birmingham. RRK6813, IRAS 271410.)

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HCRW.approvals@wales.nhs.uk

12 December 2019

Dear Mr Roberts

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**The development of a metabolomic test to diagnose and quantify pancreatic exocrine insufficiency.**
IRAS project ID:	**271410**
Protocol number:	**RRK6813**
REC reference:	**19/WM/0358**
Sponsor	**University Hospital Birmingham**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

Please now work with participating NHS organisations to confirm capacity and capability, in line with the instructions provided in the "Information to support study set up" section towards the end of this letter.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report

R&D Governance Office University Hospitals Birmingham NHS
NHS Foundation Trust

(RPAv46)

Dr Keith Roberts
GI Surgery
QEHB

UHB Research Governance Office
1st Floor, Institute of Translational Medicine
Heritage Building
Queen Elizabeth Hospital Birmingham
Mindelsohn Way
Edgbaston
Birmingham B15 2TH
Tel. 0121 371 4185

Research Project Authorisation

Project reference:**RRK 6813**

Main Ethics Committee Reference	19/WM/0358
IRAS Project ID	271410

24th August 2020

Dear Dr Roberts,

The DEvelopmenT of a mEtabolomiC Test to dIagnOse and quantify paNcreatic exocrine insufficiency

Thank you for submitting details of your proposed research project, which I am happy to authorise on behalf of University Hospitals Birmingham; this includes confirmation of Capacity and Capability under the HRA Approval process.

Approval covers the following site(s) only: **Queen Elizabeth Hospital Birmingham**

The following main document versions were reviewed (note this is not a complete list of all documents submitted):

Protocol - version: V1.2 07/11/19

Participant information sheet (main) - version: V1.2 03/12/19

Participant consent form (main) - version: V1.2 03/12/19

Acv3/19

Sponsorship
University Hospital Birmingham NHS Trust has agreed to act as sponsor for this study.

Indemnity arrangements.
Researchers who hold substantive or honorary contracts with University Hospital Birmingham (UHBT) will be covered against claims of negligence by patients of UHBT under the Clinical Negligence Scheme for Trusts (CNST). This scheme does not cover 'no fault' compensation and the Trust is precluded from taking out separate insurance to cover this. Any patient or volunteer taking part in the study is entitled to know that if

Health Research Authority

West Midlands - Black Country Research Ethics Committee

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

21 December 2020

Miss Sarah Powell-Brett
University Hospital Birmingham
B15 2TH

Dear Miss Powell-Brett

Study title:	**The development of a metabolomic test to diagnose and quantify pancreatic exocrine insufficiency.**
REC reference:	**19/WM/0358**
Protocol number:	**RRK6813**
Amendment number:	**Substantial Amendment 01**
Amendment date:	**04.11.20**
IRAS project ID:	**271410**

The above amendment was reviewed 17 December 2020 by the Sub-Committee in correspondence.

Ethical opinion

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*
Completed Amendment Tool [Amendment tool]	1.2	04 November 2020
Research protocol or project proposal [DETECTION Protocol]	1.3	04 November 2020

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

NHS
Health Research Authority
West Midlands - Black Country Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0207 104 8141

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

01 September 2021

Miss Sarah Powell-Brett
University Hospital Birmingham
B15 2TH

Dear Miss Powell-Brett

Study title:	**The development of a metabolomic test to diagnose and quantify pancreatic exocrine insufficiency.**
REC reference:	**19/WM/0358**
Protocol number:	**RRK6813**
Amendment number:	**Substantial Amendment 02**
Amendment date:	**10 August 2021**
IRAS project ID:	**271410**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

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*
Completed Amendment Tool [Locked Amendment tool]	1.0	10 August 2021
Participant information sheet (PIS) [DETECTION PIS (tracked changes)]	1.3	07 August 2021
Participant information sheet (PIS) [DETECTION PIS (Clean)]	1.3	07 August 2021
Research protocol or project proposal [DETECTION Protocol (tracked changes)]	1.3	07 August 2021
Research protocol or project proposal [DETECTION Protocol (Clean)]	1.3	07 August 2021

Appendix 2: Collated results from PPIE work conducted through the Birmingham Heartlands Hospital CRAG group

NHS
University Hospitals Birmingham
NHS Foundation Trust

Feedback form coffee morning 12 June 2012

Presenter: Sarah Powell-Brett
Presentation Title: Development of a metabolomic test to diagnose and quantify pancreatic exocrine insufficiency (The DETECTION study)
Contact: Sarah.powell-brett1@nhs.net

Please tick as appropriate	Strongly agree	Agree	Neither agree nor disagree	Disagree	Strongly disagree
Was the presentation easy to understand.	8	3			
Trial expectations and patient worth					
There is a need for a more acceptable diagnostic test	5	6			
There is a need to target treatment dosing	6	5			
A blood test is preferable to a faeces test	5	5	1		
This body of work could be valuable to the target population	6	5			
Study day acceptability					
A start time of 7am is acceptable		8	2	1	
Is a nutritional drink acceptable for breakfast	4	7			
6-hour starvation (after a set breakfast) is acceptable	1	7	1	2	
Hourly blood tests for 6 hours is acceptable			5	5	1
Breath Sampling for 6 hours is acceptable	6	5			
Patient facing documents					
The patient information leaflet is easy to understand	1	3	3	4	
The patient information leaflet contains all relevant information	1	8	2		
The consent form is easy to understand and complete	4	7			
General feedback					

Update: 5.6.18
Review date: 4.6.19

Chair: Rt Hon Jacqui Smith
Chief Executive: Dame Julie Moore

For the finalised test what would you consider an acceptable timeframe for testing: 1, 2 or 3 hours etc. after a fatty meal	4 hours maximum if starving 4 hours ideally max 2 but could be 3/4 and still be useful 4 or 5 limit hard to say maybe 3 or 4 hours uncertain depends if can eat and drink and move around, if no then probably only 2 or 3 hours 1/2 I wouldn't want to not eat for more than 3 or 4 hours shorter the better
Are there any barriers to recruitment for this study	patients with palliative disease or very old might find the day too difficulty (long, blood tests, food) need for no food for 6 hours and if person has had lots of chemo they may not wat many blood tests you cohort may be frail, recruitment electronically may not work, better face to face or on the phone no long day for this group of people multiple blood tests - NO TEA FOR 6 HOURS the study day may be difficult for some, may be coming a long way, parking is expensive at QE, especially for that long. Would you reimburse parking or pay for the study travel cost, arrival time and how long not eating or drinking
What further patient/public involvement do you think this trial design needs	you talked about a test meal, I don't like all of those drinks should we do a repeat CRAG day testing the different flavour information leaflet lay summary needs some changes, I will email teresa with my thoughts the meal needs testing and the information leaflet needs shortening would like to see the results, exciting work meal used could be trialled with. us continue involvement through whole trial - nice presentation, look forward to the end results need to find out how much patient expenses you could afford to pay would be nice to try out different meals if its the only thing they will eat for all morning

Appendix 3: Patient facing documents.

DETECTION-Study. Consent Form: Version 1.2
3/12/2019

INFORMED CONSENT FORM

Study Title: The development of a metabolomic test to diagnose and quantify pancreatic exocrine insufficiency. (The DETECTION Study)

Principle Investigator: Mr Keith Roberts

Participant Number: _____

		INITIALS
1	I have read and understood the Patient Information Sheet/Healthy control information sheet version 1.2; dated 03/12/2019 for the above study and I confirm that the study procedures and information has been explained to me. I have had the opportunity to ask questions and I am satisfied with the answers and explanations provided.	☐
2	I understand that my participation in this study is voluntary and that I am free to withdraw at any time, without giving a reason and without my medical care or legal rights being affected. I understand that data collected up to my time of withdrawal may be used.	☐
3	I understand that sections of my medical notes or information related directly to my participation in this study may be looked at by responsible individuals from the sponsor, regulatory authorities and research personnel where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
4	I give permission that my samples taken as **part of this study** will be stored for analysis. No personal information will be sent to another laboratory.	☐
5	I agree to participate in the DETECTION study.	☐
6	I give permission for the results of my blood samples, breath samples and patient questionnaires to be used **in other future ethically approved studies (once made anonymous)**.	☐

_____ _____ _____
Name of patient Signature Date

_____ _____ _____
Name of person taking consent Signature Date

1 copy for the patient, 1 copy for the study team, 1 copy to be retained in the hospital notes.

University Hospital Birmingham NHS
NHS Foundation Trust

Patient information sheet

Title of Project

Study Title: The development of a metabolomic test to diagnose and quantify pancreatic exocrine insufficiency (The DETECTION Study)

An Invitation to Take Part in Research

Before you decide to take part you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully.

What Is The Research Study About?

Pancreatic exocrine insufficiency (PEI) is very common in chronic pancreatitis, cystic fibrosis and pancreatic cancer; it results in a failure of the enzymes produced by the pancreas to aid in the digestion of food and leads to weight loss, malnutrition and gastrointestinal symptoms. The current widely used test has low accuracy, takes days for results to be available and is disliked by patients. It performs particularly poorly after surgery and cannot be used to guide treatment dose with pancreatic enzymes. This proposal is to use the study of metabolites (small molecules in the blood) to develop a 'fingerprint' of PEI from a person's blood. This will ultimately let us develop a relatively quick and easy blood test that can diagnose PEI and can be used to ensure patients are on the correct dose of enzyme therapy.

Early diagnosis of PEI and correction of malnutrition is a basic and fundamental way to ensure well-being. Treatment with enzyme corrects malnutrition, weight loss, improves symptoms and length of survival. Yet many patients do not receive enzyme therapy. One major barrier to treatment are the failings of current tests which mean they are used infrequently leading to lack of appropriate diagnosis. Thus, the development of a simple blood test to diagnose PEI will improve diagnosis and treatment of pancreatic failure which will in turn improve patients' lives.

Do I Have To Take Part?

It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show that you have agreed to take part. You are free to withdraw

Appendix 4: Full search terms for SR of 13C MTGT methodology and accuracy
Note search updated when written up for publication in 2022

("exocrine pancreatic insufficiency"[MeSH Terms] OR "pancreas, exocrine"[MeSH Terms] OR "pancreatic exocrine insufficiency"[Title/Abstract] OR "pancreatic exocrine failure"[Title/Abstract] OR "exocrine insufficiency"[Title/Abstract] OR "exocrine failure"[Title/Abstract] OR "pancreatic function"[Title/Abstract] OR "pancreatic failure"[Title/Abstract] OR "PEI"[Title/Abstract] OR "EPI"[Title/Abstract] OR "pancreatic dysfunction"[Title/Abstract]) AND ("breath tests"[MeSH Terms] OR "breath tests/methods"[MeSH Terms] OR "13C"[Title/Abstract] OR "13C-MTG"[Title/Abstract] OR "13-Carbon"[Title/Abstract] OR "13CO2"[Title/Abstract] OR "MTBT"[Title/Abstract] OR "MTGT"[Title/Abstract] OR (("mixed"[All Fields] OR "mixes"[All Fields] OR "mixing"[All Fields] OR "mixings"[All Fields]) AND "tryglyceride"[Title/Abstract]))

Pubmed terms – 279 results (17 additional since 2020 July, 4 for inclusion)

Embase <1974 to 2022 August 03>

1 13C.mp. or exp carbon 13/ 36209
2 13C-MTG.mp. 32
3 13C mixed triglyceride.mp. 61
4 MTBT.mp. 34
5 MTGT.mp. 19
6 breath test.mp. or exp breath analysis/ 23102
7 1 or 2 or 3 or 4 or 5 or 6 55572
8 exp pancreas exocrine insufficiency/ or exocrine insufficiency.mp. or exp exocrine pancreatic insufficiency/ 4333
9 exocrine failure.mp. 78
10 pancreatic function.mp. or pancreas function/ 7794
11 exocrine pancreas.mp. or exp exocrine pancreas/ 4997
12 8 or 9 or 10 or 11 15746
13 7 and 12 324

EMBASE 324 results, (30 additional since July 2020 – 2 for inclusion)

Appendix 5: Excel spreadsheet autocalculate with worked example from P1

Study ID	P1						
13C MTG (mg)	250			**BSA (m2)**	Haycock	1.768587282	
Height	160			**VCO2 (mmol/h)**	Shreeve	530.5761847	
Weight	69			**Tracer dose (mmol)**	13CMTG(mg)/	0.332889481	
Date	05/08/2020			**Tracer enrichment**	(99-1.11) x 10	979000	

RAW RESULTS

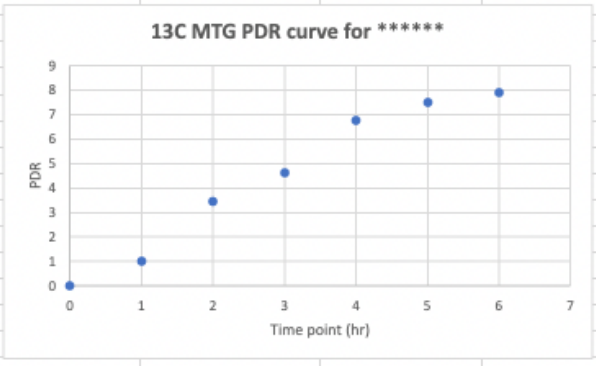
Sample label	Time pt (hr)	$\delta^{13}C_{V\text{-}PDB}$ (‰)	Atom%
P1 0hr A	0	-26.7	1.08188
P1 1hr A	1	-26.14	1.0825
P1 2hr A	2	-24.76	1.08401
P1 3hr A	3	-24.13	1.08471
P1 4hr A	4	-22.92	1.08604
P1 5hr A	5	-22.52	1.08648
P1 6hr A	6	-22.29	1.08674

$$PDR\ h^{-1} = \frac{VCO_2(mmol.h^{-1})\ x\ Breath\ CO_2\ enrichment\ (ppm\ 13C\ excess) x\ 100}{Tracer\ dose\ (mmol)\ x\ Tracer\ enrichment\ (atom\ \%\ excess\ x\ 10^4)}$$

Raw data
Intermediate
Results

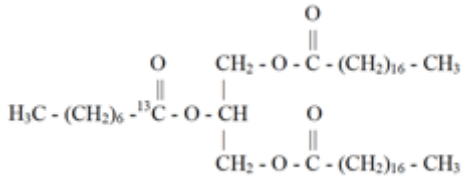
Time point	Atom % (AP) 13C in sample	STEP 1: Convert AP to PPM	Step 2: calculate PPM excess	Step 3: Top of PDR equation	Step 4: bottom of PDR equation	Step 5: PDR	Step 6: cPDR
(hr)		Atom % x 10,0000	sample ppm-baseline ppm	VCO2 x ppm excess x 100	Tracer dose x Tracer enrichment	Step 3/Step 4	
0	1.08188	10818.8	0	0	0	0	0
1	1.0825	10825	6.2	328957.2345	325898.8016	1.009384609	0.504692305
2	1.08401	10840.1	21.3	1130127.274	325898.8016	3.467724545	2.743246882
3	1.08471	10847.1	28.3	1501530.603	325898.8016	4.60735233	6.780785319
4	1.08604	10860.4	41.6	2207196.929	325898.8016	6.772645121	12.47078404
5	1.08648	10864.8	46	2440650.45	325898.8016	7.488982586	19.6015979
6	1.08674	10867.4	48.6	2578600.258	325898.8016	7.912272906	27.30222564

Time point	PDR
0	0
1	1.009384609
2	3.467724545
3	4.60735233
4	6.772645121
5	7.488982586
6	7.912272906



Appendix 6: **Summary of formula**

Molecular formula of the synthetic triacyglycerol used for the 13C MTGT (2-OCTANOYL-1,3-DISTEARIN)



Calculating Atom percent (to measure absolute isotopic concentration)

- atom % $(AP)\ 13C\ = \frac{[13C]}{[12C]+[13C]}\ x\ 100$

Calculating Atom percent excess

- Atom % excess (APE) $= (atom\ \%)_E\ -\ (atom\ \%)_B$

- $(atom\ \%)_E$ is the abundance of the enriched sample.
- $(atom\ \%)_B$ is the abundance of the baseline sample.

Calculating VCO2 – using formula by Shreeve et al

- $VCO_2(mmol.h^{-1}) = 300\ x\ body\ surface\ area$

Calculating BSA – Using the Haycock formula

- $BSA\ (m^2) = (weight\ (kg)^{0.5378})\ x\ (height(cm)^{0.3964})\ x\ 0.024265$

Calculating Tracer dose

- $Tracer\ dose\ (mmol) = \frac{weight\ substrate\ (mg)}{molecular\ weight\ substrate}$

Calculating percent dose recovery at that time point

- $PDR\ h^{-1} = \frac{(300\ x\ BSA)\ x\ Breath\ CO_2\ enrichment\ (ppm\ 13C\ excess)\ x\ 100}{(\frac{13MTG(mg)}{751})\ x\ (97.9\ x\ 10^4)}$

Appendix 7: Statistically significant metabolites at baseline

Using a one-way ANOVA comparing phenotype, top 15 most significant

Metabolite	UHPLC assay	BH adj.p-value
PE-Cer(d14:1(4E)/20:1(11Z))||PE-Cer(d14:2(4E,6E)/20:0)||PE-Cer(d16:2(4E,6E)/18:0)||PE-Cer(d14:1(4E)/20:0)||PE-Cer(d16:1(4E)/18:0)||16:1 Cholesterol ester||16:1 Cholesterol ester||5-dihydroergosteryl-pentadecylate||CE(16:1(9Z))||Vitamin D3 palmitate	HILIC Positive	0.001195
PE-Cer(d14:2(4E,6E)/21:0)||PE-Cer(d15:2(4E,6E)/20:0)||PE-Cer(d16:2(4E,6E)/19:0) || SM(d18:1/14:1(9Z))||SM(d18:2(4E,14Z)/14:0)||SM(d18:2/14:0)||16:2 Campesterol ester||episteryl palmitoleate||fecosteryl palmitoleate||16:2 Campesterol ester||episteryl palmitoleate||fecosteryl palmitoleate||16:2 Campesterol ester||episteryl palmitoleate||fecosteryl palmitoleate	HILIC Positive	0.001195
5,6-Dihydrouridine||Creatine riboside	HILIC Positive	0.0025185
3-Hydroxy-N6,N6,N6-trimethyl-L-lysine	HILIC Positive	0.0033202
PC(14:0/20:3(5Z,8Z,11Z))||PC(14:0/20:3(5Z,8Z,11Z))||PC(14:0/20:3(8Z,11Z,14Z))||PC(14:0/20:3(8Z,11Z,14Z))||. PC(14:1(9Z)/20:2(11Z,14Z))||PC(14:1(9Z)/20:2(11Z,14Z))||PC(16:0/18:3(6Z,9Z,12Z))||PC(16:0/18:3(6Z,9Z,12Z))||PC(16:0/18:3(9Z,12Z,15Z))||PC(16:0/18:3(9Z,12Z,15Z))||PC(16:1(9Z)/18:2(9Z,12Z))||PC(16:1(9Z)/18:2(9Z,12Z))||PC(17:1(9Z)/17:2(9Z,12Z))||PC(17:2(9Z,12Z)/17:1(9Z))||PC(18:2(9Z,12Z)/16:1(9Z))||PC(18:2(9Z,12Z)/16:1(9Z))|	HILIC Positive	0.0039725
2-Hepteneoylglycine||3-Hepteneoylglycine||4-Hepteneoylglycine||5-Hepteneoylglycine||Ecgonine||Carnitine	HILIC Positive	0.0054177
PE-Cer(d14:2(4E,6E)/21:0)||PE-Cer(d15:2(4E,6E)/20:0)||PE-Cer(d16:2(4E,6E)/19:0)||SM(d18:1/14:1(9Z))||SM(d18:2(4E,14Z)/14:0)||SM(d18:2/14:0)||16:2 Campesterol ester||episteryl palmitoleate||fecosteryl palmitoleate	HILIC Positive	0.0054177
N1-Acetylspermidine||N8-Acetylspermidine	HILIC Positive	0.0067239
PC(0:0/PGE2)||PKOHA-PE||Kanokoside D||SQMG(18:0/0:0)||Oxyacanthine||PKOOA-PA	HILIC Positive	0.0067239
Cer(d40:2)	HILIC Positive	0.0069471
alpha-Carotene||gamma-Carotene	HILIC Positive	0.007984
Betaine aldehyde	HILIC Positive	0.0082032
alpha-Carotene||gamma-Carotene	HILIC Positive	0.0084406
PE-Cer(d14:1(4E)/20:0)||PE-Cer(d16:1(4E)/18:0)||PE-Cer(d14:1(4E)/20:0)||PE-Cer(d16:1(4E)/18:0)||PE-Cer(d14:1(4E)/20:0)||PE-Cer(d16:1(4E)/18:0)||PE-Cer(d14:1(4E)/20:0)||PE-Cer(d16:1(4E)/18:0)	HILIC Positive	0.0084406
3-Sialyl-N-acetyllactosamine||6-Sialyl-N-acetyllactosamine	HILIC Positive	0.010792

Appendix 8 : Statistically significant metabolites: interaction between phenotype and time

Metabolite	Metabolite class	Interaction(BH adj.p-value)
(23R)-3alpha,5beta,7alpha,23-Tetrahydroxycholan-24-oic Acid||3beta,4beta,7alpha,12alpha-Tetrahydroxy-5beta-cholan-24-oic Acid||3a,7b,12a-Trihydroxyoxocholanyl-Glycine||3a,7b,12a-Trihydroxyoxocholanyl-Glycine||Glycocholate||Glycocholic Acid||	Sterol and steroid metabolism	0.000000000000257400
Pseudouridine||Uridine	Water-soluble metabolites	0.000000000138800000
N1-Methyl-2-pyridone-5-carboxamide||N1-Methyl-4-pyridone-3-carboxamide||2-Amino-4-hydroxy-3-methylpentanoic acid||2-amino-6-hydroxyhexanoic acid	Water-soluble metabolites	0.000000000152460000
S-Glutaryldihydrolipoamide	Water-soluble metabolites	0.000000013781000000
N-Phenylacetyl pyroglutamic acid||tetradecadienoic acid	Mixed class	0.000000027126000000
hexacosatrienoic acid	Fatty acid	0.000000075042000000
Chenodeoxyglycocholic acid||Glycochenodeoxycholic acid||Glycoursodeoxycholic acid	Bile acid	0.000000113060000000
LysoPG(O-18:0)	Lysoglycerophospholipid	0.000000336600000000
hexenedioylcarnitine||3-Methylglutarylcarnitine||O-Adipoylcarnitine	Acyl carnitines	0.000000413820000000
2-Pentanamido-3-phenylpropanoic acid	Water-soluble metabolites	0.000001075800000000
Cholesterol sulfate	Sterol and steroid metabolism	0.000001679000000000
Pyruvic acid||Serine	Water-soluble metabolites	0.000002428800000000
2-Formylglutarate||2-Methyl-4-oxopentanedioic acid||2-Oxoadipate||3-Oxoadipic acid|2-Aminomuconate semialdehyde	Water-soluble metabolites	0.000002725800000000
Glycocholic acid||Glycohyocholic acid	Bile acid	0.000003154800000000

Appendix 9

Publications resulting from PhD and background work (abstracts not included)

Comprehensive, long-term evaluation of pancreatic exocrine insufficiency after pancreatoduodenectomy - 1st Author, Accepted 'Pancreatology' October 2023	Submitted Sep. 2023
A standardised nutritional drink as a test meal for the ^{13}C mixed triglyceride breath test for pancreatic exocrine insufficiency. A randomised, 2-arm crossover comparative study. - 1st Author. 'JHND' 2023, DOI:10.1111/jhn.13237	Published Aug. 2023
Casting a Wider NET: PEI Induced by Somatostatin Analogues among Patients with Neuroendocrine Tumours? - 2nd Author. 'Cancers' 2023, 15(7), PMID: 37046594	Published Mar. 2023
A systematic review and meta-analysis of the accuracy and methodology of the ^{13}C mixed triglyceride breath test for the evaluation of pancreatic function. - 1st Author. Published Pancreatology 2023 Feb. S1424-3903(23)00040-6. PMID 36805050	Published Feb. 2023
Survival benefit of PERT in patients undergoing treatment of pancreatic neuroendocrine tumours - Middle author, 'HPB'. 2022 Jun. doi.org/10.1016/j.hpb.2022.06.001	Published Jun. 2022
Achieving 'Marginal Gains' to Optimise Outcomes in Resectable Pancreatic Cancer - 1st Author (joint). Cancers 2021 Apr 1;13(7):1669. PMID: 33916294	Published Apr. 2021
Understanding pancreatic exocrine insufficiency and replacement therapy in pancreatic cancer. - 1st Author. 'Eur J Surg Oncol'. PMID 32178962	Published Mar. 2021
Management of pancreatic exocrine insufficiency. - 1st Author. 'Textbook of pancreatic cancer'. Chapter 43. ISBN: 978-3-030-53786-9	Published Feb. 2021
Pancreatic exocrine insufficiency after pancreaticoduodenectomy: Current evidence and management - Middle Author. 'World J Gastrointest Pathophysiol'. PMID 32318312	Published Apr. 2020

Presentations resulting from PhD and background work (Posters not included)

Oral: Investigating the Metabolome of PEI in pancreatic cancer - Invited speaker PSGBI conference: Sheffield 2023	Nov. 2023
Oral: Novel standardised test meal for the ^{13}C MTGT. randomised 2-arm crossover comparative study - PSGBI conference: Lord Aston Medal presentation: Leeds 2022 – First prize	Nov. 2022
Oral: Improving PEI diagnosis and management in pancreatic cancer - Invited speaker VIATRIS ® Webinar on the future of pancreatic cancer	Nov. 2022
Oral: Development of 13CMTG breath test for use in a U.K. research setting - Invited speaker RCS pancreatic symposium London 2022	May. 2022
Oral: The DETECTION study: Pilot results and recruitment - Invited speaker RCS pancreatic symposium London 2022	May. 2022
Oral: The DETECTION study: Pump priming grant update - Invited speaker AUGIS UGI Congress Belfast 2021	Oct. 2021
Oral: PEI in pancreatic cancer, status, and future. - Invited Presentation at YOUPPIE section of the European Pancreatic Club Virtual Congress 2021	Jul. 2021