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Contemporary approaches to the diagnosis and management of

Pancreatic Ductal Adenocarcinoma, examining the role of

biomarkers in aiding early diagnosis

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THESIS ABSTRACT

Whilst other cancers have seen improvements in survival over recent decades, Pancreatic Ductal Adenocarcinoma (PDAC) remains a disease with poor outcomes. At present no screening tests exist to detect pancreatic cancer at an early stage in the asymptomatic population. There is an increasing interest in novel ways to detect pancreatic cancer at an earlier stage in the disease process when a potential cure is more likely to be achieved.

A literature review was undertaken of the current understanding and management of this devastating disease, focussing on aetiology, current methods of cancer diagnosis and staging, and therapeutic options. A feasibility study was then undertaken to evaluate the diagnostic accuracy of a selection of novel candidate biomarkers to differentiate between plasma and urine obtained from participants with and without pancreatic cancer, comparing them with the current gold standard biomarker, Ca19-9, which is often used with a cut-off concentration of 37U/L. Enzyme-Linked-Immunosorbent Assay (ELISA) was used to quantify concentrations of Ca19-9, Thrombospondin-2 (THBS2) and Human Chitinase 3-like 1 (YKL-40). Samples were analysed using Fourier Transform Infrared (FTIR) spectroscopy, with the spectra of cancer and non-cancer specimens being compared, allowing a machine-learning diagnostic model to be created.
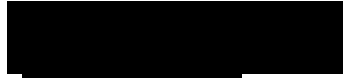
In isolation, plasma Ca19-9 had the greatest ability to discriminate between cancer and non-cancer (AUC = 0.885). However, a multi-analyte panel (comprising plasma Ca19-9, plasma THBS2 and urinary THBS2) was found to have a greater diagnostic accuracy to discriminate between the 2 groups when compared to using the widely used Ca19-9 cut-off of 37U/L (83.33% vs 76.6%). A diagnostic model using FTIR spectroscopy had a diagnostic accuracy of >90%.

Pancreatic cancer remains a disease with poor outcomes, but there are promising new strategies to diagnose patients at an earlier stage. The initial results from these investigations are promising, but require validation with a larger test cohort

DECLARATIONS

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.
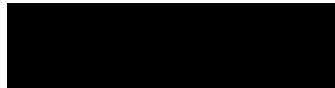
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This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.
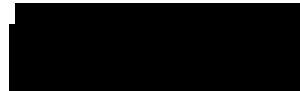
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ACKNOWLEDGEMENTS

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ABBREVIATIONS

ABMU	Abertawe Bro Morgannwg University Health Board
AJCC	American Joint Committee on Cancer
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BD-IPMN	Branch Duct Intraductal Papillary Mucinous Neoplasm
BMI	Body Mass Index (Weight (kg)/Height(m)^2)
BoP	Body of Pancreas
BSA	Bovine Serum Albumin
CA19-9	Carbohydrate Antigen 19-9
CA	Coeliac Axis
CBD	Common Bile Duct
CEA	Carcinoembryonic Antigen
CHA	Common Hepatic Artery
CHD	Common Hepatic Duct
CT	Computed Tomography
CNH	Centre for NanoHealth (ILS 2, Swansea University)
D1	1st part of the duodenum
D2	2nd part of the duodenum
D3	3rd part of the duodenum
D4	4th part of the duodenum
DCCa	Distal Cholangiocarcinoma
DGE	Delayed Gastric Emptying
DJF	Duodenojejunal Flexure
ELISA	Enzyme Linked Immunosorbent Assay
ERAS	Enhanced Recovery After Surgery
ERCP	Endoscopic Retrograde Cholangiopancreatography
ESPAC	European Study Group for Pancreatic Cancer
EUS	Endoscopic Ultrasound
FNA	Fine Needle Aspiration

FOLFIRINOX	Combination chemotherapy regimen consisting of Folinic acid, 5-Fluorouracil, Irinotecan and Oxaliplatin
FTIR	Fourier Transform Infrared Spectroscopy
GDA	Gastroduodenal Artery
GemCap	Combination chemotherapy regimen consisting of Gemcitabine and Capecitabine
GOO	Gastric Outlet Obstruction
GPC-1	Glypican-1
HGD	High-Grade Dysplasia
HoP	Head of Pancreas
HPB	Hepato-Pancreato-Biliary
HRP	Horse Radish Peroxidase
ICF	Informed Consent Form
ILS1	Institute of Life Sciences 1, Swansea University
ILS2	Institute of Life Sciences 2, Swansea University
IRE	Irreversible Electroporation
ISGPS	International Study Group on Pancreatic Surgery
IVC	Inferior Vena Cava
JSRC	Joint Scientific Research Committee
LAPC	Locally Advanced Pancreatic Cancer
LFT	Liver Function Test
LGD	Low Grade Dysplasia
LNR	Lymph Node Ratio
LSPLP	Laparoscopic Spleen Preserving Left Pancreatectomy
MCN	Mucinous Cystic Neoplasm
MD-IPMN	Main Duct Intraductal Papillary Mucinous Neoplasm
MDT	Multidisciplinary Team
MH	Morriston Hospital, Swansea
MPD	Main Pancreatic Duct
MRCP	Magnetic Resonance Cholangiopancreatography
NET	Neuroendocrine Tumour
NoP	Neck of Pancreas
OGD	Oesophago-Gastro-Duodenoscopy (Upper GI endoscopy)
PanIN	Pancreatic Intra-epithelial Neoplasia (graded 1a, 1b, 2, 3)

PBS	Phosphate Buffered Saline
PIS	Participant Information Sheet
PCN	Pancreatic Cystic Neoplasm
PDAC	Pancreatic Ductal Adenocarcinoma
PET-CT	Positron Emission Tomography - CT
PG	Pancreatico-Gastrostomy
PJ	Pancreatico-Jejunostomy
pNET	Pancreatic Neuroendocrine Tumour
POPF	Post-Operative Pancreatic Fistula
PPH	Post Pancreatectomy Haemorrhage
PPPD	Pylorus Preserving Pancreaticoduodenectomy
PTBD	Percutaneous Transhepatic Biliary Drain(s)
PTC	Percutaneous Transhepatic Cholangiography
RCPath	Royal College of Pathologists
REC	Regional Ethics Committee
RLP	Radical Left Pancreatectomy
SBUHB	Swansea Bay University Health Board
SCA	Serous Cyst Adenoma
SMA	Superior Mesenteric Artery
SMV	Superior Mesenteric Vein
SUMS	Swansea University Medical School
THBS2	Thrombospondin-2
TNM	Tumour Node Metastasis Staging system
ToP	Tail of Pancreas
UICC	Union for International Cancer Control
YKL-40	Human Chitinase 3-Like 1

CHAPTER 1

CONTEMPORARY APPROACHES TO THE DIAGNOSIS AND MANAGEMENT OF PANCREATIC DUCTAL ADENOCARCINOMA

1.1 SUMMARY

Pancreatic Ductal Adenocarcinoma (PDAC) remains a devastating disease, associated with dismal outcomes, despite several advances in pre-operative staging, surgical techniques and oncological strategies which have increased the number of patients able to undergo potentially curative surgery.

This chapter sets out to review the current understanding of the clinicopathological aspects of PDAC and how these affect outcomes amongst these patients. It will examine how the management of PDAC is evolving both for patients with potentially curable and non-curable diseases and present the evidence base for the contemporary management of this disease.

1.2 PANCREATIC STRUCTURE AND FUNCTION

The pancreas is an accessory organ of the gastrointestinal (GI) tract (Figure 1.1), producing a variety of enzymes which are key in the digestion and absorption of food. It is a foregut structure which lies behind the stomach, in a slightly oblique, horizontal orientation, within the retroperitoneum in the upper abdomen at the level of the 1st lumbar vertebra (the transpyloric plane). The left-sided tail lies slightly superior to the right-sided head (1).

In utero, the pancreas develops initially as 2 separate buds arising from the foregut gut tube in the 4th week (2). The larger, dorsal bud extends from the left-hand side of the gut. The smaller ventral bud branches off from the right side of the gut, sharing a common duct with the gallbladder which will ultimately develop into the Common Bile Duct (CBD). As the 2 buds develop, the ventral bud rotates in an anti-clockwise direction, behind the gut tube, where it then fuses with the dorsal pancreas.

Running from right to left it is divided into 4 zones: The head (and uncinate process) which lies within the duodenal C-loop. The neck overlies the Superior Mesenteric Vein (SMV) and Superior Mesenteric Artery (SMA). To the left-hand side of the SMA lie the body and tail. The distinction between the body and tail is not clearly defined. The arterial blood supply to the pancreas is derived predominantly from the branches of the Coeliac axis. The splenic artery runs along the superior border of the pancreas giving off branches to the body and tail. The proximal pancreas is supplied by a rich vascular arcade (the superior and inferior pancreaticoduodenal arteries) which forms a vascular anastomosis between the Common Hepatic Artery (CHA) – via the Gastroduodenal Artery (GDA) and the SMA respectively. Venous branches drain into the Splenic vein and SMV/Portal Vein. Understanding the relevant vascular anatomy and being aware of the different regions of the pancreas is key to understanding the principles of surgical management of different stages of pancreatic cancer (Figure 1.2).
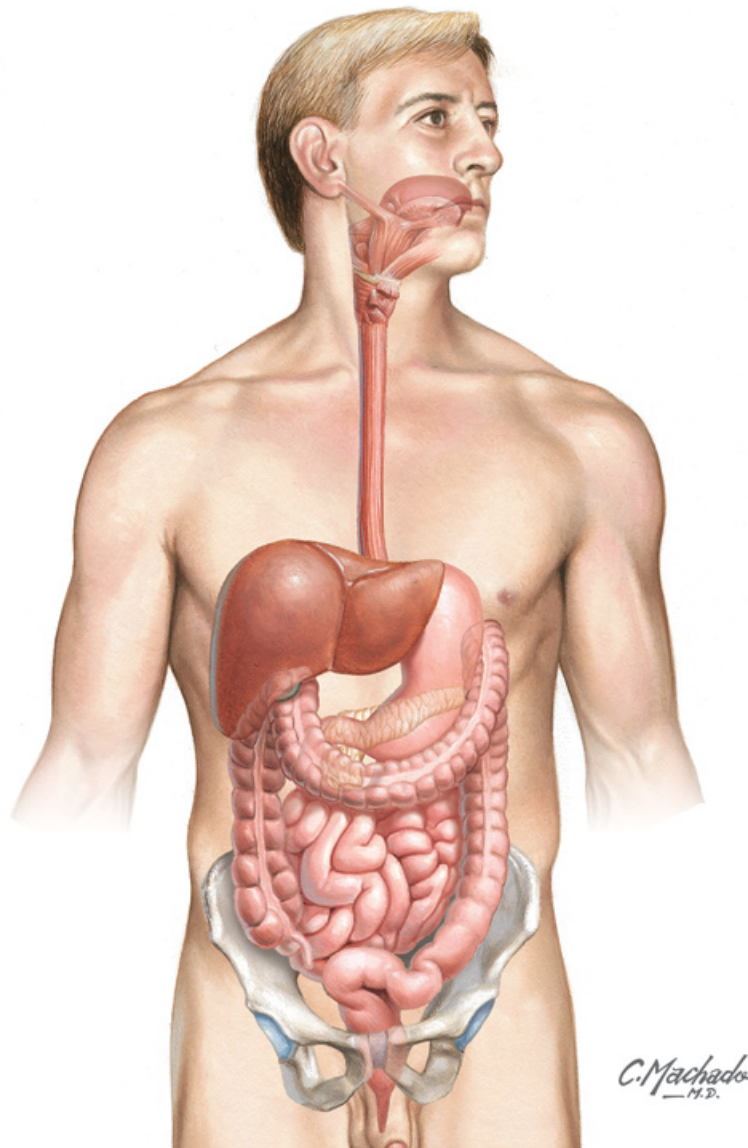
Figure 1.1 The Gastrointestinal (GI) Tract (Reproduced from Netterimages.com with permission from Elsevier).

The pancreas can be seen lying posterior to the stomach in the upper abdomen at the level of the L1 vertebra (the transpyloric plane).

Arteries of Stomach, Duodenum, Pancreas and Spleen
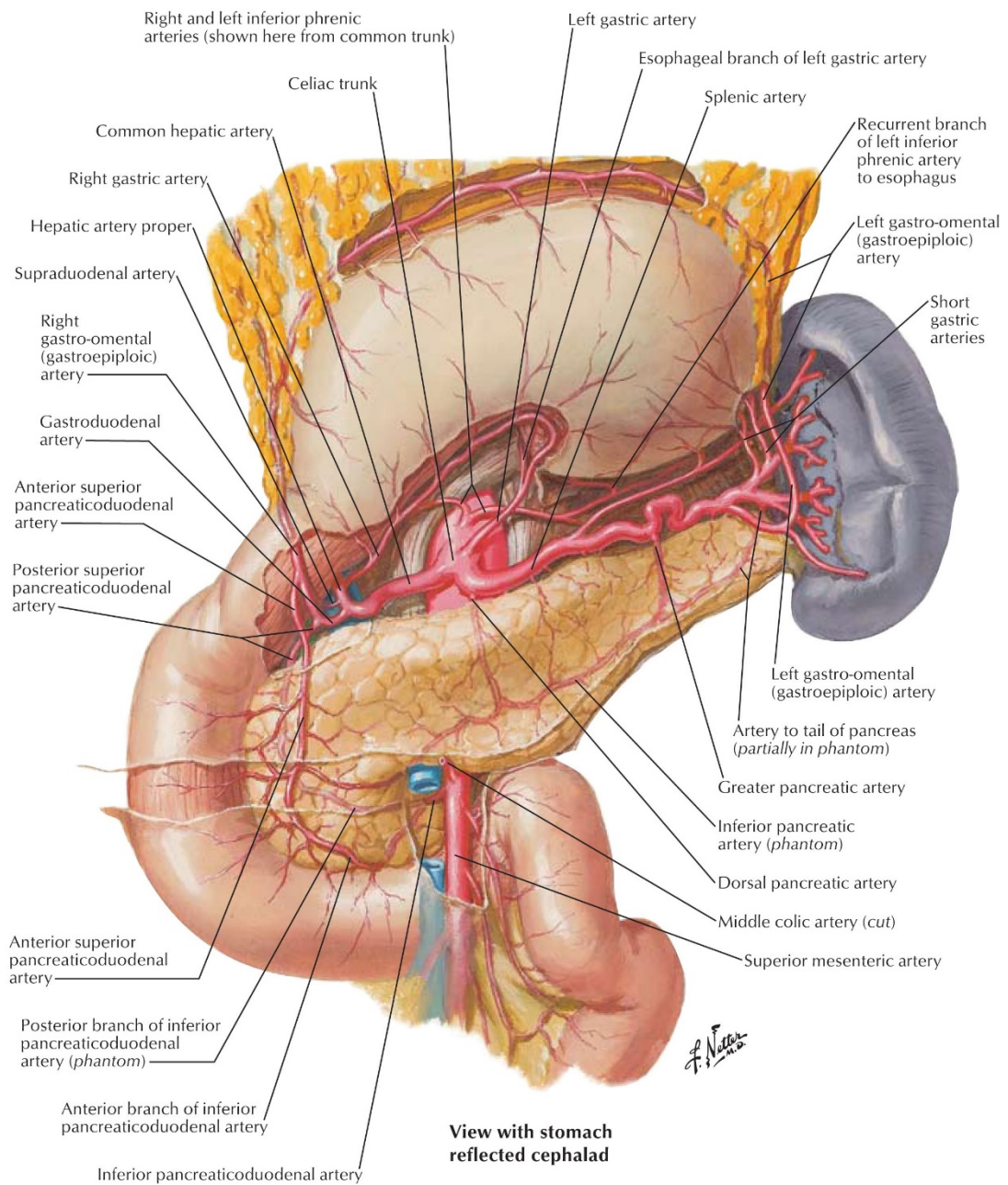
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Figure 1.2 Vascular supply of the pancreas (Reproduced from netterimages.com with permission from Elsevier Publishing).

The pancreas receives its blood from both the branches coeliac axis and the Superior Mesenteric Artery (SMA), via the rich Pancreaticoduodenal arcade and Splenic artery. Venous drainage is via the porto-mesenteric venous system

Internally, the pancreas is made up predominantly of exocrine acinar cells which form around epithelial lined ductules, which then drain into a larger Main Pancreatic Duct (MPD) or Duct of Wirsung. This too is also lined by glandular epithelium. The acinar cells synthesize and secrete a range of proteolytic enzymes which play an important role in the digestion and absorption of food. Within the head of the pancreas (HoP), in the majority of people, the MPD combines with the distal Common Bile Duct (CBD) at the Ampulla of Vater, producing a short common channel which then drains the pancreatic juice and bile into the second part of the duodenum, via the major papilla. In a minority of people, there is an incomplete fusion of the 2 ductal systems, leading to pancreas divisum.

Dispersed amongst the enzyme-producing exocrine cells are the Islets of Langerhans which consist of a variety of cells of Neuroendocrine origin which play an important role in, amongst other things, glycaemic control. These endocrine cells can lose cell regulation and develop into Pancreatic Neuroendocrine Tumours (pNET). However, these tumours have a much more indolent clinical course compared to PDAC, and the management is often different, therefore they will not be discussed further.

1.3   EPIDEMIOLOGY

Pancreatic Ductal Adenocarcinoma (PDAC) is currently the tenth most common cancer in the UK; however, it is the fifth most common cause of cancer-related mortality. Between 2016 and 2018, 10,452 new PDAC diagnoses were made in the UK, with an average of 9558 deaths from the disease annually between 2017 and 2019 (3). Of the 22 most common cancers in the UK, PDAC carries the worst prognosis (4). Median survival following diagnosis of untreated PDAC is just 4-6 months. Overall one-year survival is just 25%, with five-year survival at just 7.3% (3).

On a worldwide scale, more than 330,000 patients are newly diagnosed with PDAC each year, with nearly as many patients succumbing to the disease (5). Despite many other more common cancers showing an improvement in prognosis, overall survival for patients with PDAC appears to in fact be getting worse, and it is predicted that by 2030, it will be the second most common cause of cancer-related death within the USA (6).

The majority of patients (up to 80%) will present with advanced PDAC at the time of diagnosis, meaning it is not amenable to surgical resection and therefore is deemed incurable. This will either be due to the involvement of major visceral blood vessels by direct spread (locally advanced cancer), or due to spread to distant organs (metastatic cancer) the most common sites for this being the liver, peritoneum and lungs.

1.4   RISK FACTORS

Several factors have been identified as putting individuals at increased risk of developing PDAC, including non-modifiable genetic factors, as well as modifiable lifestyle and environmental factors.

1.4.1   Age

Like many cancers, the incidence of PDAC increases with age. The diagnosis is extremely rare in those under 30, whilst the average age of diagnosis is in the 8th decade of life (3).

1.4.2   Gender

The incidence of pancreatic cancer appears to be slightly higher amongst males compared to females (7). Whether this difference is purely due to gender is not fully established with some studies, hypothesizing that this difference may be a reflection of different risk behaviours, in particular the fact that men are more likely to be smokers compared to females (8).

1.4.3   Ethnicity

It has been shown in several epidemiological studies, that ethnicity does affect one's risk of developing PDAC. In the USA, African Americans have been found to have a significantly higher incidence and mortality from PDAC compared to other ethnic groups (9). Potentially more alarmingly, multiple studies have found that patients of black ethnicity were less likely to receive like-for-like treatment for pancreatic cancer compared to white patients (10, 11). As well as less access to treatment for pancreatic cancer amongst non-caucasian populations, there is some evidence to suggest that ethnicity does have a direct effect on disease severity and response to treatment. A recent study by Irfan and colleagues reviewed outcomes in black and white patients who had undergone potentially curative surgery with or without adjuvant chemotherapy (12). This study found that black patients were likely to present at a younger age with larger tumours suggesting a more aggressive disease. Even when they underwent curative resection with adjuvant chemo, median overall survival was

shorter compared to the white cohort who underwent the same treatment (35 vs 21 months). Further studies into the underlying genetics and pathophysiology of pancreatic cancer, coupled with the large-scale PRECISION-Panc study and the offshoot PRIMUS studies looking into personalised medicine will hopefully one day remove this inequity (13)

1.4.4   Blood Type

ABO blood type has been shown to have an effect on the incidence of pancreatic cancer. A retrospective review in 2010 (14) showed that pancreatic cancer had a significantly lower incidence amongst individuals with O-type blood compared to all other blood types. The combined Odds Ratio for all other blood types (A, B, AB) was 1.38. The risk appeared to be reduced by the presence of an 'O' allele. Each non-O allele increased the risk of cancer (i.e., further analysis of those with B-serotype blood showed BB to increase the risk more than BO). The mechanism by which blood type can protect or put one at an increased risk of PDAC is not understood.

1.4.5   Smoking

Like many cancers, cigarette smoking has been shown to increase an individual's risk of developing PDAC. The number of cigarettes smoked positively correlates with an increased risk and ex-smokers still have an increased risk of developing PDAC compared to non-smokers for at least 10 years after stopping (15).

As well as increasing the risk of developing pancreatic cancer, it has been shown in a more recent study that cigarette smoking also has a detrimental effect on survival in those who are diagnosed with PDAC (16). This study analysed over a thousand patients with pancreatic cancer and found a hazard ratio of 1.37 when current smokers were compared to never smokers. The hazard ratio increased when the intensity of the smoking habit (as defined by pack/years) was further analysed. Interestingly there was no statistically significant difference in the survival of ex-smokers and non-smokers. Unlike the effect of smoking on the incidence of PDAC, there was no lag associated with the cessation of smoking and the return to overall survival as seen in non-smokers, suggesting that smokers diagnosed with PDAC should still be encouraged to stop smoking as this may improve their survival.

1.4.6 Diabetes Mellitus

There is an ongoing debate about whether the correlation between diabetes mellitus and PDAC is a result of diabetes causing cancer, or more likely whether cancer leads to a degree of endocrine failure of the pancreas and subsequent development of diabetes. With that said it is clear that patients with diabetes are more likely to be diagnosed with pancreatic cancer compared to those who do not have diabetes. A large study by Jamal and colleagues revealed that diabetics had an odds ratio of 3.22 for developing PDAC compared to non-diabetics (17). For this reason, new-onset diabetes or unexpected worsening glycaemic control in diabetics who are above 50 is now a recognised red flag sign that should lead to appropriate investigations to rule out pancreatic malignancy.

1.4.7 Obesity

Truncal obesity has been shown to increase the risk of developing PDAC, and this may be one of the reasons behind the increasing prevalence which is being seen in the Westernised World. Several studies have shown that those with a BMI which puts them overweight or obese have an increased risk of developing PDAC, with some studies finding a threefold increase in the risk of developing PDAC in those who have a BMI above normal (18). Perhaps more interestingly, a study from the MD Anderson Cancer Centre in Texas (USA) showed that obesity at a younger age (early adult life), not only increased the risk of developing PDAC in later life, but those who did go on to develop PDAC were likely to be diagnosed at an earlier age than those who were not overweight/obese as young adults (19). This increased risk was found to be independent of concomitant diabetes mellitus.

1.4.8 Chronic Pancreatitis

Chronic pancreatitis is an inflammatory condition of the pancreas which leads to a permanent change in the architecture of the gland, with fibrosis and calcification of the gland replacing the normal functioning exocrine and endocrine cells, ultimately leading to endocrine and exocrine failure (diabetes mellitus and malabsorption). It may be the result of repeated attacks of acute pancreatitis or may develop de novo with no pre-existing pancreatic pathology. Common causes of chronic pancreatitis are excess

alcohol intake and smoking, and both of these factors are known to exacerbate the symptoms. Autoimmune Pancreatitis (AIP) is often diagnosed in younger patients presenting with recurrent attacks of acute pancreatitis with radiological and clinical features of chronic pancreatitis, without the usual risk factors, but with high serum titres of the circulating immunoglobulin IgG4.

A personal history of Chronic Pancreatitis (CP) is one of the strongest risk factors for the subsequent development of Pancreatic cancer. A large study of 2000 patients with CP revealed a lifetime risk of 4% (20). This risk is highest within the first 2 years following the diagnosis of CP, with the association decreasing as time since diagnosis increases (21). This may be due to the CP being caused by an early PDAC as opposed to the other way round. The recurrent and continued inflammation is hypothesized to drive the malignant transformation in these patients, as is seen in some other chronic inflammatory conditions of the GI tract, (i.e., Inflammatory Bowel Disease and bowel cancer, atrophic gastritis and stomach cancer).

Patients who develop PDAC on a background of Autoimmune Pancreatitis (AIP) often develop cancer at an earlier age than those who develop it sporadically. This is likely due to the fact that these patients develop inflammatory changes associated with pancreatitis in their teens or early twenties.

1.4.9 Inherited Pancreatic Cancer

Pancreatic cancer has been noted to have a familial element and is occasionally associated with several cancer syndromes including Lynch Syndrome, Peutz-Jeghers Syndrome, BRCA1 and BRCA2.

Like many cancers, there is an increased risk of developing PDAC if a first-degree relative is diagnosed, but this does not necessarily occur as a result of a known cancer syndrome. In the UK, the EUROPAC study/surveillance programme, co-ordinated by the University of Liverpool, is gathering information regarding pancreatic cancer kindreds.

1.5   PATHOPHYSIOLOGY OF PANCREATIC CANCER

Pancreatic Ductal Adenocarcinoma (PDAC) accounts for 90% of malignant neoplasms of the pancreas (22) and is commonly what is referred to as pancreatic cancer. The remaining 10% are accounted for by pancreatic Neuroendocrine Tumours (pNETs) and a whole host of rarer malignancies including those arising from the pancreatic acinar cells.

As the name suggests, PDAC arises from the glandular epithelial cells of the pancreatic ducts. Like many cancers of the GI tract, the development of PDAC is a multi-step process involving a sequence of acquired genetic and epigenetic mutations affecting a variety of Tumour Suppressor Genes (TSG) and Proto-Oncogenes. This stepwise process is comparable to the adenoma-carcinoma sequence seen in colorectal cancer. As a result of this stepwise process, there are several recognisable pre-malignant entities which will be discussed later. Whilst these pre-malignant changes can often be seen microscopically in resected specimens, they are rarely seen on cross-sectional imaging, making early identification of potentially malignant lesions difficult.

Previous studies have implicated differences in bile acid composition in the development of PDAC, with a study by Rees et al. finding a significantly greater concentration of unconjugated bile acids (in particular unconjugated cholic acid) in patients with PDAC compared to those with benign disease (23).

The most commonly implicated genetic alterations in PDAC are those affecting the oncogene KRAS, and the Tumour Suppressor Genes – Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A/p16), Tumour Protein 53 (TP53) and SMAD4 (24). More than 90% of PDACs will have a KRAS mutation (25). The next most commonly affected gene is the p16/CDKN2A gene, present in 90% of ductal adenocarcinomas, (26), followed by TP53, seen in ~70-75% of tumours (27). Finally, SMAD4 is found to be inactivated in ~55% of pancreatic cancers (28). Changes in these genes are often also identified in PDAC pre-cursor lesions.

1.6   PRECURSOR LESIONS

Like many cancers, an increased understanding of the development of pancreatic cancer has demonstrated that it is a sequence of genetic and epigenetic alterations that ultimately lead to the development of invasive cancer, with several, distinct recognisable pre-malignant lesions developing before invasion occurs. With that said, the majority of patients diagnosed with PDAC will not have a pre-existing diagnosis of a pancreatic lesion.

1.6.1   Pancreatic Intraepithelial Neoplasia (PanIN)

Whilst non-invasive pancreatic ductal lesions had been known about as distinct pathological entities for several decades, the term Pancreatic Intraepithelial Neoplasia (PanIN) and the associated classification system were not internationally accepted until after the landmark paper by Hruban and colleagues in 2001 which sought to standardise the reporting and grading of these lesions (29). PanIN develops in a stepwise process as the result of identifiable genetic and epigenetic changes, ultimately leading to invasive PDAC.

PanIN lesions tend to be less than 5mm and as such are rarely seen on currently utilised imaging modalities (including EUS). Histologically they are distinguished from invasive PDAC by the fact that they do not cross the basement membrane. They are subclassified into PanIN-1A, PanIN-1B, PaniIN-2 and PanIN-3. PanIN-1A refers to flat lesions, whilst PanIN-1B lesions are papillary (30). PanIN-1 lesions show minimal levels of atypia. PanIN-2 lesions are seen to have moderate levels of cellular atypia. PanIN-3 lesions are synonymous with Carcinoma-In-Situ. These lesions demonstrate severe atypia and are the final step before the development of invasive PDAC.

Given the stepwise progression of PanIN towards invasive PDAC, it is unsurprising that PanIN is often found in the background pancreatic parenchyma within resected specimens from patients with PDAC. One study by Hisa and colleagues looked to map where PanIN was located in relation to the invasive tumour (31). All 21 examined specimens showed evidence of concurrent PanIN. As one may expect, when PanIN-3 was present, this was often found adjacent to the invasive tumour. However, the authors were unable to definitively say whether the adjacent PanIN-3 was an extension of the invasive tumour or a de novo lesion. It was not unusual for PanIN-1 and PanIN-

2 lesions to be found at sites distant from the invasive tumour, suggesting that there may be a generalised field change within the parenchyma of the pancreas in patients who develop PDAC.

A question remains regarding the significance of PanIN at the surgical margin in resected specimens for PDAC, in particular, PanIN-3. A retrospective study of patients who had undergone a margin clear (R0) resection at Johns Hopkins, Baltimore found that the presence of PanIN at the surgical resection margin had no significant effect on patient outcome (32). However, the authors do note there are multiple limitations to this study. No published studies have been able to definitively show that the presence of PanIN at the resection margins leads to diminished survival.

Whilst the recognition and identification of PanIN have helped to improve the understanding of the underlying pathological mechanisms of the development of PDAC, its clinical relevance at present is limited until it can be readily identified and diagnosed in a non-invasive manner prior to surgical resection.

1.6.2 Pancreatic Cystic Neoplasms (PCN)

As the quality of imaging modalities continues to improve, incidental cystic lesions of the pancreas are being detected with increasing frequency. It is important to be confident in assessing these lesions and determining which may have the potential to develop into invasive cancer, and which will remain harmless. Any cystic lesion picked up on a transabdominal ultrasound scan should be further investigated with dedicated pancreatic protocol Computed Tomography (CT) or Magnetic Resonance Imaging/Cholangiopancreatography (MRI/MRCP), with MRCP having a slightly higher accuracy at differentiating between the type of cyst (33). The development of Endoscopic Ultrasound (EUS) and the ability to obtain fluid from cystic lesions for cytological and biochemical analysis via Fine Needle Aspiration (FNA) has greatly improved the decision-making process when assessing pancreatic cysts which cannot be confidently characterised by cross-sectional imaging. However, as EUS is an invasive procedure with potential morbidity it should only be undertaken when there is diagnostic doubt, or the result will change clinical management.

Commonly seen cystic lesions of the pancreas include pancreatic pseudocysts, Serous Cystadenomas (SCAs), Mucinous Cystic Neoplasms (MCN) and Intraductal Papillary

Mucinous Neoplasms (IPMN). The first question in determining the nature of a pancreatic cyst is to determine whether it is mucinous or not, as mucinous lesions are known to have malignant potential. The presence of an elevated CEA level (>173ng/ml) in the cyst fluid suggests the cyst is mucinous in origin. The fluid amylase level should also be measured to determine whether the cystic lesion is associated with the pancreatic ducts.

Non-mucinous pancreatic cysts (pseudocysts and SCAs) have no significant malignant potential so will not be discussed further. MCNs and IPMNs are mucinous lesions and therefore do have the potential to develop into invasive malignancy, which will be discussed in more detail below.

1.6.2.1 Mucinous Cystic Neoplasms

MCNs are predominantly found in the body and tail of the pancreas. They are much more prevalent in females (up to 95%) (34). Radiologically they usually appear as solitary lesions with an identifiable cyst wall and are said to have an "orange-like appearance". The mean age of diagnosis is 44 years, with the mean age of invasive MCN being a decade later at 55 years of age (35). When cross-sectional imaging is not sufficient to secure the diagnosis of MCN, EUS and FNA may be undertaken. Cyst fluid biochemistry typically reveals an elevated CEA and a low/normal amylase.

A study by Keane and colleagues reviewed the histology of 211 resected MCNs, finding a 16.1% incidence of malignancy in the resected specimens, with a further 6.2% of resected specimens showing evidence of High-Grade Dysplasia (HGD) (36). Whilst MCNs are rare in males, the risk of malignant transformation was much higher in males compared to females (33% vs 15.3%). Whilst the most recent guidance recommends resecting all MCNs greater than 4cm in diameter (33), it is interesting that the study by Keane found that five of the 34 cysts which contained invasive cancer (6.8%) were found in cysts smaller than 4cm. The study identified 4 features which individually predicted the presence of invasive malignancy on multivariate analysis, including size, elevated Ca19-9, the presence of mural nodules and a history of weight loss.

1.6.2.2 Intraductal Papillary Mucinous Neoplasms

Like MCNs, IPMNs are mucin-producing neoplasms. However, the main histological difference between the two is the absence of ovarian-type stroma in IPMNs. IPMNs can be categorized as Main Duct (MD-IPMN), Branch Duct (BD-IPMN) or Mixed Type (MT-IPMN) based on where in the ductal architecture of the pancreas they originate from. Radiologically, MD-IPMNs tend to present with a dilated main pancreatic duct and BD-IPMNs typically show a "bunch of grapes" appearance. Suspected MD-IPMNs can be further assessed with side-viewing endoscopy which will often reveal mucin extruding from the Ampulla of Vater, which will have a so-called "Fish mouth" appearance.

The potential for malignant transformation is different depending on this pathological type. MD-IPMNs carry the highest potential for malignant transformation, with invasive cancer or HGD being identified in an average of 61% of resected specimens (34). As a result, it is recommended that all cysts that are suspected to be MD-IPMNs on imaging are resected if the patient is fit to undergo major pancreatic surgery. The situation with BD-IPMNs is not so clear cut, with a mean incidence of invasive carcinoma or HGD of 31% in resected specimens. To help risk stratify those patients who are at the highest risk for malignant transformation in BD-IPMNs, several studies have identified what has been labelled as "high-risk stigmata" and "worrisome features" to aid decision-making with regards to which of these lesions should undergo surveillance and which should be resected. High-Risk Stigmata (HRS) include the presence of obstructive jaundice in IPMNs within the head of the pancreas, enhancing mural nodules >5mm, and a dilated Main Pancreatic Duct >10mm. The presence of any one of these in a cyst suspected of being a BD-IPMN warrants surgical excision if the patient is fit enough. Worrisome features include an attack of pancreatitis, or imaging suggestive of a cyst >3cm, a small enhancing mural nodule <5mm, main duct dilatation of 5-9mm, an abrupt change in MPD calibre with distal atrophy, lymphadenopathy, elevated Ca19-9 or a growth rate of ≥5mm over 2 years. The presence of any one of these worrying features should prompt further investigation with Endoscopic Ultrasound (EUS) to allow closer visualisation of the cyst and aspiration of cyst fluid for cytology and biochemistry (including amylase and CEA levels).

Patients with IPMNs who do not satisfy the criteria for upfront resection should be assessed and counselled with regard to the need for ongoing surveillance and the possible need for surgery should the IPMN develop HRS. Given that the underlying need for surveillance is the potential need for future surgery, patients who would not be fit for surgery should not undergo surveillance.

Patients who undergo resection for IPMNs should undergo regular clinical and radiological follow-up until a time when the patient is deemed no longer fit for further surgery. The updated Tanaka guidelines suggest that high-risk IPMNs (those of gastric or pancreato-biliary subtype, the presence of HGD at the resection margin or a family history of PDAC) should undergo more intensive follow-up with imaging twice a year. Those deemed to be low risk can be followed up on a yearly basis. Those patients who are found to have invasive cancer within an IPMN should be followed up and managed as per PDAC.

As access to high-quality cross-sectional imaging continues to increase, the number of patients being found to have IPMNs is only going to increase. Therefore, having robust management strategies for risk stratification, management and follow-up, such as those published by Tanaka and colleagues will be important in ensuring that only patients with a definite risk of developing invasive cancer within their cysts undergo surgical resection.

1.7   DIAGNOSIS OF PANCREATIC CANCER

1.7.1   Symptoms and Signs

As previously stated, the diagnosis of PDAC is commonly made at an advanced stage of the disease, once cancer has spread beyond its anatomical confines to involve adjacent or distant structures. The topographical location of the tumour within the pancreas will dictate which symptoms, if any, develop as a result of the tumour. Symptoms and signs of PDAC are often non-specific but may include nausea, malaise, pruritis, early satiety, anorexia and altered stools (steatorrhoea). Those tumours which develop within the proximal portion of the pancreas (head and uncinate process) are often found at an earlier stage than those of the distal pancreas (neck, body and tail). The proximal tumours are more likely to lead to biliary obstruction, causing the visible sign of jaundice, alerting the patient and doctor of some underlying pathology. A small proportion of proximal tumours will not present with biliary obstruction and jaundice but instead will lead to the development of Gastric Outlet Obstruction (GOO) due to extrinsic compression of the gastric antrum or duodenum. Distal tumours can grow more before causing any symptoms or signs that may alert the patient. These tumours are more likely to be picked up incidentally or present with back pain and constitutional symptoms once the tumour has advanced enough to invade the local coeliac plexus of nerves. For this reason, tumours of the distal pancreas are less likely to be resectable than proximal tumours. As mentioned above, a new diagnosis of diabetes mellitus in older patients or worsening glycaemic control in established diabetics should prompt further investigation.

1.7.2   Screening

At present, no formal screening programme exists within the UK to diagnose pancreatic cancer amongst the asymptomatic general public. Predominantly this is because at present pancreatic cancer doesn't meet all of the WHO Wilson-Junger criteria (37). Whilst the natural history of pancreatic cancer is well understood, and treatment at an earlier stage is known to confer a survival advantage, the main caveat preventing the development of a screening programme is the lack of an acceptable test that can readily and reliably diagnose pancreatic cancer at an early stage.

The University of Liverpool is currently co-ordinating the screening/surveillance of patients with familial pancreatic cancer and hereditary pancreatitis. Screening typically begins once relevant patients turn 40 years of age and involves a combination of regular blood tests including Ca19-9 levels, Magnetic Resonance Imaging (MRI) of the pancreas, Endoscopic Ultrasound (EUS) of the pancreas, and potentially Endoscopic Retrograde Cholangiopancreatography (ERCP) with a sampling of pancreatic juice for sequencing. The long-term informatics obtained from these studies are eagerly awaited and may lead to an international consensus on screening at-risk patients.

1.7.3 Blood Biochemistry

Biochemical analysis of the blood of patients presenting with PDAC can be both normal or abnormal meaning that current blood tests are not a reliable stand-alone diagnostic tool and should be used in conjunction with clinical suspicion and radiological investigations. As mentioned previously, proximal tumours are likely to lead to biliary obstruction, with resulting derangement of Liver Function Tests (LFTs). A usual LFT panel would include, serum bilirubin, Alkaline Phosphatase (ALP), and either Alanine Aminotransferase (ALT) or Aspartate Aminotransferase (AST). ALP is a membrane-bound enzyme that is located on the canalicular aspect of hepatocytes. When biliary obstruction occurs, one would expect that the first change seen would be an elevated Alkaline Phosphatase level. As the obstruction continues, bilirubin levels are seen to increase.

The Ca19-9 biomarker (also known as Sialyl-Lewis[A]) is currently the only widely used blood-based tumour marker for patients with suspected pancreatic cancer in clinical practice. However, up to 10% of Caucasians are Lewis antibody negative and as such will not express Ca19-9, even in the presence of an advanced pancreato-biliary malignancy (38). Conversely, serum Ca19-9 may be seen to be falsely elevated in a range of benign conditions including choledocholithiasis, pancreatitis and cirrhosis. This lack of sensitivity and specificity, unfortunately, precludes the use of Ca19-9 as a purely diagnostic test or even as a screening test in at-risk populations, and clinicians should not be tempted to use it as such unless there is a clinical or radiological suspicion of PDAC. It does however have a role in monitoring response to treatment

and in detecting evidence of relapse of disease. It can also be used to aid decision-making when considering the treatment of pancreatic lesions which are ambiguous on imaging.

The lack of a suitably sensitive and specific test for PDAC is the underlying driving force for this thesis and is discussed in more detail in the next chapter.

1.7.4   Radiology

Given the lack of reliable blood-based tests and the vagueness of clinical symptoms and signs associated with PDAC (at least in the early stages), the burden of diagnosing these patients often lies with radiological examinations which have varying degrees of sensitivity and specificity.

1.7.4.1   Ultrasound

Transabdominal Ultrasound Scanning (USS) is often the first radiological test undertaken in a patient presenting with jaundice. It should be able to delineate between an obstructive cause of jaundice, as seen in pancreatic/peri-ampullary neoplasms, stone disease and strictures, compared to intrinsic hepatic causes of jaundice. If a large pancreatic mass is present, it may be seen, but it is not uncommon for the pancreas not to be fully visualised due to overlying bowel gas or increased patient adiposity. Whilst the benefits of USS include its relative ease of access and lack of ionising radiation, it is a very user-dependent diagnostic test, reducing its use in making a definite diagnosis of pancreatic cancer, although the recognition of a dilated biliary tree in the absence of gallstones, should prompt further diagnostic tests in the form of high definition cross-sectional imaging such as Computed Tomography (CT) or Magnetic Resonance (MR).

1.7.4.2   Pancreatic Protocol Computed Tomography

CT with intravenous contrast has become the gold standard radiological test for the diagnosis of pancreatic cancer. Ideally, the scan should include both and arterial and portal venous phase to allow accurate interpretation of vessel involvement/encasement, as well as a non-contrast phase. Due to the dense stroma

seen in PDAC tumours, they appear hypovascular compared to normal pancreatic parenchyma and therefore are easier to identify in the late arterial phase, where they may appear as hypodense lesions. A discrete mass may not always be apparent with smaller tumours, but the presence of the so-called "double duct sign" in which there is dilatation of both the bile and pancreatic ducts, should alert one to the possibility of a small peri-ampullary mass which should be further assessed with Magnetic Resonance Imaging (MRI) or Endoscopic Ultrasound (EUS).

1.7.4.3   Magnetic Resonance Imaging

The lack of ionizing radiation makes MRI an attractive option in patients who require multiple imaging studies and in patients with an allergy to iodine-based contrast. It is for this reason that surveillance of those patients deemed to be at high risk of developing pancreatic cancer (family history/ pre-cursor lesions) is often undertaken with MRI instead of CT. If an abnormality is detected on MRI, it can be further investigated with a CT.

Contrast enhanced MRI can be a useful adjunct to use alongside CT when secondary signs of PDAC such as a double duct sign are seen without an obvious pancreatic mass.

1.7.4.4   Endoscopic Ultrasound

Endoscopic Ultrasound (EUS) has developed over the preceding decades to become an invaluable tool in the evaluation of pancreatic lesions and subsequent diagnosis of PDAC.

A flexible endoscope with an incorporated linear ultrasound probe is passed orally down the oesophagus until it lies within the stomach/duodenum. The operator is then able to obtain ultrasound images of the pancreas and peri-ampullary region, taking advantage of the proximity in which, the stomach and duodenum lie to the pancreas. Any lesions identified at EUS can then be biopsied (if solid) or aspirated (if cystic), with the obtained tissue being sent off for histological/cytological and potentially biochemical analysis. A large meta-analysis by Puli and colleagues in 2013 included 41 studies, with a total of 4766 patients and found that EUS-guided FNA had a pooled sensitivity and specificity of 86.8% and 95.8% respectively for assessing solid pancreatic lesions (39).

1.7.5   Cytology and Histopathology

Whilst cytology and histopathology can aid in the decision-making regarding the management of PDAC, a tissue diagnosis is not always a requirement prior to attempted resection if radiology is suspicious for cancer which is deemed to be operable by the pancreatic Multi-Disciplinary Team (MDT). Endo-biliary brushings can be taken at the time of ERCP and stent placement. Whilst the sensitivity for detecting cholangiocarcinoma (cancer arising directly from the bile duct wall) using endo-biliary brushings can be up to 80%, (40), the sensitivity for picking up pancreatic cancers is much lower, ranging from 15% to 65% (41, 42), meaning that they cannot be relied upon to detect pancreatic cancer. Percutaneous approaches to obtaining a biopsy in patients with potentially operable tumours are best avoided due to the possibility of tumour seeding along the biopsy tract, which could subsequently render a potentially operable tumour inoperable.

Patients with borderline or locally advanced disease being considered for neoadjuvant therapy should have a confirmed tissue diagnosis of PDAC prior to starting any oncological treatment. Ideally, this should be achieved via endo-biliary brushings at ERCP or trans-luminally via EUS.

Patients with metastatic pancreatic cancer should have a tissue diagnosis if it is intended that they will receive palliative chemotherapy. Tissue can be obtained via percutaneous or transluminal approaches to the primary tumour, or a percutaneous approach to an accessible metastasis (liver or peritoneal). For those in whom it has been decided by the patient or by the Multidisciplinary Team (MDT) that the best supportive care is appropriate, attempts at obtaining tissue for diagnosis should not be undertaken as they are not without risk, and ultimately would be of no benefit to the patient.

1.8   STAGING OF PANCREATIC CANCER

It is imperative that all patients with a new diagnosis of PDAC, who may benefit from treatment (whether curative or palliative), should undergo complete staging to establish the extent of the disease to allow informed decision-making at the relevant Multi-Disciplinary Team (MDT) meeting. Accurate staging of disease prior to the commencement of treatment will allow reliable assessment of response to treatment, especially in those patients who may undergo neoadjuvant treatment in an attempt to convert non-operable locally advanced disease into an operable disease.

1.8.1   Staging Investigations

1.8.1.1   CT of the Thorax, Abdomen and Pelvis (CT TAP)

As soon as a diagnosis of PDAC is suspected, NICE recommend that complete staging should be obtained with a CT of the chest, abdomen and pelvis (43). This aims to detect metastatic disease, which is present in up to 50% of patients at the time of initial diagnosis of PDAC.

1.8.1.2   Positron Emission Tomography (PET-CT)

The utility of [18]Fluoro-Deoxy-D-Glucose (FDG)–PET scanning in the staging of cancer has been recognised for some time. There has been increasing evidence that PET-CT scanning has a superior positive predictive value for detecting metastatic disease compared to traditional CT scanning (44). As a result of this emerging evidence, the National Institute for Health and Care Excellence (NICE) now recommend that all patients with localised PDAC on standard CT TAP (resectable or locally advanced) should undergo PET-CT prior to treatment to reduce the number of patients with occult metastatic disease undergoing unnecessary treatments (43).

1.8.1.3   Laparoscopy

A select subset of patients who have disease that has been deemed potentially resectable on high-quality cross-sectional imaging and PET-CT will undergo laparoscopy as a further staging investigation to rule out occult hepatic or peritoneal metastases. The exact criteria for which patients undergo laparoscopy varies in each

centre. Whilst some centres will perform a staging laparoscopy on all patients who will undergo resection, some centres may use a more selective approach such as performing a laparoscopy if the patient has a particularly elevated serum Ca19-9. Equivocal lesions of the liver or peritoneal surface demonstrated on the other imaging require further evaluation and potentially biopsy can be assessed with laparoscopy and potentially laparoscopic ultrasound. If used appropriately, staging laparoscopy can reduce the number of patients undergoing non-therapeutic laparotomy. There is little evidence or guidance to suggest whether routine staging laparoscopy is worthwhile for all patients with potentially resectable PDAC. A study by Schnelldorfer evaluated the role of staging laparoscopy in patients with resectable PDAC (45). They found that 2% of 136 patients had metastasis not seen on pre-operative imaging. However a further 12 patients were subsequently found to have metastatic disease at the time of laparotomy which had not been seen on pre-operative imaging or staging laparoscopy, leading the authors to conclude that there is a role for staging laparoscopy, however, it should be extensive to include opening the lesser sac and examining the root of the mesentery – something which is not routinely done in all centres.

1.8.2   Defining the operability of Pancreatic Cancer

Pancreatic cancer can more descriptively be divided into 4 categories: 1) Upfront resectable, 2) Borderline resectable, 3) locally advanced, and 4) metastatic. As the name suggests, patients with upfront resectable tumours are those whose tumour is clear of major vascular structures and adjacent organs, and therefore suitable for surgery as the primary treatment. Locally advanced tumours are those in which the tumour is encasing one of the nearby major arteries, i.e., the Coeliac axis, the Hepatic artery or the Superior Mesenteric Artery. Traditionally these arteries cannot be excised with the tumour and the tumour should not just be carved off the vessels as this would lead to residual tumour being left behind which would incur no survival benefit to the patient. However, it must be noted that in some centres across the world, arterial resection is being undertaken for locally advanced disease. Metastatic disease is apparent when there is evidence of tumour spread to distant organs. Borderline resectable disease is a more recent concept and essentially covers a wide range of patients whose tumour is not deemed upfront resectable as it touches but doesn't invade the local major vessels. Whilst these tumours can technically be removed, there

is a higher chance that the resection margin will be found to have cancer. The management approaches to these different stages of pancreatic cancer will be discussed later in this chapter.

1.8.3 TNM Staging Classification

Due to its ease of use, the TNM staging system developed by the Union for International Cancer Control and the American Joint Committee on Cancer (UICC and AJCC) has become the most commonly implemented staging system used for PDAC. It can be used to pre-operatively stage disease based on imaging as well as provide a definitive stage following histological assessment. The widespread use of the TNM system standardises the staging of the disease, to enable better comparisons to be made.

The T stage refers to information about the primary Tumour, whilst the N stage refers to the presence or absence of involved regional lymph Nodes. The M stage simply refers to the presence or absence of distant metastases.

The current incarnation of TNM is the 8th edition, being brought into use on 1st January 2018. Key changes between the 7th and 8th editions include a change in what defines a T3 and a T4 lesion. The 8th edition also now includes a modification of the N stage. Whilst the 7th edition only differentiates between the absence (N0) or presence (N1) of lymph node metastasis, TNM 8 divides the old N1 category into N1 and N2 dependent on the number of involved lymph nodes. Figures 1.3 and 1.4 show the current criteria and classification of the different stages based on the 8th edition.

1.8.3.1 Tumour (T) Stage

Like other GI tract tumours, the T stage in PDAC ranges from 1-4. In PDAC the T stage refers to the size of the tumour or its involvement of adjacent visceral arteries. In the current 8th edition of the TNM staging manual, T1-3 are assigned based on tumour size (<2cm, 2-4cm, >4cm), whilst T4 is assigned to tumours with evidence of arterial involvement (Figure 1.3). This differs from the previous edition, in which T1 and T2 were based on size (<2cm or ≥2cm). T3 tumours were those which extended

beyond the pancreas, without involving adjacent vascular structures, whilst T4 tumours involved the Coeliac Axis or SMA.

Of the three elements of the TNM stage, the T stage appears to have the least impact on predicting survival in pancreatic cancer. Whilst some studies have shown the T stage to have no prognostic value (46), others have shown limited value, such as a multicentre retrospective review by Marchegiani and colleagues which demonstrated that a tumour size of less than 2cm (i.e. T1 lesions) independently incurs an improved prognosis compared to larger tumours (47).

1.8.3.2   Nodal (N) Stage

The nodal stage is an independent predictor of disease recurrence. To ensure an accurate assessment of lymph node status, an adequate lymph node harvest must be undertaken. The most recent guidance by the Royal College of Pathologists stipulates that a minimum of 15 lymph nodes should be excised and assessed with pancreatic resections to ensure an adequate assessment of the nodal status can be made.

Prior to the 8th edition of the TNM, lymph node status was staged as either N0 or N1, with the only discriminator being the absence or presence of lymph node involvement. Several studies looking at prognostic factors following potentially curative resection have shown that it is not simply the arbitrary presence or absence of lymph node metastasis that affects survival, but the burden of lymph node disease. The importance of Lymph Node Ratio (LNR) has been described by several studies including Robinson and colleagues (48). More recently it has been shown that the absolute number of involved lymph nodes has greater prognostic importance than the LNR number (49, 50). The study by Baldwin and colleagues found that survival amongst patients without lymph node involvement was not significantly different to those with one or two involved nodes (median OS 25.5 months vs 21.0 months). However, patients with three or more involved lymph nodes had a significantly reduced median overall survival of just 12.3 months. Studies such as these have contributed to the change in the N stage in the 8th edition of TNM, so the importance of lymph node burden is recognised.

1.8.3.3   Metastasis (M) Stage

The M Stage of PDAC has constantly been divided into M0 and M1 referring to the absence or presence of metastatic disease. It does not discriminate between the sites of metastases. It should be noted that lymph node metastasis to non-regional lymph node basins represents non-curable disease, and therefore are recorded in the M stage as opposed to the N stage. The development of metastases signifies inoperable disease. If detected on pre-operative staging, exploratory surgery should not be undertaken.

	T	N	M
0	No evidence of pancreatic tumour	No involved lymph nodes	No evidence of distant metastasis
1	Tumour smaller than 2cm	1-3 involved lymph nodes	Metastatic disease
2	Tumour >2 but ≤4cm	≥4 involved lymph nodes	
3	Tumour greater than 4cm		
4	Tumour involves Coeliac Axis, SMA and/or Common Hepatic Artery		

Figure 1.3   Tumour, Nodal status, and Metastases definitions as per TNM8.

The Tumour stage (T) is dependent on the size of primary tumour, whilst the Nodal and Metastatic stages refer to the presence of cancer cells within the regional lymph nodes (N) or distant sites (M).

Stage	T	N	M
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1, T2, T3	N1	M0
III	T1, T2, T3 T4	N2 Any N	M0
IV	Any T	Any N	M1

Figure 1.4   Pancreatic cancer disease stage by TNM8.

Different combinations of T,N and M stages are combined to give the overall stage of cancer.

1.8.4 Resection Margin (R) Status

The R status is assigned following surgical excision and refers to the resection margin(s) of the excised specimen. All cancer resections should aim to achieve margins that are microscopically clear of cancer cells. This is defined as an R0 resection. Microscopic involvement of the resection margin is referred to as an R1 resection. The Royal College of Pathologists has declared that a distance of less than 1mm between viable tumour cells and the margin constitutes a microscopically involved margin (R1). The exception to this is the anterior margin which is an anatomical margin as opposed to a surgical margin. In this instance, there has to be a direct breach of the margin to be deemed as an R1 resection. When there is macroscopic evidence of a tumour at the margin this is deemed to be an R2 resection. An R2 margin is deemed not to be an acceptable outcome in an oncologically correct resection as it signifies that macroscopic viable cancer has been left in situ.

Whilst the formal report of the margin status can only be made following fixation of the resected specimen, the use of an intra-operative Fresh Frozen Section of the resection margin has traditionally been used to guide the extent of resection. If either of the transection margins (pancreatic duct, bile duct) reveal evidence of tumour involvement at the frozen section, then a further margin can potentially be taken or even a completion pancreatectomy to remove the rest of the gland may be undertaken. However, there is emerging evidence that the addition of a further resection to achieve an R0 resection, may not have prognostic benefits, leading there to a question mark of the role that the frozen section of the resection margins may play in the future (51-53).

Margin involvement with invasive tumour as classified by the R status is an independent predictor of survival (54). However, as alluded to previously, the presence of pre-invasive tumour (i.e. PanIN) at the surgical margin has not been shown to negatively impact on patient survival (32).

1.9   PERI-OPERATIVE CARE

1.9.1   Prehabilitation

The concept of prehabilitation (or prehab) is becoming an increasingly important element of the peri-operative care of surgical patients, especially for those who are planned to undergo major resections as is the case for patients with pancreatic cancer. The underlying principle of prehabilitation is to improve the patient's ability to withstand the significant physiological (and psychological) insult of major surgery, by making meaningful interventions prior to undergoing surgery. The most commonly quoted approach to prehabilitation is the Tri-modal model which addresses physical fitness and strength, nutrition and mental health/anxiety related to cancer treatments. The timing and duration of any prehabilitation are key to its success. If the duration is too short, it is unlikely to provide any patient benefit, but if it is too long the risk is that the patient will lose interest and motivation. To address the physical aspect of prehabilitation, patients undergo daily exercise routines lasting no longer than an hour to improve cardiovascular health and improve muscle bulk. Muscle bulk is key, particularly in pancreatic cancer. It is an extremely catabolic disease with patients often reporting significant weight loss leading up to diagnosis and treatment. In a variety of cancers, relative sarcopenia has been shown to independently have a negative impact on patient outcomes. Pancreatic cancer patients should be assessed by a specialised dietician to ensure their calorific requirements are being met. Pancreatic Enzyme Replacement Therapy (PERT) should be prescribed for all patients with PDAC to replace the enzymes that the pancreas would usually secrete, ensuring maximal absorption of nutrients. The psychological aspect of prehabilitation may be easily overlooked but is just as important as the other two aspects. Reducing anxiety can lower levels of catecholamines and glucocorticoids (stress hormones) which can lead to improved glycaemic control and subsequent healing. Psychological interventions utilised in prehabilitation include Cognitive Behavioural Therapy (CBT) and Sensory description – talking the patients through the physical sensations they are going to feel around the time of their surgery, i.e., describing the various tubes and drips the patient will have so they seem less frightening and alien when the patient comes to theatre.

1.9.2 Enhanced Recovery After Surgery

Enhanced Recovery After Surgery (ERAS) has gained momentum over the preceding decades and has now become a standard of care following many types of major surgery. ERAS encompasses a multimodal approach with evidence-based pre-operative, intra-operative and post-operative interventions, all with a common aim of reducing peri-operative morbidity and reducing Length of Stay (LoS) in hospital. Common themes that have emerged from the development of several ERAS protocols include pre-operative carbohydrate loading, the avoidance of opiate analgesia, prevention of over-administering IV fluids, the avoidance if possible of leaving surgical drains and nasogastric tubes (or early removal if avoidance is not possible), early mobilisation and early return to oral intake. Whilst these interventions have been shown to improve functional recovery in other forms of surgery, not all of the above-mentioned interventions easily lend themselves to patients undergoing pancreatic resection (in particular the lack of nasogastric tubes and surgical drains due to the risk of Delayed Gastric Emptying (DGE) and Post-Operative Pancreatic Fistula (POPF) respectively).

There is an increasing evidence base confirming that ERAS programmes are safe for patients undergoing pancreatic resection for cancer. However, a 2016 review by Pecorelli found that the majority of published studies looking at ERAS, were retrospective in nature, and often ERAS patient cohorts were compared to more historical control cases, making meaningful interpretation of the outcomes difficult (55). Retrospective cohort studies have reported reduced length of stay and reduced complications in patients undergoing both Pancreaticoduodenectomy (PD) and distal Pancreatectomy (DP) (56, 57). As many of these studies are non-comparative, they can therefore only state that ERAS is safe and feasible but cannot truly compare the outcomes to patients who have a more traditional peri-operative course.

A large, single-centre, prospective study by Agarwal and colleagues looked closer at how increasing compliance with an ERAS programme affected outcomes for patients, directly comparing patients who had a compliance of >80% with the ERAS interventions with those patients who had intervention compliance of <80% (58). The two groups were well-matched concerning demographics and surgical procedures. This study demonstrated that outcomes were significantly improved when there was greater compliance with the ERAS interventions, in particular, there was reduced

return to theatre rate, development of major complications (including Post-Operative Pancreatic Fistula) and reduced mortality. Interestingly there was a significantly greater rate of minor complications seen in the increased compliance group. Whilst the authors suggest that ERAS may be responsible for these improved outcomes, it is important to note that those patients who achieved compliance of <80% were more likely to have had increased intra-operative blood loss, leading to an increased transfusion demand. This in itself may be the cause of reduced compliance with ERAS, as well as being an important factor in increasing the risk of developing the aforementioned complications.

At present, the lack of prospective randomised control trials directly comparing outcomes between ERAS pathways and traditional post-operative recovery pathways makes it difficult to say with absolute certainty that an ERAS pathway leads to improved outcomes following pancreatic resection for cancer.

1.10   SURGICAL TREATMENT OF PANCREATIC CANCER

1.10.1 General Concerns

Since the first reported cases of resections of the pancreas, surgery to treat PDAC has filled the surgeon with dread due to the technical challenges of operating on a relatively inaccessible organ, a rocky post-operative course and poor long-term outcomes. Anatomically, the pancreas is difficult to access due to its location within the retroperitoneum and its proximity to adjacent major vascular structures – meaning any attempts at resection risk significant, life-threatening bleeding. Even after successful resection of part of the pancreas, there is a constant worry about leakage of pancreatic juice with its proteolytic enzymes which may lead to the development of Post-Operative Pancreatic Fistulas (POPFs), with the potential for delayed haemorrhage, intra-abdominal abscesses risking subsequent organ failure and potentially death. In surgery of the proximal pancreas, the need to resect the duodenum +/- gastric antrum and therefore subsequent need to re-establish continuity of the gastrointestinal tract can lead to delayed gastric emptying. This can then lead to poor nutrition in the post-operative period when the patient is already in a catabolic state following major surgery.

1.10.2   Pancreaticoduodenectomy (Kausch-Whipple and PPPD)

One of the most famous eponymous operations in abdominal surgery is the Whipple procedure or one-stage pancreaticoduodenectomy, attributed to the American surgeon Alan Oldfather Whipple.

The most significant modification made to the Whipple procedure was the preservation of the gastric antrum and pylorus. The Pylorus Preserving Pancreaticoduodenectomy (PPPD) was popularised by Traverso and Longmire (59), although the technique was first reported some 34 years earlier by a British surgeon for a periampullary carcinoma (60). Whilst preservation of the pylorus reduces the incidence of bile reflux into the stomach, there is an increased incidence of post-operative Delayed Gastric Emptying (DGE).

The underlying principle behind both the classic Whipple procedure and the PPPD remains the same - En bloc removal of the tumour with the surrounding head/neck of the pancreas, duodenum, distal bile duct +/- gallbladder, and regional lymph nodes

with clear macroscopic margins (Figure 1.5). There are many different methods of restoring the continuity of the GI tract after resection (61). The first decision to make is what to do with the pancreatic remnant. The two most popular ways to reconnect the pancreas to the GI tract are by anastomosing it to either the back of the stomach (Pancreatico-Gastrostomy/PG) or by anastomosing it to a loop of the jejunum (Pancreatico-jejunostomy/PJ). There have been several studies and meta-analyses comparing these two methods, with a particular focus on the rates of POPF (62). Ultimately no significant difference between these two techniques has been demonstrated, therefore the decision to perform a PJ or PG is left to the discretion of the surgeon. Within the PJ group, several different techniques have been described. The most commonly described techniques are modifications of the duct-to-mucosa anastomosis. The principle of this is precise apposition of the transected pancreatic duct to the mucosa of the jejunum, with additional sutures between the seromuscular layer of the jejunum and the parenchyma of the pancreas, to take tension off of the anastomosis. The Cattell-Warren Anastomosis is a commonly used technique which involves a posterior and anterior layer of pancreatico-jejunal sutures over a duct-to-mucosa anastomosis (63). More recently, Blumgart described a simpler method in which through and through pancreatico-jejunal sutures or U-stitches are used to cover a duct-to-mucosa anastomosis. The PANasta trial sought to identify whether there were significant differences in POPF rate between the Cattell-Warren and the Blumgart techniques, but none was apparent (64)
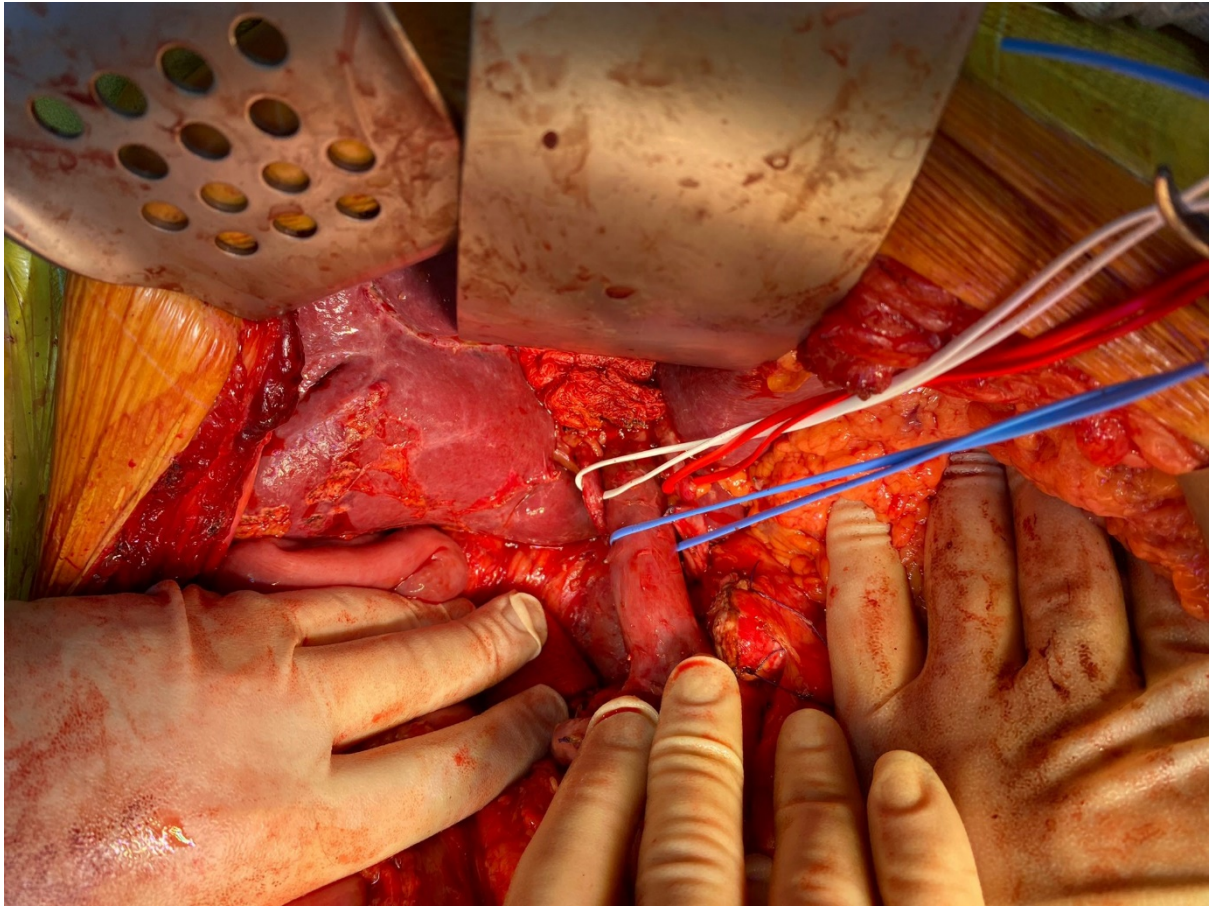
Figure 1.5 – Intra-operative photo of the appearance of the upper abdomen after the resectional stage of pancreaticoduodenectomy, prior to reconstruction.

The pancreas has been divided at the neck, and the head of pancreas, duodenum, bile duct and gallbladder have been removed and sent for pathological examination. Note this patient had slightly aberrant anatomy with a replaced right hepatic artery arising from the SMA (white sling). The portal vein (blue sling) and Common Hepatic Artery/ Left Hepatic Artery (red sling) are both clearly identified.

1.10.3 Radical Distal/Left Pancreatectomy

Tumours of the body and tail of the pancreas are technically easier to resect and may be treated with a radical distal (or left) pancreatectomy. The lesser sac is opened and a tunnel behind the neck of the pancreas is developed, allowing the pancreas to be transected at this point (65). The Splenic artery is ligated and divided proximally, and the splenic vein is divided at the confluence with the SMV. The body and tail are then dissected out of the retroperitoneum and removed en bloc with the surrounding lymph nodes and the spleen. To ensure a clear margin, the left adrenal gland and part of Gerota's fascia may be excised en bloc with the specimen as well.

1.10.4 Extended Pancreatectomy and Vascular Resections

The idea of extended pancreatectomy was first publicised by Fortner in 1973 with the preliminary report of a patient in whom he had undertaken what he described as a "Regional Pancreatectomy" which involved resection of the tumour with en bloc excision of the portomesenteric confluence as well as a portion of the Hepatic Artery and Superior Mesenteric Artery (66). In 1977, he and his colleagues then published their series of the first 18 patients who had undergone pancreatectomy with en bloc vascular resection. Since then, there has been a great interest regarding the risk/benefit ratio of such resections. Many studies have revealed the morbidity to be greatly increased in the presence of a vascular resection. However, if a clear surgical margin can be achieved, then the long-term outcomes are similar to those of patients undergoing curative resection and are much better than the outcomes seen in those patients who undergo palliative bypass. With this in mind, the International Study Group for Pancreatic Surgery (ISGPS) published a consensus statement confirming that en bloc venous resection should be undertaken in cases of pancreatic resections for cancer if a clear margin can be obtained (67). As a result, more and more centres are routinely undertaking pancreatectomy with en bloc venous resection.

Arterial resection for pancreatic cancer is less commonly undertaken compared to venous resection, owing to reduced options for reconstruction, leading to significant morbidity, however, some centres do undertake regular arterial resection and reconstruction.

1.10.5 Laparoscopic Pancreatectomy

As with most other aspects of abdominal surgery, laparoscopic or "minimally invasive" techniques are playing an increasingly important role in surgery for pancreatic cancer. In 1994 Gagner and Pomp described the technique for performing a laparoscopic PPPD in a patient with chronic pancreatitis affecting the head of the gland (68). Whilst the procedure had no major complications, the authors did note that whilst technically feasible, the laparoscopic approach to a Whipple procedure may not improve overall outcome or shorten post-operative stay. Given this, the uptake of laparoscopic pancreaticoduodenectomy was fairly slow to begin with. Two years later, Gagner and colleagues then published a small case series of patients undergoing laparoscopic pancreatic resections for neuroendocrine tumours. This series included patients who had undergone either laparoscopic enucleation or laparoscopic distal pancreatectomy (69). The patients who underwent a laparoscopic distal pancreatectomy had a significantly shorter length of stay compared to those patients who underwent conversion to open (however it should be noted that 2 of the converted patients required en bloc partial gastrectomy of the greater curvature). A more recent study from South Korea compared outcomes amongst patients undergoing open or laparoscopic left pancreatectomy for PDAC (70). This propensity-matched study showed no significant difference in overall survival, margin status or fistula formation. However, the length of stay and return to normal diet were significantly shorter in the laparoscopic group, leading the authors to conclude that laparoscopic left pancreatectomy is a "feasible, safe and effective" approach for the treatment of left-sided pancreatic adenocarcinomas. The Dutch randomised controlled "LEOPARD" trial compared open and laparoscopic distal pancreatectomy in 108 patients and similarly found no decrease in the overall complication rate. However, there was a lower incidence of delayed gastric emptying and a shorter time to functional recovery (71). Due to the relative ease of distal/left pancreatectomy compared to pancreaticoduodenectomy, it is not surprising that the laparoscopic left pancreatectomy is now seen as the gold standard approach for distal/left-sided tumours. Over the last 10 years, many centres have begun to publish their results of laparoscopic pancreaticoduodenectomies. Initial anecdotal reports were encouraging, with less bleeding and a shorter return to functional recovery. The Dutch LEOPARD-2 trial set out to objectively compare outcomes of open and laparoscopic

pancreaticoduodenectomy (72). However, the trial was terminated early due to an unexpectedly high mortality rate amongst the laparoscopic group (10% vs 2%), although initial reports did show similar outcomes with regard to time to functional recovery and morbidity (73).

Whilst there is a clear benefit to laparoscopic resection of left-sided tumours as demonstrated in randomized trials, this benefit has yet to be conclusively shown in the management of proximal pancreatic tumours requiring pancreaticoduodenectomy.

1.10.6 Complications following Pancreatic Resection

The post-operative course following pancreatectomy can be a rocky one, with significant potential for complications which can be life-threatening. Due to the more complex nature of resections of the proximal pancreas, pancreatoduodenectomy is associated with significantly more morbidity compared to left-sided resections. Whilst pancreatic resections carry all of the risks associated with other major intra-abdominal procedures (wound infection, venous thromboembolic events, respiratory tract infection and cardiac problems), they also are associated with more specific complications which will be discussed further below.

1.10.6.1   Postoperative Pancreatic Fistula (POPF)

Leakage of pancreatic secretions from either the main duct or the pancreatic parenchyma following pancreatectomy results in a POPF. The diagnosis and severity grading of POPF can be made based on the 2016 updated ISGPS classification of POPF (74). A drain fluid sample with an amylase level of at least 3 times the upper limit of serum amylase by the 3rd post-operative day is diagnostic of a leak. If there are no clinical sequelae to this and the drain can be removed in under 3 weeks, then this is deemed as a "Biochemical Leak" and is not technically deemed to represent a POPF as per the updated guidelines. This would have originally been defined as a Grade A POPF under the original ISGPS guidelines of 2005 (75). The persistence of the leakage of amylase-rich pancreatic fluid beyond the 3rd post-operative week or the need for non-surgical intervention (such as drain-repositioning/radiological drain placement, intravenous antibiotics or parenteral feeding) would be classified as a Grade B POPF.

Patients who develop organ failure, require surgical re-exploration, or die as a result of the leak are deemed to have a Grade C POPF.

Uncontrolled POPF can ultimately lead to catastrophic bleeding and sepsis and as such prompt diagnosis and early intervention (where required) are key to improving outcomes for patients who develop this complication. Very rarely, patients will require further surgical intervention for an uncontrolled fistula, often in the form of a completed pancreatectomy in which the remaining pancreas is resected. Morbidity and mortality are significant in this patient group.

The majority of clinically relevant fistulae can be managed non-operatively with antibiotics, and patience. Route of nutrition in the presence of POPF is a hotly debated topic with some centres advocating keeping the patient Nil By Mouth (NBM) and providing nutrition intravenously with Total Parenteral Nutrition (TPN) whilst others feel oral nutrition doesn't impact fistula healing or subsequent complications. Drain volumes and fluid amylase levels are closely monitored until it is evident that the fistula has healed, at which point oral intake can be re-instated.

1.10.6.2   Haemorrhage

Post-pancreatectomy haemorrhage (PPH) continues to be probably the most feared complication following pancreatectomy, with significant associated morbidity and mortality. The incidence of PPH varies between series, with one large series from Germany stating an incidence of 7.9% (76). The mortality seen in patients who develop PPH seems to vary widely from 3 — 28% (76, 77).

As with POPF, a unified definition was previously lacking, until the ISGPS produced their consensus definition in 2007 (78). This sought to better define PPH based on timing after surgery, site of the bleed (including whether the bleed is intraluminal or extraluminal) and the severity of the bleed. The clinical impact of the bleed is then graded as A, B or C.

With regards to timing, the ISGPS divided PPH into early, referring to those bleeds which occur within 24 hours of surgery, and later, referring to any bleed that occurs more than 24 hours after surgery. Whilst early bleeds are usually attributed to a technical failure at the time of surgery, late bleeds are often the result of a postoperative complication such as a collection or a POPF.

Bleeding may occur from a range of locations, both intra- and extra-luminal. Intraluminal bleeds may be due to peptic ulcer disease or marginal ulceration at the site of any of the enteric anastomoses. Extraluminal bleeds may develop from the stump of the divided gastroduodenal artery, or splenic artery or may occur following pseudoaneurysm formation secondary to the leakage of pancreatic enzymes from the pancreatic anastomosis.

The severity of bleeding is described as mild or severe. Mild bleeding has no significant clinical impact, whilst severe bleeding is noted if there is a transfusion requirement of more than 4-6 units of blood within 24 hours, a drop of haemoglobin of greater than 4g/dL or the need for intervention in the form of endoscopy, interventional radiology or re-laparotomy.

1.10.6.3 Delayed Gastric Emptying

Delayed Gastric Emptying (DGE) is a recognised complication of pancreaticoduodenectomy, manifesting with high nasogastric aspirates (if an NG tube is still present) or nausea and vomiting in the post-operative period and therefore the inability to maintain oral intake.

As with other significant post-operative complications that may occur following pancreaticoduodenectomy, the International Study Group on Pancreatic Surgery (ISGPS) has sought to provide a consensus definition and a standardized grading system (79). The ISGPS have therefore defined DGE as "*The inability to return to a standard diet by the end of the first postoperative week and includes prolonged nasogastric intubation of the patient.*" Before labelling a patient as having DGE, it is important to ensure that there is no mechanical cause for the high nasogastric aspirates. It is therefore recommended that the gastro-enteric anastomosis should be assessed either under direct vision using endoscopy or by using a contrast study.

The severity of DGE can be classified as Grade A-C, based on 1) the duration of Nasogastric tube placement, 2) the time to return to a solid diet 3) the presence of vomiting/distension and 4) the need for pro-kinetics.

Several studies have tried to identify risk factors which may increase the risk of the patient developing DGE in the post-operative period.

The effects of DGE on nutrition in the post-operative period can be extremely detrimental to recovery to function and time to subsequent adjuvant treatment, therefore early recognition and provision of alternative routes of nutrition are key with this complication.

1.11 ADJUVANT THERAPY FOLLOWING PANCREATIC RESECTION

Adjuvant therapy is given after surgical resection of a malignant tumour. The aim of this is to kill any microscopic systemic disease which may not have been picked up on pre-operative staging.

1.11.1 Chemotherapy

Whilst surgical resection of pancreatic cancer with clear margins offers the only chance of long-term cure, surgery can be complemented with the addition of adjuvant oncological therapy to achieve the best long-term outcomes. Current practices in adjuvant therapy in the UK and Europe have been shaped by the ESPAC trials which began in the early 2000s. The debate regarding the role of adjuvant chemotherapy vs chemoradiotherapy was the basis for the ESPAC-1 trial (80). This trial randomised 289 patients who had undergone a potentially curative pancreatic resection to receive either chemoradiotherapy, chemotherapy with 5-Fluorouracil (5FU), chemoradiotherapy or observation alone. The results showed a 5-year survival rate of 21% amongst the chemotherapy-only group, whilst patients who received chemoradiotherapy had a 5-year survival rate of 10%. As a result of this trial, it became the standard of care in the UK to give adjuvant chemotherapy to all patients who underwent potentially curative surgery, assuming their post-operative performance status was sufficient to receive treatment (81).

The ESPAC-3 trial (82) subsequently went on to compare adjuvant chemotherapy using 5-Fluorouracil (5FU) (as used in ESPAC-1) against the use of Gemcitabine, which had been showing improved outcomes in the palliative setting. Initially, the trial had a 3rd "observation" arm. However, due to the overwhelming evidence from ESPAC-1 that adjuvant chemotherapy improved survival, this arm was stopped prior to the conclusion of the trial. The ESPAC-3 trial showed similar survival outcomes between the 2 interventions, but the Gemcitabine regimen had a better toxicity profile, and as a result, Gemcitabine became the chemotherapeutic agent of choice for adjuvant therapy following pancreatic resection.

A further question that ESPAC attempted to answer was whether the addition of capecitabine (an oral pro-drug of 5FU) to adjuvant gemcitabine could improve survival further. The ESPAC-4 confirmed that combination adjuvant therapy improved overall

survival compared to single-agent gemcitabine (83). Ultimately Gemcitabine and Capecitabine (GemCap) combination chemotherapy became the recommended adjuvant chemotherapy regime

1.11.2 Radiotherapy

The role of adjuvant radiotherapy following pancreatic resection with curative intent remains a subject of debate. As previously mentioned, the ESPAC-1 trial confirmed the role of adjuvant chemotherapy, however, the results showed that adjuvant radiotherapy had a deleterious effect on outcomes following resection with curative intent (80). As a result of this, adjuvant radiotherapy is not recommended by NICE, and as such is not used outside of clinical trials in the UK. Despite this, elsewhere in the world, adjuvant radiotherapy is still administered and there are emerging reports that suggest that adjuvant radiotherapy may provide some survival benefit (84-86). However, there are conflicting reports as to whether the location of the tumour within the pancreas influences the outcome.

1.12 NEOADJUVANT THERAPY AND BORDERLINE RESECTABLE PANCREATIC CANCER

The term "neoadjuvant therapy" refers to oncological treatment being administered prior to surgical intervention. Whilst adjuvant treatment aims to kill any remaining occult disease, neoadjuvant aims to reduce tumour burden pre-operatively by reducing tumour volume and making a clear margin more likely to be obtained at the time of surgery. This is particularly important in pancreatic resections given the high number of microscopically involved surgical margins (R1 resections). The exact role of neoadjuvant treatment for patients with upfront resectable pancreatic cancer is still being established. Neoadjuvant chemoradiotherapy has been shown to improve the likelihood of an R0 resection in some series. Some established pancreatic cancer units, such as Glasgow offer neoadjuvant chemoradiotherapy to those with upfront resectable, borderline resectable and locally advanced PDAC (assuming they are fit to undergo surgical exploration). However, at present NICE only recommends the use of neoadjuvant therapy in patients with resectable or borderline resectable disease in the context of clinical trials (43). One concern patients have with undergoing neoadjuvant therapy when their tumour is upfront resectable or borderline resectable is that their disease will progress and become inoperable while they are receiving their treatment. Whilst it is true then that some patients will progress before surgery, this period of systemic therapy acts as a good test of tumour biology. We know that pancreatic cancer is an aggressive disease, and it is likely that those who progress on neoadjuvant therapy would likely have presented with local recurrence or metastatic disease early after resection, mitigating any benefit that surgical resection may offer in this situation. In a way, it weans out those who will likely have the longest disease-free intervals after surgery.

Over the last few years, there has been a shift in which patients are offered neoadjuvant treatment. This has come about following the preliminary results of the ESPAC-5 trial. This was a four-arm, randomised control trial which aimed to provide the evidence required to prove whether there is a benefit to neoadjuvant chemoradiotherapy for borderline resectable PDAC. Enrolled patients with borderline resectable disease will be randomised to either upfront surgery, neoadjuvant chemotherapy with Folinic Acid, 5-Fluorouracil, Irinotecan and Oxaliplatin (FOLFIRINOX), neoadjuvant chemotherapy with Gemcitabine and Capecitabine or neoadjuvant chemoradiotherapy

with induction Gemcitabine/Capecitabine followed by radiotherapy with capecitabine as the sensitiser. The preliminary results have shown that 1-year survival is significantly increased in the neoadjuvant groups compared to the upfront surgery group (77% vs 40%) (87, 88). The greatest survival benefit was seen in those patients who received neoadjuvant FOLFIRINOX chemotherapy. Interestingly there was no significant difference between the two groups in obtaining a clear microscopic resection margin (R0). With these impressive preliminary results, there is likely to be a shift towards offering more patients neoadjuvant therapy prior to pancreatectomy.

1.13   LOCALLY ADVANCED PANCREATIC CANCER

As previously alluded to, 20-30% of patients with a new diagnosis of PDAC will have locally advanced disease at the time of diagnosis. By definition, these tumours are traditionally not amenable to upfront surgical resection. However, survival amongst this patient group is better than that of patients with metastatic disease. For those patients with locally advanced disease but no evidence of metastatic disease, who are deemed to have a good performance status (ECOG PS0-1), systemic chemotherapy should be undertaken as per NICE guidance. Chemoradiotherapy is also commonly used in this group, with radiotherapy being given after induction chemotherapy, assuming the disease is stable or responding. Multiple regimens of different chemotherapy agents and radio-sensitizers have been trialled to establish which is best at controlling the disease. The SCALOP trial (89), demonstrated that capecitabine as a radiosensitizer may be better than Gemcitabine. However, the authors do note that the patient numbers were small and this difference did not reach statistical significance. New regimens that show improved survival within the cohort of patients with metastatic disease often make their way into trials to assess their benefit in locally advanced patients. One such example is the increasing use of the FOLFIRINOX regimen (Folinic Acid, 5FU, Irinotecan and Oxaliplatin) in locally advanced patients. Whilst this is a fairly toxic regimen, the initial results from several studies are promising. A meta-analysis by Suker and colleagues reviewed 13 studies, finding a pooled median overall survival of 24.2 months (10.0-32.7) (90). Single-agent gemcitabine had been the regimen of choice previously for locally advanced PDAC. However, overall survival in these patients is much less than that demonstrated with FOLFIRINOX by Suker and colleagues. One study by Chauffert and colleagues showed a median overall survival in patients treated with gemcitabine alone to be just 13 months (91).

Whilst systemic therapy primarily aims to control disease in locally advanced PDAC, some patients will have such a marked tumour response to chemotherapy or chemoradiotherapy, that the tumour becomes resectable. Rates of conversion to resectable disease vary, but in the meta-analysis by Suker et al., 28% of all patients analysed underwent resection, with an incredible R0 rate of 74%. However, it should be noted that a later series published by Suker and colleagues from their single institution revealed just 2 patients (9%) undergoing successful resection following

systemic treatment. When counselling patients regarding the rationale for systemic treatment on the background of locally advanced disease, it is important to explain that whilst, some patients may respond enough to ultimately undergo surgery, this remains the exception rather than the rule.

Whilst systemic chemotherapy +/- chemoradiotherapy continues to form the backbone of current treatment for locally advanced PDAC, new technologies are developing and being evaluated for their potential role as an adjunct to current treatment modalities. One such development is Irreversible Electroporation (IRE). The principle of IRE is that it produces high-voltage pulsations that lead to non-thermal injury to the tumour cells, ultimately leading to cell death. It can be used percutaneously as well as surgically (having been used in both open and laparoscopic settings). Due to its non-thermal mode of action, it is safe to use near vascular structures, thus lending itself to be used in locally advanced pancreatic cancer.

1.14   METASTATIC PANCREATIC CANCER

As mentioned previously, up to 50% of patients will already have metastatic disease at the time of the first diagnosis, placing oncology and palliative care at the centre of treating this patient group. Pancreatic cancer can metastasize to a variety of locations, both inside and outside of the peritoneal cavity. The most commonly affected sites are the peritoneum (42.3%), liver (41%), lungs (13.9%) and lymph nodes (9%) outside of the field of resection (92).

With improvements in cross-sectional imaging, most patients with metastatic PDAC will be diagnosed prior to undergoing laparotomy. However, a small proportion of patients will be found to have occult metastatic disease at the time of surgery.

Due to the aggressive nature of PDAC, patients with metastatic disease have a median survival of just 3-6 months from the time of diagnosis. Developments in the treatment of metastatic disease often predate those seen in locally advanced PDAC and early pancreatic cancer. In the late 90s, Gemcitabine monotherapy was found to confer a modest survival benefit compared to 5FU, making it the agent of choice in metastatic PDAC (93). Since then, gemcitabine-based combination therapies including the addition of the tyrosine kinase inhibitor, Erlotinib, have been shown to improve overall survival compared to monotherapy gemcitabine. A Canadian study in 2007 showed that a combination of Gemcitabine and Erlotinib significantly improved survival compared to monotherapy Gemcitabine from 5.91 to 6.24 months (94). The PRODIGE 4/ACCORD 11 trial (95), showed a greatly improved overall survival for patients treated with FOLFIRINOX compared to single agent Gemcitabine (11.1 vs 6.8 months).

Two years later, the MPACT trial (96) showed that the addition of nab-Paclitaxel to Gemcitabine conferred a significant survival benefit over gemcitabine alone (8.5 vs 6.7 months).

At present, no randomised trials have been undertaken to compare FOLFIRINOX against a combination nab-Paclitaxel and Gemcitabine in the treatment of patients with metastatic PDAC. A few retrospective studies have been undertaken to compare outcomes, but the results from these are contradictory with some showing FOLFIRIONX to be superior (97) and others showing nab-paclitaxel/gemcitabine to have improved overall survival (98).

Given the available evidence as demonstrated in the above studies, at present current NICE guidance advises FOLFIRINOX should be offered as first-line treatment in metastatic PDAC for patients with performance status 0-1 who are deemed fit enough to tolerate its high toxicity profile. If FOLFIRINOX cannot be tolerated, combination nab-paclitaxel/gemcitabine should be offered over monotherapy Gemcitabine which should only be offered to those who cannot tolerate combination chemotherapy.

1.15   PALLIATION OF ADVANCED PANCREATIC CANCER

In patients with advanced PDAC, symptoms tend to arise due to the local extension of the primary tumour into surrounding tissues and organs. As a result, the most common symptoms experienced by patients with advanced PDAC are jaundice with associated pruritis and cholangitis due to biliary obstruction; vomiting and malnutrition from gastric outlet obstruction; and pain due to perineural spread into the nearby coeliac plexus of nerves. Each of these can be addressed by a variety of modalities, and the timing of such interventions will depend upon the severity of the symptoms, the patient's fitness and ultimately the presumed prognosis based on disease burden.

1.15.1 Biliary Obstruction

Biliary obstruction on the background of PDAC may be due to compression by the primary tumour itself or may occur as a result of nodal disease within the porta hepatis. Biliary obstruction leads to jaundice due to the deposition of bile salts within the subcutaneous tissues. Pruritis is often present before the patient is visibly jaundiced and this can be a difficult symptom to manage. Medical management should be initiated in the first instance, and the use of bile salt sequestrants such as cholestyramine may alleviate symptoms. The mere presence of jaundice alone in the asymptomatic patient should not necessarily lead to biliary drainage due to the potential risks associated with ERCP/PTC/Surgical bypass. However, biliary drainage is indicated if the patient is a candidate for palliative chemotherapy (many oncologists will not give chemotherapy to patients with significant hyperbilirubinaemia), or if the patient is suffering from intractable pruritis or recurrent cholangitis. Patients who develop recurrent cholangitis/biliary obstruction following endo-biliary stenting may be started on the medication Ursodeoxycholic Acid (UDCA). UDCA is a secondary bile salt which reduces the cholesterol saturation of bile, theoretically leading to the thinning of bile/biliary sludge. Whilst many patients with biliary stents on the background of malignant biliary obstruction are given UDCA to prevent stent occlusion, the evidence base for this is currently lacking (99), with some evidence to suggest a detrimental effect as the result of the administration of UDCA(100).

The role of the surgical biliary bypass as a primary procedure in patients with advanced PDAC has now been superseded by improvements in endoluminal therapy and the

development of metal endo-biliary stents. The majority of patients presenting with biliary obstruction on the background of advanced PDAC will be able to undergo successful ERCP with the insertion of a metal stent. In those patients found to have advanced disease at the time of exploratory laparotomy, a prophylactic hepaticojejunostomy is typically performed to prevent biliary obstruction as the disease progresses. However, in those patients who have had a fully covered, removable metal stent placed pre-operatively, it is deemed reasonable to leave this stent in and not perform a biliary bypass, as these newer stents are less prone to blocking and will likely last beyond the patient's lifespan.

1.15.2 Gastric Outlet Obstruction

The development of gastric outlet obstruction amongst patients with advanced PDAC results from local tumour growth into the duodenum, until luminal obstruction occurs. Patients will typically complain of early satiety after eating and epigastric discomfort followed by a large, effortless vomit. The clinical examination may reveal upper abdominal distension, which is dull to percussion and the presence of a succussion splash on auscultation. Any patient with known pancreatic cancer presenting with the new development of vomiting should be assumed to have GOO until proven otherwise. Patients presenting with GOO often have grossly deranged electrolytes and acid: base balance, with the finding of a hypokalaemic, hypochloraemic metabolic alkalosis being pathognomonic of GOO. These imbalances should be aggressively corrected, and the patient appropriately resuscitated prior to any planned intervention. Strict fluid management using a urinary catheter should be used. A large bore nasogastric tube should be used to decompress the stomach. If the gastric contents prove to be thick, then gastric lavage should be undertaken. A plain Abdominal X-ray may show a large gas-filled stomach, or if there is a large volume of fluid within the stomach, then a paucity of gas may be noted. Cross-sectional imaging with CT should be undertaken to confirm the diagnosis, once the patient's renal function is sufficient to administer intravenous contrast.

Once the diagnosis is confirmed and the patient has been appropriately resuscitated, then a multidisciplinary, patient-centred approach to managing the patient should be instigated. The main decision to be made is whether the goal of treatment is to allow

enteral nutrition so that the patient may be able to go on to have further palliative therapy or whether a purely palliative approach is to be taken. Options for allowing enteral nutrition include surgical bypass and endoluminal stenting.

A surgical bypass in the form of a gastro-jejunostomy can be undertaken either by a laparotomy or as is increasingly the case via the laparoscopic root. Regardless of the approach, a surgical bypass involves anastomosing a loop of jejunum onto the greater curvature of the stomach.

As is the case with endo-biliary stenting for obstructive jaundice, Self-Expanding Metal Stents (SEMS) have been developed to allow stenting of the duodenum for patients with malignant GOO. Initially, there were concerns that the duodenal stenting was not appropriate for patients with a prognosis of longer than a couple of months (101), but more recent studies have shown that stents may in fact have the same level of long-term success as gastro-jejunostomy (102). In patients who are still candidates for palliative chemotherapy, the reduced morbidity and quicker recovery offered by duodenal stenting make this method of treatment an increasingly attractive prospect for patients with malignant GOO.

In patients who are clearly approaching the end of their life and will not be receiving any more treatment, then it may be appropriate to do nothing (best supportive care). If the patient is clearly approaching the end of their life, then palliation from the distress of vomiting may be achieved with ongoing nasogastric drainage or palliative venting gastrostomy.

1.15.3 Pain

Due to the location of the pancreas and its proximity to the neurovascular bundles around the SMA and Coeliac axis, as pancreatic cancer advances it will often lead to abdominal/back pain that is difficult to treat due to a combination of peri-tumoural inflammation and direct invasion into the parasympathetic nerves of the coeliac plexus. Early input of specialist palliative care has been shown to improve the management of pain in these patients. As with all causes of pain, analgesia should be carefully titrated to the patient's needs, as per the WHO analgesic ladder, starting with simple analgesics such as paracetamol, gradually increasing the potency, working towards strong opioids, and adding adjuvants such as anti-emetics as required (103). In those patients

in whom oral/subcutaneous analgesia is not sufficient to control pain, or there are adverse side effects associated with the opioid usage, consideration should be given to the use of coeliac neurolysis/nerve block, which can be performed via EUS or percutaneously (43). As with biliary obstruction and gastric outlet obstruction, if the patient is found to have advanced disease at the time of laparotomy, intra-operative neurolysis can be performed under direct vision.

1.16   CONCLUSIONS

Despite advances in surgical techniques, peri-operative care and oncological approaches, Pancreatic Ductal Adenocarcinoma (PDAC) continues to be a death sentence for nearly all patients in whom it is diagnosed. Early diagnosis remains the exception as opposed to the rule due to the insidious onset and vagueness of the symptoms of pancreatic cancer. Whilst several risk factors have been identified as putting people at a higher likelihood of developing PDAC, being able to screen these people with a reliable test seems a long way off. Increasing interest in the identification of biomarkers in various cancers may allow detection at an earlier stage and potentially lead to improved outcomes.

1.17 AIMS AND HYPOTHESES OF THIS THESIS

The aims of this thesis are to:

- Review the current role of biochemical biomarkers in the diagnosis and monitoring of Pancreatic Ductal Adenocarcinoma whilst gaining an insight into which markers are currently being looked at in the experimental setting by undertaking a review of the contemporary published literature in this field.

- Experimentally determine if a selection of other biomarkers possess diagnostic accuracy, using blood samples to allow them to be used instead of or alongside Ca19-9 in identifying early PDAC

- Review the role of spectroscopy in the diagnosis of PDAC

The hypotheses are:

- Plasma Ca19-9, with its currently widely used cut-off of 37U/L, is not the best available biomarker for detecting pancreatic cancer.
  - o This hypothesis was tested by using ELISA to quantify levels of selected biomarkers (Ca19-9, Thrombospondin-2 and YKL-40) in plasma samples obtained from consenting participants with and without pancreatic cancer. Receiver Operating Characteristic (ROC) curves were constructed for each analysed biomarker and compared to the gold standard of Ca19-9.

- Urinary biomarkers have the potential to be used to differentiate between plasma samples obtained from participants with pancreatic cancer and those without cancer
  - o Urine samples collected from consenting participants were used in the ELISAs to quantify whether the biomarkers are present in urine. The correlation between the biomarker concentrations in the paired plasma and urine samples was assessed.

- A multi-analyte biomarker panel will have more diagnostic accuracy at differentiating between samples obtained from the cohorts with and without pancreatic cancer than a single biomarker, namely plasma Ca19-9 with a cut-off of 37U/L.

  - A selection of multi-analyte panels was constructed by incorporating the quantified biomarker concentrations in plasma and urine. Cut-off levels for each of these biomarkers were determined using the previously constructed ROC curves. Diagnostic accuracy of each of these models. Accuracy was determined by combining the true positive and true negative proportions for each panel.

- FTIR Spectroscopy can differentiate between plasma samples obtained from participants with pancreatic cancer and those without cancer.
  Plasma samples obtained from all of the cohorts were subjected to FTIR spectroscopy, and the spectra were compared between the groups. Machine learning was then used to create a model to analyse the spectra

CHAPTER 2

REVIEW OF THE LITERATURE REGARDING BIOMARKERS CURRENTLY USED TO DETECT PANCREATIC DUCTAL ADENOCARCINOMA IN CLINICAL AND NON-CLINICAL SETTINGS

2.1 SUMMARY

Early detection of Pancreatic Ductal Adenocarcinoma (PDAC) is key to increasing the percentage of patients diagnosed with the less advanced and potentially curable disease. Currently, there are no truly diagnostic biochemical tests that can reliably diagnose PDAC in asymptomatic patients. Therefore, the diagnosis requires radiological imaging which is often only instigated after the development of symptoms which occur as a result of disease progression. Carbohydrate Antigen 19-9 (Ca19-9/Sialyl-Lewis Antigen A) is often used indiscriminately as a tumour marker to attempt to detect pancreato-biliary malignancies in patients with vague symptoms such as weight loss and anorexia. However, it lacks the sensitivity and specificity required to allow it to be used routinely to screen asymptomatic patients.

This chapter reviews the current literature surrounding current biochemical methods of detecting PDAC in clinical and pre-clinical practice. A literature search was undertaken using the Medline database. Search terms of "early pancreatic cancer," "diagnosis," and "biomarker" were combined, yielding approximately 400 abstracts. These abstracts were screened, and papers which described biomarkers which frequently appeared in different studies from this search and those which seemed to have an excellent accuracy in discriminating pancreatic cancer from non-cancer or had been trialled in clinical practice were then further interrogated and discussed below. This amounted to approximately 70 papers regarding biomarkers in PDAC (clinical and pre-clinical).

2.2 BLOOD-BASED MARKERS

The idea of a blood-based diagnostic tool for the diagnosis of early pancreatic cancer is an appealing one for several reasons. The current pathways for diagnosing pancreatic cancer rely on a high clinical index of suspicion (due to the often ambiguous symptoms) coupled with high-quality cross-sectional imaging. Whilst a radiological diagnosis is sufficient to proceed to potentially curative surgical resection, radical and palliative oncological therapies will often not be considered in the absence of a tissue diagnosis, something that invariably involves an invasive procedure (Endoscopic Retrograde Cholangiopancreatography [ERCP], Endoscopic Ultrasound [EUS] or surgical biopsy). Even patients with a high Ca19-9 and suspicious imaging are likely to be turned down by oncologists due to concerns about the lack of sensitivity of the biomarker.

The early 90s saw several candidate biomarkers being identified all with the hope of being the one which will lead to early diagnosis of Pancreatic Ductal Adenocarcinoma (PDAC). These included analysis of tissue as well as bodily fluids (blood, bile, pancreatic juice, ascites fluid) to identify potential markers. Whilst several of these previously investigated markers have been found to lack the sensitivity and specificity needed to diagnose PDAC, new candidate biomarkers are being identified regularly.

A literature search using the Medline database revealed a multitude of different candidate biomarkers which have been evaluated over the past few decades. A handful of the most promising of these are discussed below.

2.2.1 CA19-9

Carbohydrate Antigen19-9 (Ca19-9, Sialyl Lewis Antigen A) was first identified in 1979 by Koporwski and colleagues (104), whilst trying to identify markers for colon cancer. It is now most commonly used when looking at epithelial malignancies arising from the pancreato-biliary tract and as such is the current gold standard to which new biomarkers are compared. As mentioned in the previous chapter, Ca19-9 is not secreted in 10% of Caucasians leading to a significant false negative rate. Conversely, there is a wide range of benign and non-pancreato-biliary pathologies which can lead to a falsely elevated Ca19-9, including cirrhosis and choledocholithiasis. As such the sensitivity has been noted to be anywhere between 79% and 95% with a specificity of

82% and 91% (105). The relatively high false positive and false negative values mean that it is not suitable to be used in isolation when trying to diagnose pancreatic cancer, even in the symptomatic patient, let alone the asymptomatic population. It is best used as a marker of disease recurrence in those patients who have had a tissue diagnosis of PDAC.

2.2.2 CEA

Carcinoembryonic Antigen (CEA) is a glycoprotein that was first identified in 1965 by Gold and Freedman (106), having been isolated from colonic cancer tissue. Whilst predominantly used as a tumour marker in patients with suspected or confirmed colonic cancer, its role in aiding with the diagnosis of other cancers, including pancreatic cancer, has been previously investigated. Whilst serum/plasma levels of CEA offer little help with diagnosing pancreatic cancer, cyst fluid levels of CEA have been found to strongly correlate with the presence of mucinous cystic tumours of the pancreas (Mucinous Cystic Neoplasms [MCN] and Intraductal Papillary Mucinous Neoplasms [IPMN]), both of which are known to harbour a potential for malignant transformation. Currently, the main role of monitoring CEA levels is in monitoring response to treatment in patients with colorectal malignancies.

2.2.3 Glypican-1

A 2015 paper by Melo, put Glypican-1 (GPC-1) under the spotlight as an exciting new potential marker for detecting pancreatic cancer (107). The study identified GPC-1 positive exosomes in the serum of patients with pancreatic cancer and proclaimed absolute sensitivity and specificity in distinguishing PDAC samples from healthy controls and those with benign pancreatic pathologies. Further studies have assessed the role of exosomal GPC-1 as a diagnostic marker with encouraging results (108, 109). Interestingly, however, other studies have failed to demonstrate a difference in the expression of GPC-1 between PDAC samples and samples obtained from patients with benign pancreatic pathology (110). As a result of these conflicting studies, it is unclear whether GPC-1 will prove itself to be a useful biomarker in pancreatic cancer diagnosis or not.

2.2.4 Thrombospondin-2

Thrombospondin-2 (THBS2) is a glycoprotein which had been implicated in the downregulation of angiogenesis. It has been found to play a key role in pancreatic cell invasion, being secreted by tumour-derived pancreatic stellate cells (111). Its potential role in clinical diagnostics was evaluated by Kim and colleagues, who identified it as a potential candidate biomarker that was secreted by premalignant PanIN organoids in their study (112). This study showed that THBS2 could be used to differentiate between the serum of patients with PDAC and healthy controls with high specificity (99%). However, the sensitivity was low at 52%. They also showed that combining levels of THBS2 with that of Ca19-9 increased the sensitivity to 87% (with a specificity of 98%).

These promising results are why we opted to include THBS2 as a biomarker in our study. Encouragingly, several other studies have been published during the time our study was underway, which have confirmed the utility of THBS2 (alone or in combination with ca19-9) in discriminating between PDAC and control serum (113-115). As well as its potential as a diagnostic biomarker in PDAC, a few studies have looked at its role as a prognostic marker, showing that patients with a higher serum THBS2 level are likely to have a worse prognosis compared to those with lower levels (113, 116). Initial studies are showing that Thrombospondin-2 may soon be heading towards clinical applications in pancreatic cancer.

2.2.5 Human Chitinase 3-Like 1 Protein

Chitinase 3-Like 1 Protein, also referred to as YKL-40, is a highly conserved glycoprotein, which plays a role in inflammation. It has been found to be secreted by macrophages, as well as a variety of cancer cells including PDAC (117). Initial immunohistochemistry studies in our lab with PDAC cell samples prompted us to look further into its potential role as a blood-based biomarker.

A review of the literature revealed just a handful of studies looking at YKL-40 in pancreatic cancer, with the majority focusing on its potential as a marker of prognosis. Just two studies, looked at YKL-40 as a diagnostic marker, both evaluating it as part of a biomarker panel in combination with Ca19-9 and other biomarkers (118, 119). The study by Schultz concluded that as a diagnostic marker, it was less reliable than

Ca19-9, but did highlight its potential role as a prognostic marker(118). Interestingly the study by Ma did show a significant difference in serum levels of YKL-40 in PDAC patients compared to healthy controls (119). Clearly, from these contrasting outcomes, it is difficult to conclude at this point whether YKL-40 will have a role in the future as a diagnostic biomarker for PDAC.

Whilst the jury is out on the utility of YKL-40 as a diagnostic marker, there appears to be a consensus that it can be used as a prognostic marker, with studies consistently showing that elevated serum tissue levels of YKL-40 translate into poorer outcomes (120, 121) suggesting that quantitative assessment of YKL-40 levels may have a role in guiding therapeutic strategies.

2.2.6   K-Ras

As previously discussed in chapter 1, K-Ras mutations are present in 61%-90% of pancreatic cancers (25, 122). Being able to detect these mutations in the plasma of patients with pancreatic cancer and utilise them as a biomarker is therefore an attractive prospect. As in most cancers in which K-Ras mutations have been identified, a mutation at Codon 12 is most commonly seen, being the culprit in up to 90% of K-Ras mutations seen in pancreatic cancer (123). The most common codon 12 mutations seen in pancreatic cancer are a single base switch from GGT to either GAT or GTT (122). Less frequently, mutations at codon 13 or 61 have been identified in PDAC. The frequency of codon 12 mutations allows it to be targeted when undertaking analysis of plasma samples, as well as making it a target for specific drugs.

Castells and colleagues analysed plasma K-Ras mutations in a small study, finding them to present in just 27% of patients with pancreatic cancer, being more likely to be present in those patients with more advanced disease (124). These findings are similar to those shown in an earlier study by Yamada, in which 9 out of 21 patients with pancreatic cancer (42.9%) had detectable plasma K-Ras mutations. Again, the presence of K-Ras mutations in plasma samples signalled a more aggressive disease (125).

Interestingly, however, a prospective case series by Dianxu found that plasma K-Ras mutations could be detected in 70.7% of patients with pancreatic cancer - a similar frequency to those with an elevated Ca19-9 (126). This study also showed that 90% of

patients with pancreatic cancer had either an elevated Ca19-9 or K-Ras mutation, suggesting the utility of K-Ras analysis to detect a subset of patients who do not have an elevated Ca19-9.

2.2.7   PIVKA-II

A more recently identified potential biomarker, which has shown promise in a couple of studies using small sample sizes, is Protein Induced by Vitamin K Absence II (PIVKA-II). It is an abnormal prothrombin which had previously been identified to be elevated in other GI malignancies (particularly liver). Tartaglione and colleagues used chemiluminescent enzyme immunoassay to quantify levels of PIVKA-II in the serum of patients with PDAC and benign diseases, also using ELISA to analyse levels of Ca19-9, CEA and C242 (127). They found a significant difference in concentration between the 2 patient groups, and subsequent receiver operating characteristic (RoC) curves showed PIVKA-II to be better at differentiating between the 2 groups than the other analysed traditional markers of PDAC. The group subsequently added to this research by showing that PIVKA-II is expressed in PDAC tissue, and serum levels of PIVKA-II drop significantly after surgical resection. This poses a very promising potential biomarker for PDAC (128).

2.3 NON-BLOOD BASED MARKERS

Whilst blood-based biochemical tests form the mainstay of the diagnostic workup for many conditions, both benign and malignant, some have begun to focus on analysing other bodily fluids to aid with diagnosis/screening. Whilst obtaining a blood sample is fairly non-invasive on the spectrum of medical investigations, it is still unpleasant for the patient and can lead to pain and bruising. Also, given some patients suffer from trypanophobia, more commonly referred to as "needle-phobia," the ability to investigate a patient and obtain a diagnosis through less/non-invasive methods compared to blood sampling can only be deemed as a positive step. Obtaining a urine sample is extremely easy and is associated with no morbidity or significant patient distress.

Due to its ease of collection, several studies have sought to analyse urine samples to see if they can be used to diagnose multiple cancers. Given urine is produced in the kidneys via ultrafiltration of the blood, it is a fair assumption that at least some of the markers which have been identified in blood samples may be isolated from urine samples.

A multi-analyte urinary biomarker panel has been developed by a London-based research team to predict the risk of an individual developing PDAC, allowing other methods of investigation to be initiated. PancRISK was developed by retrospectively analysing urine samples from patients with PDAC and healthy controls (129). Large-scale validation of this panel is still awaited, however.

As well as analysing urine for proteins and circulating tumour DNA (ctDNA), there has been some interest in differences in concentrations of metals within the urine of those with and without pancreatic cancer. An interesting small study by Schilling has shown that patients with PDAC were found to have significantly higher concentrations of urinary copper and zinc compared to healthy controls (130). PDAC patients were also found to have a lower urinary concentration of calcium and magnesium.

The adult pancreas produces up to 2 litres of "juice" each day. This fluid is rich in electrolytes, amylase, lipase and proteolytic enzymes to aid with digestion. The enzymes are produced by the exocrine, acinar cells of the pancreas, in a bicarbonate-rich fluid which is secreted into the small branch ducts, before draining into the Main

Pancreatic Duct (MPD). From there the juice drains into the second part of the duodenum via the ampulla of Vater. The pancreas is stimulated to produce pancreatic juice and release it into the duodenum by the peptide hormone secretin, which is secreted by the S cells in the duodenum. IV infusion of synthetic secretin has been shown to artificially cause the pancreas to increase pancreatic juice secretion, and as such is being used to increase the yield of pancreatic juice which can be collected at endoscopy. Given pancreatic juice is in direct contact with the malignant ductal cells in PDAC, it is unsurprising that pancreatic juice may be able to detect pancreatic cancer at a much earlier stage than blood-based biomarkers. Several studies have looked to profile candidate biomarkers in pancreatic juice, including micro RNAs (miRNAs) (131), and proteins (132, 133). However, one of the difficulties in the utilisation of pancreatic juice as a diagnostic tool is standardising how to collect the pancreatic juice. An interesting small study by Levink and colleagues looked to identify the optimal method of pancreatic juice collection, looking at the yield of biomarkers with 2 different methods (endoscopic suction vs catheter retrieval) over different time points from secretin infusion (0-4, 4-8 and 8-15 minutes) (134). This has shown a greater yield of biomarkers including mutant KRAS is higher if endoscopic suction is used for up to 8 minutes after secretin infusion). It is hoped by standardising the method of pancreatic juice collection, further research can be undertaken in this promising area of PDAC diagnosis.

2.4   SPECTROSCOPY

As well as traditional methods of analysing tissue/body fluids (ELISA, Immunohistochemistry etc) there has been an increasing interest in utilising spectroscopy. Using the underlying principle that each cancer has a spectroscopic "fingerprint," it is hoped that spectroscopy may be able to differentiate between cancer samples and healthy controls, whilst not being necessarily able to identify the markers which given the unique spectroscopic appearance, the development and recognition of this unique fingerprint, would open the door to allow increased application of Artificial Intelligence (AI) in cancer diagnostics. Initial studies looking at the role of spectroscopy in diagnosing pancreatic cancer are promising (135, 136), though most machine learning models require full validation.

2.5   CONCLUSIONS

It is apparent from the above review of the available literature that biomarkers have been a source of interest in pancreatic cancer for many decades. Whilst many candidate biomarkers have been identified and investigated in both laboratory and clinical settings, it is apparent that none have the sensitivity and specificity required to be used as a diagnostic tool in isolation from radiology and histological diagnoses.

Previous immunohistochemistry work in our lab looking at the expression of YKL-40 in pancreatic cancer tissue samples has been promising and therefore we decided to further explore the utility of this protein as a plasma and urine-based biomarker. We also decided to further interrogate Thrombospondin-2 (THBS2) and Glypican-1 (GPC-1) as potential biomarkers due to some promising results mentioned in the literature. As Ca19-9 remains the gold standard biomarker in PDAC, we decided to compare the ability of the above biomarkers to differentiate between PDAC and non-PDAC samples with Ca19-9, in isolation and potentially as a biomarker panel. The increasing interest and potential utility of spectroscopy are why we elected to undertake Fourier transformation-infra red (FT-IR) spectroscopy on our samples as a proof of principle and to see if we could develop a machine learning diagnostic model.

CHAPTER 3

MATERIALS AND METHODS

3.1 SUMMARY

This chapter sets out the logistical and practical aspects of undertaking a study to develop a potential multi-analyte biomarker panel which may be used to diagnose Pancreatic Ductal Adenocarcinoma. It examines the process from study conception and design, through the various committees through which it must be approved and finally the practical laboratory-based aspects of the study.

3.2 MATERIALS

3.2.1 Sample Collection, Processing and Storage

Item	*Manufacturer*
6ml Heparinised Vacutainer blood collection tube (Green top)	*Becton Dickinson and Company, Franklin Lakes, NJ, USA.*
Universal container (White top)	*Greiner Bio-One, Stonehouse, UK*
Bench Top Centrifuge 5810R	*Eppendorf UK Ltd, Stevenage, UK*
Variable Volume Pipette Tips (100-1000µl)	*Greiner Bio-One, Stonehouse, UK*
Filtered Pipette Tips	*Greiner Bio-One, Stonehouse, UK*
Microcentrifuge Tubes (0.5ml, 1.5ml)	*Eppendorf UK Ltd, Stevenage, UK*
Innova U725 -80ºc Freezer	*New Brunswick Scientific UK, St Albans, UK*

3.2.2 Enzyme-Linked Immunosorbent Assay

Item	*Manufacturer*
Hardware and Consumables	
Tabletop Microcentrifuge 5415D	*Eppendorf UK Ltd, Stevenage, UK*
Variable Volume Pipettes (2-10µl, 10-100µl, 20-200µl,100-1000µl)	*Gilson, Middleton, WI, USA*
8 tip Multichannel Pipette	*ThermoScientific, Waltham, MA, USA*
Filtered Pipette Tips	*Greiner Bio-One, Stonehouse, UK*
Microcentrifuge Tubes (0.5ml, 1.5ml, 2.0ml)	*Eppendorf UK Ltd, Stevenage, UK*
Conical Centrifuge Tubes (15ml and 50ml)	*Greiner Bio-One, Stonehouse, UK*
POLARstar Omega Microplate Reader	*BMG LABTECH, Ortenberg, Germany*
Incubator	*ThermoFisher Scientific, Loughborough, UK*
Vortexer Genie II	*Scientific Industries, New York, NY, USA*

R&D Systems Ancillary Reagent Kit 2	*Bio-Techne, Abingdon, UK*
96 well high binding microplate	
Plate-Coating Buffer (1X PBS)	
Reagent Diluent 2 (10X BSA)	
Wash Buffer Concentrate (25X PBS + Tween® 20)	
Colour Reagent A (Hydrogen Peroxide)	
Colour Reagent B (Tetramethylbenzadine)	
Stop Solution (2N Sulfuric Acid)	
R&D Systems Thrombospondin-2 DuoSet	Bio-Techne, Abingdon, UK
Mouse Anti-Human Thrombospondin-2 Capture Antibody	
Biotinylated Goat Anti-Human Thrombospondin-2 Detection Antibody	
Recombinant Human Thrombospondin-2 Standard	
Streptavidin conjugated with Horse Radish Peroxidase	
R&D Systems Human Chitinase 3 Like 1 DuoSet	*Bio-Techne, Abingdon, UK*
Rat Anti-Human Chitinase 3-Like 1 Capture Antibody	
Biotinylated Goat Anti-Human Chitinase 3-Like 1 Detection Antibody	
Recombinant Human Chitinase 3-Like 1 Standard	
Streptavidin conjugated with Horse Radish Peroxidase	
R&D Systems Glypican-1 DuoSet	*Bio-Techne, Abingdon, UK*

Goat Anti-Human Glypican 1 Capture Antibody	
Biotinylated Goat Anti-Human Glypican 1 Detection Antibody	
Recombinant Human Glypican 1 Standard	
Streptavidin conjugated with Horse Radish Peroxidase	
Novus Biologicals Human Ca19-9/Sialyl Lewis A ELISA Kit (Colorimetric)	*Bio-Techne, Abingdon, UK*
Pre-coated 96-well Assay plate	
Standard	
Horse Radish Peroxidase Conjugate	
Wash Buffer (20X)	
Substrate A	
Substrate B	
Stop Solution	
Other Stock Reagents	
Bovine Serum Albumin	*Sigma-Aldrich, St Louis, MO, USA*
Phosphate Buffered Saline (PBS)	*Sigma-Aldrich, St Louis, MO, USA*
Tween® 20	*Sigma-Aldrich, St Louis, MO, USA*
De-ionised water	*Milli-Q*

3.2.3 FTIR Spectroscopy

Item	*Manufacturer*
Spectrum TWO FTIR Spectrometer	*PerkinElmer Inc, Waltham, MA, USA*
Calcium Fluoride Slide	*Crystran, Poole, UK*

3.2.4   PREPARATION OF STOCK REAGENTS

Ancillary Reagent Wash buffer (25X)

20ml Wash Buffer Concentrate

480ml Distilled water

Made at the time of coating plates and stored at 4ºC overnight

Reagent Diluent (10X Bovine Serum Albumin [BSA])

10ml Reagent Diluent Concentrate

90ml Distilled water

Made fresh on assay day

Bovine Serum Albumin (BSA) 1%

1g BSA powder (no protease)

100ml Phosphate Buffered Saline

Made fresh on assay day

Wash Buffer II – PBS and Tween 10X

2.5ml Tween® 20 in 500ml PBS

50ml of the above mix

450ml distilled water

Made when coating plates and stored at 4ºC overnight

3.3 STUDY METHODOLOGY

3.3.1 Study Concept and Initial Protocol

As previously alluded to, Pancreatic Ductal Adenocarcinoma (PDAC) remains an elusive disease with a depressingly poor prognosis due to a range of clinic-pathological factors, not least the lack of a reliable biomarker which can be readily used to aid diagnosis in the asymptomatic/minimally symptomatic patient. Whilst serum Ca19-9 levels are frequently employed as a blanket biomarker to attempt to detect pancreato-biliary malignancy in the patient with vague, non-specific symptoms of underlying malignancy such as weight loss and decreased appetite, its lack of sensitivity and specificity as a stand-alone biomarker render it near useless as a tool to detect early, treatable disease, especially in the screening environment.

The development of the "CancerSEEK" multi-analyte biomarker panel by the Johns Hopkins group (137) has led to a renewed interest in the idea that using a combination of biomarkers to detect (or rule out) cancer may improve the accuracy of a diagnostic test. We, therefore, sought to develop a small panel of biomarkers to use in combination with Ca19-9 levels to see if we could improve the diagnostic accuracy of this test. We decided to look at three biomarkers: Thrombospondin 2 (THBS2), Human Chitinase 3-Like 1 (YKL-40) and Glypican-1(GPC-1) to see if they could be used to discriminate between samples obtained from participants with pancreatic cancer (both early and advanced), benign pancreatic conditions (acute pancreatitis, chronic pancreatitis, and pancreatic cysts) and control participants with no evidence of pancreatic disease or malignancy. Each of the selected biomarkers has been previously shown to be raised in people with pancreatic cancer to varying extents (as previously discussed in chapter 2). We decided to focus on blood and urine samples as these can be obtained with relative ease. The main aim was to investigate the blood samples. The urine would be investigated to see if any positive results obtained with the blood work could be demonstrated with urine, providing a potentially less invasive way of detecting pancreatic cancer. We initially hoped to analyse bile aspirates from patients with cancer and those with benign gallstone disease and this was included in our protocol. However, obtaining bile samples and transporting them to the laboratory in ILS1 in a timely manner often proved not possible, meaning that just a few samples were collected and were not enough to add any value to our results.

3.3.1.1 Reviewing Bodies and Funding

The protocol for the study was drawn up using a template provided by the ABMU R&D department by myself. I also acted as Principal Investigator (PI) for the study. Professor Bilal Al-Sarireh, Consultant Hepato-Pancreato-Biliary Surgeon at Morriston Hospital acted as Chief Investigator (CI). The study sponsor was the ABMU R&D department.

The study (including the protocol and associated documents) was discussed and evaluated by the Joint Scientific Review Committee (JSRC) of Swansea University and Abertawe Bro Morgannwg University Health Board. After some adjustments to the protocol, it was finally approved by the JSRC on 18th January 2019.

The protocol for the study and the associated documents, including Participant Information Sheets (PIS), Informed Consent Forms (ICF) and CRF forms were submitted for review by the South West Wales Regional Ethics Committee (REC) number 7 using the online Integrated Research Application System (IRAS). The IRAS number given to the study is **252525** and the REC reference is **19/WA/0064**. The ethics committee meeting was held on 19th February 2019. A favourable decision was given on 4th March 2019 after a few minor amendments, which were reviewed by the chair of the committee.

Approval by the Health Research Authority (HRA) and Health and Care Research Wales (HCRW) was granted on 7th March 2019. The final protocol to be approved and put into practice was version 3.4, dated 27/2/19.

An ABMU Pathway to Portfolio Bid was submitted to the sponsoring health board and was successful in securing £12,000 of funding to cover the costs of technical help, reagents and antibodies for the experiments.

3.3.1.2 Amendments to protocol/ study documents

A Non-Substantial Amendment (NSA) to the protocol and the associated documents was submitted to the REC on 7/11/2019. This was approved on December 12th. This amendment sought to broaden the application of spectroscopy to allow Fourier-Transform Infrared (FTIR) spectroscopy to be used to analyse samples instead of/as well as Raman spectroscopy. This also led to a change in the name of the study to a

Feasibility study to identify the potential role of spectroscopic techniques and ELISA analysis in identifying biomarkers to reliably detect early pancreatic cancer.

Other NSA's were submitted to allow extension to the recruitment period, which had to be extended due to the impact of the COVID-19 pandemic which led to a halt of all non-COVID-related studies for over a year. A further NSA was submitted to increase the potential number of participants recruited in each group.

3.3.2   Study Procedures

3.3.2.1   Participant groups

As the primary aim of the study was to determine if other biomarkers can be used instead of/to complement Ca19-9 to improve the diagnosis of pancreatic cancer, we sought to enrol participants with suspected or histologically confirmed PDAC into the study. We split these participants into 2 groups depending on whether at the time of enrolment, they were deemed to have early, potentially resectable disease (Group 1), or advanced disease not amenable to curative therapy (i.e., locally advanced or metastatic disease). Patients with advanced disease were labelled as group 2. Given the aggressive nature of PDAC and the potential for patients to develop advanced disease whilst waiting for surgery, or to be found to have advanced disease at the time of attempted surgery, some participants ultimately moved from group 1 to group 2. Similarly, as a tissue diagnosis is not required prior to surgery, there was a potential for patients who were initially felt to have a resectable tumour to undergo surgery, only for the final histology to reveal a benign diagnosis. In this case, the participants were moved from group 1 to group 3.

To establish whether any changes in biomarker concentrations were specific to pancreatic cancer or just pancreatic disease, we planned to include 2 non-cancer groups in our study. We sought to enrol participants with benign pancreatic pathology, namely acute pancreatitis, chronic pancreatitis and pancreatic cysts. It should be noted that, as previously mentioned, chronic pancreatitis and some pancreatic cysts are known to be pre-malignant. However, when these patients were screened, it was ensured that up-to-date radiology had shown no suspicion of developing malignancy. Patients with benign pancreatic pathology were allocated to group 3. The final group of participants

to be enrolled on the study were our true controls, i.e., those without a current or past history of the pancreatic disease (whether benign or malignant).

We set an initial target of enrolling 100 participants in the study given the time-critical nature of the study. As this was a feasibility study, the number of participants was decided based on the likelihood of completing recruitment within a 12-month period as opposed to being based on power calculations. We aimed to recruit 20 participants for group 1. This was based on the number of pancreatic cancer resections undertaken at Morriston Hospital in a 12 month period. Approximately 100 pancreatic resections (pancreaticoduodenectomy and distal pancreatectomy) were undertaken by the department of pancreato-biliary surgery at Morriston Hospital in 2017. Thirty three of these were for PDAC, the remaining being for a variety of other malignant and benign conditions (distal cholangiocarcinoma, ampullary carcinoma. Given the high proportion of patients presenting with advanced disease, we decided to recruit 20 participants with advanced PDAC, giving us a total of 40 patients with PDAC. Acute pancreatitis is an increasingly common diagnosis on acute surgical intake and due to increased utilisation of high-quality cross-sectional imaging, more and more patients are being found to have pancreatic cysts. We, therefore, hoped to recruit 30 patients with benign pancreatic pathology and we matched this with 30 control participants to give a grand total of 100 participants.

3.3.2.2   Screening and Recruitment

Potential participants were identified from a variety of sources. Those with confirmed or suspected PDAC who were to be recruited to groups 1 or groups 2 were identified from the weekly South Wales Pancreatic Cancer Multidisciplinary Team (MDT) meeting and the subsequent Pancreatic Outpatient clinic (both held at Morriston Hospital, Swansea). Some participants with advanced disease would be recruited when they attended Morriston or Singleton hospitals to undergo further investigations, such as Endoscopic Ultrasound (EUS) or percutaneous biopsy. Those with benign pancreatic conditions (acute pancreatitis (AP), chronic pancreatitis (CP), pancreatic cysts (PCN)) were identified from either the outpatient clinic (CP, PCN) or the inpatient wards (AP, CP). The final group of participants were those with no history

of pancreatic pathology. These participants were predominantly identified from day-case elective surgery lists.

Potential participants were screened for eligibility to enrol in the study by myself. Inclusion and exclusion criteria were applied as shown below in table 3.1 and were dependent on the potential study group in which the participants were to be enrolled.

Table 3.1. Inclusion/exclusion criteria for enrolment into the study

Inclusions	Exclusion
Aged 18 years or older	Under the age of 18
Capacity to provide informed consent	Lacks the capacity to consent
Groups 1 and 2 Confirmed/suspected diagnosis of PDAC where tissue confirmation can potentially be obtained - either by surgery or biopsy	Pregnant/breastfeeding women
Group 3 (AC) Diagnosis of acute pancreatitis	Recurrence of a previously treated PDAC
Group 3 (CP) Diagnosis of chronic pancreatitis as evidenced by radiological changes +/- pancreatic endocrine or exocrine insufficiency	Patients who have undergone neoadjuvant therapy for PDAC
Group 3 (Cyst) A diagnosis of a pancreatic cyst with no definite clinical, radiological or cytological evidence of malignancy as discussed at the regional pancreatic MDT	Vulnerable adults
Group 4 No diagnosis to place participants in groups 1-3	Previous or concurrent non-pancreatic malignancy
	Suspected PDAC but no tissue diagnosis is possible
	Suspected neuroendocrine tumour of the pancreas

3.3.2.3 Informed Consent Procedures

Once suitable participants had been identified, they were approached about whether they would be willing to take part in the study. The aims and methods of the study were discussed with the potential participant by a member of the research team and they were given a Participant Information Sheet (PIS) to read through in more detail. After the participants had been allowed a suitable time to read the PIS they were re-approached by a member of the research team and were given the opportunity to ask any questions they might have about the study. If they were happy to proceed with enrolling in the study, then they were subsequently asked to complete an Informed Consent Form which was signed by the participant and the research team member. The consent form was then copied, with the original being retained for the site file, 1 copy was given to the participant and another copy was filed in the participant's medical records. Enrolled participants were subsequently allocated a unique Participant ID number which they were informed of in case they needed or wanted to discuss anything further with the research team. A Case Report Form (CRF) would then be completed by the research team member and filed in the site file. For those participants in group 1 who had further samples taken post-operatively, consent would be re-confirmed with the patient and a further CRF documenting their post-operative course would be completed before taking blood and urine samples. If patients declined to give further samples they would be thanked for their input and asked if they were still happy for their original samples to be analysed. If they were no longer happy for previously obtained samples to be used, then these would be destroyed in accordance with the Human Tissue Act (2004).

3.3.2.4 Data Handling and Processing

As mentioned above, relevant participant information was collected on a standardised Case Report Form (CRF), specific to the participant visit.

Two databases were created using Microsoft Excel (Microsoft, Redmond, WA, USA). The first database contained participant demographics and linked their NHS number to their unique Participant ID which had been allocated to them at the time of enrolment of the study. The Second database contained the information taken from the CRFs and linked the participant ID to the specimen ID. This database also contained

information about the samples which had been obtained including the time they were obtained, centrifuged (if applicable) and placed into the freezer. Both of these databases were password protected with only the Chief Investigator (CI) and Principal Investigator (PI) privy to the passwords.

Hard copies of the signed Informed Consent Forms and Case Report Forms were stored in the Study Site File which was kept in a locked filing cabinet in the office of the CI which was locked when no one was in the office.

3.3.2.5 Statistical Analysis

Once samples had been processed and biomarker concentrations established, standard descriptive statistics were to establish median biomarker concentrations for each biomarker in each of the four participant groups. Median biomarker concentrations were also compared between participant gender and age. Multivariate analysis was undertaken using SPPSS (IBM, Armonk, NY, USA) on each biomarker to assess if the perceived differences in median concentrations between the different patient groups were confounded by participant age and gender or whether the perceived differences could be purely put down to the presence or lack of pancreatic cancer.

To compare the diagnostic value of the different analysed biomarkers, Receiver Operating Characteristic (ROC) curves were constructed. ROC curves plot the sensitivity against 1-sensitivity when different biomarker concentrations are used as cut-off values to distinguish between cancer and non-cancer specimens. The closer the apex of the curve lies to the upper left corner of the graph, the higher the diagnostic accuracy of the biomarker in distinguishing cancer from non-cancer samples, as reflected by a greater Area Under the Curve (AUC) value. A diagnostic test with an AUC of between 0.7 and 0.8 is seen as acceptable, whilst an AUC of greater than 0.8 is considered excellent. The AUC of each of the biomarkers were compared to establish which biomarker individually had the greater diagnostic accuracy.

Sensitivity equates to the percentage of people with a certain condition who will have a positive test, whilst specificity is the percentage of people without a condition who will have a negative test. Sensitivity and specificity for each chosen biomarker concentration can be calculated by constructing a 2 x 2 table as seen below.

	Has condition	Does not have condition
Positive Test (≥x)	True positive (A)	False Positive (B)
Negative Test (<x)	False Negative (C)	True Negative (D)

From this table, Sensitivity can be calculated as [A/(A+C)] x 100, and specificity as [D/(B+D)] x 100. The Positive Predictive Value (PPV) of a test is therefore equal to [A/(A+B)] x 100 and the Negative Predictive Value (NPV) is equal to [D/(C+D)] x 100. The overall accuracy of a chosen biomarker concentration can is the combination of the true positive and true negative rate [(A+D)/(A+B+C+D)] x 100.

The calculated sensitivity and specificity values for each measured biomarker concentration which were used in the construction of the ROC curves were then also used to establish potential concentration cut-offs for each of the biomarkers (dependent whether the focus should be on high sensitivity, high specificity, or overall accuracy). Once cut-off concentrations had been decided upon for each biomarker, a model was created combing the cut-off for each of the new biomarkers with the Ca19-9 concentration cut-off.

3.4 EXPERIMENTAL METHOD

3.4.1 Handling of samples

3.4.1.1 Obtainment of Samples

Once participants had given their informed, written consent to participate in the study they were asked to provide relevant tissue samples. All patients were requested to provide a blood and urine sample. Patients undergoing surgical intervention where access to the biliary tree was anticipated were asked if a sample of bile may be taken at the time of surgery.

All blood samples were taken by someone who was appropriately trained in venepuncture after the participant had been counselled regarding the study and given their informed consent by signing the appropriate consent form. Blood samples from participants initially enrolled into groups 2, 3 and 4 were all obtained from peripheral venous cannulation. Pre-operative samples obtained from participants initially enrolled on Group 1 were obtained either from peripheral or central venous cannulation arterial catheter in the radial artery ("art line").

Blood was drawn into 6ml heparinized, green top BD Vacutainer tubes (Becton, Dickenson and Company, Franklin Lakes, NJ, USA). The tubes were gently inverted at least 10 times to allow the heparin sodium anticoagulant within the tube to fully mix with the obtained blood. Blood bottles were labelled with the relevant unique specimen number which was linked to the donor's unique participant number. Once appropriately labelled, blood bottles were placed in a sealed, chilled transport bag.

Participants were asked to provide a urine sample in a white-topped universal container. All specimens were appropriately labelled with a unique specimen number linked to the donor's unique participant number and then placed in a sealed, chilled transport bag.

As obtaining bile requires an invasive procedure, only those patients undergoing surgery where the bile duct could be accessed were asked for bile specimens. This included participants undergoing attempted resection of confirmed or suspected PDAC and patients undergoing cholecystectomy for gallstones where the operating surgeon would undertake a cholangiogram for clinical reasons. Participants undergoing pancreatoduodenectomy would have a sample of bile aspirated directly from the bile duct at the time of transection of the duct. For those patients found to be inoperable at

the time of surgery and in whom a biliary bypass was undertaken, a sample of bile would be obtained at the time of choledochotomy. Those patients with gallstone disease undergoing a cholecystectomy with intra-operative cholangiogram would have bile aspirated via the cholangiogram catheter. All obtained bile specimens were transferred to a white top sterile universal contained and appropriately labelled with a unique specimen number which was linked to the donor's unique participant number. Labelled specimens were then placed in a sealed, chilled transportation bag.

3.4.1.2 Transport of Samples

All specimens were transported from the hospital of collection to the 2nd-floor laboratories in the Institute of Life Sciences 1 (ILS1) at Swansea University in a chilled, sealed bag in accordance with the Human Tissue Act, via a private vehicle.

3.4.1.3 Processing of Samples

Once in the ILS1 2nd-floor laboratory, the labelled blood tubes were placed in an Eppendorf Centrifuge (5810R). If an odd number of specimens had been collected, a vacutainer tube containing 6ml of water was used as a balance. Samples were centrifuged at 4ºC for 15 minutes at 2000 xG as per Thermo-Fisher Scientific protocol to obtain platelet-depleted plasma (138).

Samples were then carefully removed from the centrifuge and placed in a standard test tube rack inside a Mars Primary Hood. The resulting plasma was then transferred from the vacutainer blood tubes into sterile microcentrifuge tubes using a variable-volume pipette with filter tips. All Eppendorf tubes were labelled with the anonymous specimen number that was originally assigned to the donor.

Once in the ILS1 2nd-floor laboratory, urine samples were transported to the Mars Primary hood. Urine was then transferred from the universal container into 15ml conical centrifuge tubes. Samples were then spun at 5000 xG for 5 minutes at 4ºC to pellet any debris within the sample. The spun urine was then transferred into sterile microcentrifuge tubes, giving a total of 4.5ml of urine from each participant. Each tube was individually labelled with the anonymous sample number which had been assigned to the donor.

In the ILS1 2nd-floor laboratory, bile samples were transported to the Mars Primary hood. Bile was then transferred from the universal container to 1.5ml sterile Eppendorf tubes. Each Eppendorf tube was individually labelled with the anonymous sample number which had been assigned to the donor.

3.4.1.4 Storage of Samples Prior to Analysis

All plasma, urine and bile samples were frozen prior to analysis. Each labelled micro-centrifuge tube was placed in a labelled storage box and placed in one of the -80ºC freezers in the ILS1 2nd-floor laboratory until they were required for analysis. The position of each sample in the storage boxes was noted to allow ease of retrieval at a later date. All samples were processed and placed in the freezer within 4 hours of obtaining them.

3.4.1.5 Preparation of samples for assaying

On the day of the assay, the required samples were removed from the freezer and placed in a microcentrifuge rack to allow them to thaw. Once thawed the samples were placed in a bench-top microcentrifuge and spun at 10000xG for 5 minutes to pellet any debris. The relevant volume of the sample was then pipetted into a fresh microcentrifuge tube, in which the relevant reagent diluent was added to make up the appropriate volume and concentration of the sample. The prepared sample was then placed on the Vortex mixer Genie II (Scientific Industries, New York, NY, USA) to ensure adequate mixing of the sample and the diluent. The remaining samples were then placed back into the -80ºC freezer for use with further experiments. Typically, samples were out of the freezer for no more than 2 hours

3.4.1.6 Long-term storage and disposal of samples

The initial study protocol stipulated that samples would be held for 5 years. Following ELISA/spectroscopic analysis, all relevant materials (specimens) were/ will be disposed of in clinical waste bins within the hospital or laboratory.

3.4.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Biomarker quantification was undertaken using a quantitative sandwich ELISA method technique on the processed samples. Once the protocol(s) had been optimised, each plasma/urine sample was assayed once (in duplicate on the same 96 well plate) for each of the biomarkers being assessed. There were no replicates undertaken (n=1).

Standard procedure for DuoSet ELISA (THBS2, YKL-40 and GPC1)

Commercially available DuoSet ELISA kits (R&DSystems, Minneapolis, MN, USA) were used for quantifying concentrations of Thrombospondin-2, Human Chitinase 3-Like 1 (YKL-40) and Glypican 1 in the collected samples. The antibodies in these kits were used alongside reagents supplied in the Ancillary Reagent Kit 2 (also supplied by R&DSystems). A standard protocol (Figure 3.1) for undertaking these DuoSet ELISAs' was as follows:

1) The relevant capture antibody would be diluted to the recommended working concentration using 1x Phosphate Buffered Saline (PBS). Each well on a high protein binding plate would then be coated with 100μl of the capture antibody solution. The plates were then sealed and left on the bench top overnight at room temperature.

2) The following day the capture antibody would be removed from the wells and the wells would be washed with 400μl of wash buffer solution (supplied in the Ancillary Reagent Kit 2) three times. The residual wash buffer was removed by gently tapping the plate onto the blotting paper.

3) Each well would then be filled with 300μl 1x Bovine Serum Albumin (BSA) to act as a blocking buffer. The plates were again sealed and left for an hour at room temperature on the bench top. After one hour, the BSA was removed and the wells were again washed 3 times with 400μl of wash buffer.

4) One hundred microlitres of standard or prepared samples were then pipetted into the wells in duplicate. One hundred microlitres of 1x BSA was placed in 2 wells to act as the blank. The plates were again covered and left on the bench top for 2 hours at room temperature before the samples were removed and the plates washed as previously.

5) The appropriate reconstituted biotinylated detection antibody was diluted down to the recommended working concentration, and 100μl of this was placed in each well. The plates were covered again and left for a further 2 hours at room temperature, before being washed as above.

6) Streptavidin-Horse Radish Peroxidase (Strep-HRP) was diluted in 1x BSA to the appropriate concentration as recommended by the supplier. One hundred microlitres of diluted Strep-HRP was placed in each well. The plates were covered and placed in a dark area, where they were left at room temperature for 20 minutes before the Strep-HRP was removed and the plates were washed as described previously.

7) Just prior to removing the Strep-HRP, a substrate solution was made using equal volumes of stabilized hydrogen peroxide and tetramethylbenzidine which were mixed together. Once the wells had been washed following the removal of the Strep-HRP, 100μl of the substrate solution was placed in each well. The plates were covered and left in the dark for a further 20 minutes.

8) After the 20 minutes had elapsed, 50μl of 2N Sulfuric acid was added to each well to stop the further activity of the Strep-HRP on the substrate solution. The plates were gently tapped to ensure adequate mixing of the substrate and stop solutions.

9) Plates were then analysed using a POLARstar Omega plate reader (BMG LABTECH, Ortenberg, Germany) which read the plates at 450nm, as well as 540nm and 570nm. Absorbance was analysed using Mars software and data was then exported on an Excel spreadsheet (Microsoft, Redmond, WA, USA).
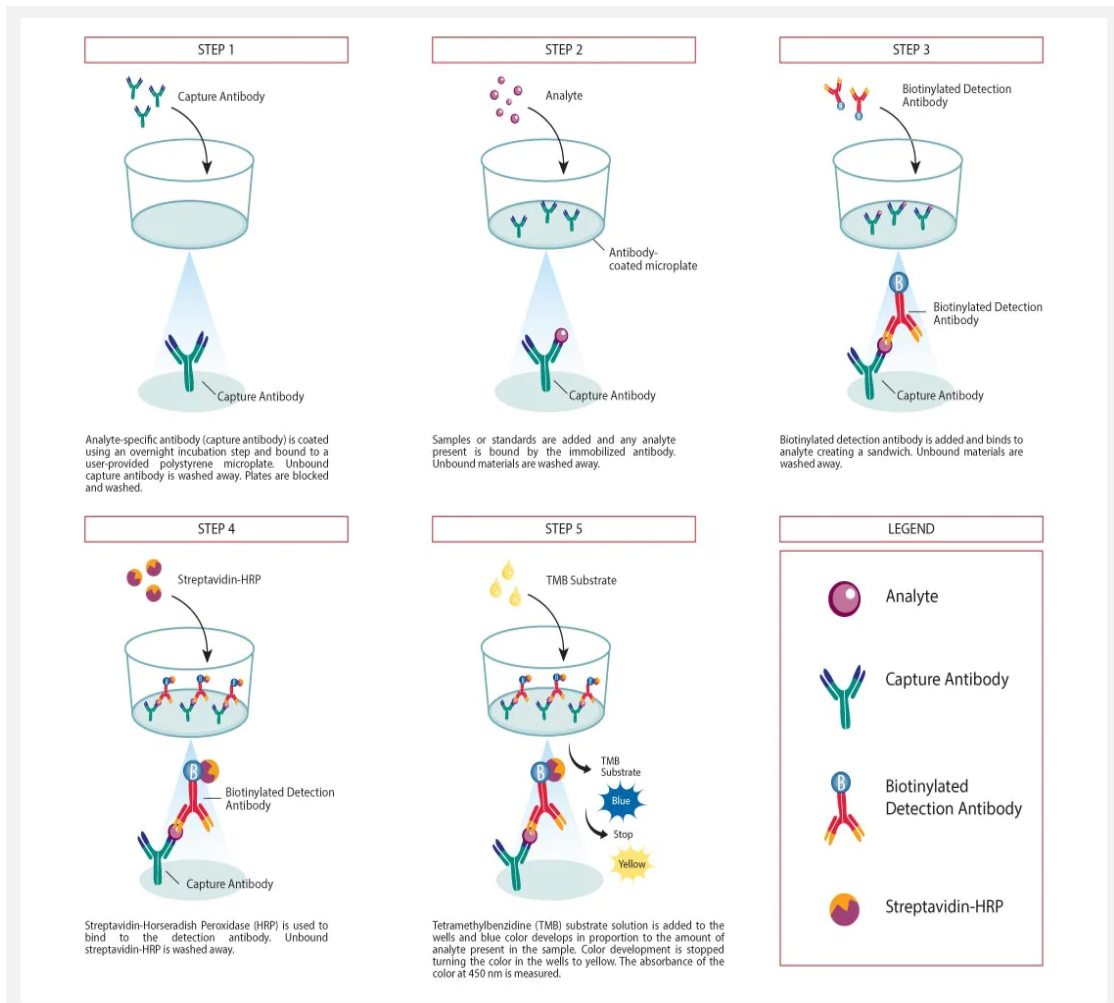
Figure 3.1 – Schematic demonstrating the key steps of the Bio-techne R&D Systems DuoSet ELISA *(Resources owned and approved use from Bio-techne).* The plate is first coated with capture antibody and subsequently washed and then blocked with BSA for 1 hour, before being washed again. Sample/standards are then placed in the wells for 2 hours before the plate is washed again. The capture antibody is then added to the well and left for a further 2 hours before the plate is again washed. Streptavidin-HRP is then added to the plates and left for 20 minutes (covered from light) before the plates are washed a final time. Substrate A and Substrate B are combined to make the substrate solution which is then added to each well and left for 20 minutes. After the 20 minutes have elapsed, the "stop solution" is added to the wells. The plate is then analysed.

3.4.2.1 Thrombospondin-2 (THBS-2)

A Thrombospondin-2 DuoSet ELISA kit (R&DSystems, Minneapolis, MN, USA) was used to quantify sample concentrations of Thrombospondin-2 (THBS2). Seven hundred and twenty micrograms of mouse anti-human thrombospondin 2 capture antibody was provided. This was then reconstituted with 1ml of PBS. The recommended working concentration was 4μg/ml, which equated to a 1 in 180 dilution. This was diluted to the working concentration using PBS and pipetted into the wells as described above.

Recombinant human thrombospondin 2 was used to create a seven-point standard curve. The recombinant THBS2 was first reconstituted in 500μl of 1x BSA, creating a concentration of 250ng/ml. This was then diluted in 1x BSA to create 1000μl of 10,000pg/ml standard. Serial 2-fold dilutions were then performed until the lowest concentration on the standard curve was 156pg/ml.

A biotinylated goat anti-human thrombospondin 2 antibody was used for the detection of captured thrombospondin 2. This was initially reconstituted with 1ml of 1x BSA to produce 1ml of 36μg/ml of detection antibody. The recommended working concentration was 200ng/ml which was achieved by diluting the antibody in 1x BSA at a ratio of 1:180.

Streptavidin-HRP was diluted 200-fold for use with this kit.

3.4.2.2 Human Chitinase 3-like 1 (YKL-40 [CH3L1])

A Human Chitinase 3-like 1 (YKL-40) DuoSet ELISA kit (R&DSystems, Minneapolis, MN, USA) was used to quantify sample concentrations of YKL-40. Three hundred and sixty micrograms of rat anti-human YKL-40 capture antibody was provided. This was then reconstituted with 1ml of PBS. The recommended working concentration was 2μg/ml, which equated to a 1 in 180 dilution. This was diluted to the working concentration using PBS.

Recombinant human YKL-40 was used to create a seven-point standard curve. The recombinant THBS2 was first reconstituted in 500μl of 1x BSA, creating a concentration of 290ng/ml. This was then diluted to 1:145 with 1x BSA to create

1000µl of 2,000pg/ml standard. Serial 2-fold dilutions were then performed until the lowest concentration on the standard curve was 31.3pg/ml.

A biotinylated goat anti-human YKL-40 antibody was used for the detection of captured YKL-40. This was initially reconstituted with 1ml of 1x BSA to produce 1ml of 36µg/ml of detection antibody. The recommended working concentration was 200ng/ml which was achieved by diluting the antibody in 1x BSA at a ratio of 1:180.

Streptavidin-HRP was diluted 200-fold for use with this kit.

3.4.2.3   Glypican 1 (GPC-1)

A Glypican-1 DuoSet ELISA kit (R&DSystems, Minneapolis, MN, USA) was used to quantify sample concentrations of Glypican-1 (GPC-1). Fifty micrograms of goat anti-human Glypican-1 capture antibody was provided. This was then reconstituted with 0.5ml of PBS to produce a concentration of 100µg/ml. The recommended working concentration was 800ng/ml, which equated to a 1 in 125 dilution. This was diluted to the working concentration using PBS.

Recombinant human Glypican-1 was used to create a seven-point standard curve. The recombinant GPC-1 was first reconstituted in 500µl of 1x BSA, creating a concentration of 270ng/ml. This was then diluted at a ratio of 1:9 in 1x BSA to create 1ml of 30,000pg/ml standard. Serial 2-fold dilutions were then performed until the lowest concentration on the standard curve was 469pg/ml.

A biotinylated goat anti-human Glypican-1 antibody was used for the detection of captured GPC1. This was initially reconstituted with 1ml of 1x BSA to produce 1ml of 12µg/ml of detection antibody. The recommended working concentration was 200ng/ml which was achieved by diluting the antibody in 1x BSA at a ratio of 1:60.

Streptavidin-HRP was diluted 40-fold for use with this kit.

3.4.2.4   Ca19-9/Sialylated Lewis A

A Colorimetric ELISA kit was used to quantify Ca19-9 levels in the samples. These kits came with a 96-well microplate in which the wells had been pre-coated with a

suitable capture antibody. Pre-made standards were supplied with the kit, with concentrations of 10U/ml, 40U/ml, 100U/ml and 150U/ml.

Two wells were left empty to allow the calculation of the blank. Fifty microlitres of standard or sample was added to each well in duplicate, along with 50µl of detection antibody which had been conjugated to HRP. The wells were then covered and placed in an incubator to keep them at 37ºC for 1 hour. After the hour had elapsed, the plate was removed from the incubator and the wells were aspirated. The plates were washed with a supplied wash buffer, which had been diluted from 20X to 1X using de-iodised water. Each well was vigorously washed 3 times, and any liquid in the remaining wells at the end of the 3rd wash cycle was expelled by tapping the plate onto blotting paper. Once the wells had been thoroughly washed, 50µl of Substrate A and Substrate B was added to each well and the plate was gently tapped to allow the mixing of the 2 substrates. The plate was covered and placed back in the incubator for a further 15 minutes at 37ºC. After this time, the plate was removed from the incubator and 50µl of stop solution was placed in each well. The plate was then placed in the POLARstar Omega microplate reader (BMG Labtech, Orternberg, Germany), and readings were taken at 450nm. As with the DuoSet ELISA kits, absorbance was analysed using MARS software and the results were exported to an Excel Spreadsheet (Microsoft, Redmond, WA, USA).

3.4.2.5   Quantifying biomarker concentrations based on Absorbance

Absorbance values were then opened in Excel (Microsoft, Redmond, WA, USA). Absorbance values at 540nm were subtracted from the readings taken at 450nm. The average absorbance of the blank wells was then calculated, before being subtracted from each of the wells containing sample and standard (in duplicate). These values were then exported to GraphPad Prism Version 8.4 (GraphPad Software, San Diego, CA, USA). Four Parameter Logistic Regression was then undertaken using the absorbance values of the known concentration standards to produce a standard curve, which was then used to calculate the concentrations of the samples.

Once the concentrations of the samples had been determined using the standard curve, the values were then exported back to the Excel spreadsheet (Microsoft, Redmond, WA, USA). Concentrations were then multiplied by the dilution factor (if the samples

had been diluted prior to being assayed) to provide the actual biomarker concentrations. These values were then added to an SPSS workbook to allow statistical analysis of the results.

3.4.2.6   Determining concentration cut-off values

Once the data had been transferred to SPSS, Receiver Operator Characteristic (ROC) curves were then constructed for each of the sample biomarkers, and the Area Under the Curve (AUC) was determined to assess the utility of each of the biomarkers in discriminating between PDAC and non-PDAC samples.

3.4.3   Spectroscopy

Plasma and urine samples were prepared as above and aliquoted into micro-centrifuge tubes and stored in the -80ºC freezers in ILS1 until they were required for spectroscopy. Samples were subsequently transferred to the department of chemistry at Swansea University to undergo FTIR spectroscopy, which was kindly undertaken by Dr Deb Roy (Senior Lecturer, Swansea University) and Dr Edward Duckworth (PhD candidate, Swansea University).

Each biofluid sample was first filtered through a 100kDa filter, with both filtrate (permeate) and concentrate (retentate) being collected (Figure 3.2), and the filtrate being moved on to further filtering using 50, 30, 10 and 3kDa filters until 6 subsets of the plasma samples were produced. The subset windows 0-3, 3-10, 10-30, 30-50, 50-100, >100kDa and whole plasma were initially analysed for comparison and test of principle. For the final analysis of all samples, a 10kDa filter was used, allowing the analysis of both whole and <10kDa plasma. For the FTIR measurement, each fraction was diluted in a 1:24 ratio with MilliQ ultrapure water before 500µl of the diluted sample was then placed on a 25mm diameter calcium fluoride slide (Crystran, Poole, UK), ensuring the surface was covered to the edges. The slides were then left to dry overnight for analysis.

FTIR spectra were then acquired using the PerkinElmer 'Spectrum Two' FTIR spectrometer used in transmission mode. The resolution was 4 cm-1 and spectra were acquired for 5 seconds with 10 accumulations over a range of 750-4000 cm-1.
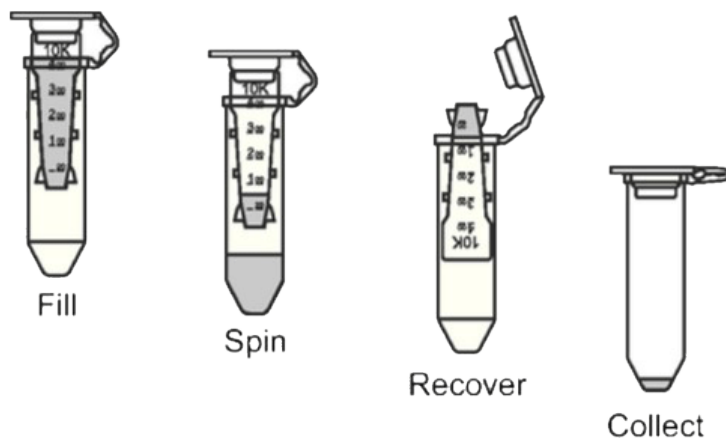
Figure 3.2 Schematic showing filtration of obtained samples prior to FTIR spectroscopy (using a10kDa filter as an example).

Obtained plasma/urine is added to the appropriate filter within an Eppendorf tube and then spun on the centrifuge. The retentate is then recovered from the filter and diluted down with MilliQ ultrapure water. The filtrate is then added to the next size filter and the process is repeated to allow the collection of the different subset windows for analysis.

CHAPTER 4

A FEASIBILITY INVESTIGATION INTO POTENTIAL NOVEL BIOMARKERS TO DETECT PANCREATIC CANCER, INCLUDING THE USE OF MULTI-ANALYTE MODELS AND FTIR SPECTROSCOPY

4.1   SUMMARY

This chapter describes the outcomes of the main feasibility study undertaken at Swansea Bay University Health Board looking to determine new ways of diagnosing pancreatic cancer.

It describes the patient factors and experimental results obtained from a series of lab-based investigations which sought to identify new biomarkers to differentiate plasma and urine samples from patients with and without pancreatic cancer. Candidate biomarkers were identified after reviewing the contemporary literature. Quantitative sandwich ELISAs were then undertaken to measure the plasma and urinary concentrations of the selected biomarkers.

Based on these results, several biomarker panels were constructed, the diagnostic accuracy of which was compared to the gold standard biomarker of plasma Ca19-9.

Simultaneous to the ELISA work, colleagues in the chemistry department undertook FTIR spectroscopy analysis of the samples to see if machine learning could be used to distinguish between cancer and non-cancer specimens.

Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) remains a disease with poor outcomes. At present, Ca19-9 is the only biomarker routinely used in clinical practice. However, it lacks the accuracy to be used as a stand-alone screening/diagnostic tool. We aimed to assess the feasibility of quantifying a selection of biomarkers in plasma and urine samples comparing them to and combining them with Ca19-9.

Methods

A study was set up with relevant ethical approval being granted. Blood and urine samples were obtained from consenting participants who were divided into 4 groups (Early PDAC, Advanced PDAC, Benign pancreatic pathology and Control). Plasma and urine Levels of Ca19-9, Thrombospondin-2 and YKL-40 were quantified using a Sandwich ELISA technique. Samples were also analysed using FTIR spectroscopy.

Results

A multi-analyte biomarker panel consisting of plasma Ca19-9 (cut off 42.05U/L), plasma thrombospondin-2 (cut off 24.50ng/ml) and urinary thrombospondin-2 (cut off 1.21ng/ml) was found to be more accurate in distinguishing between pancreatic cancer and non-cancer in our cohort than the gold standard of plasma Ca19-9 with a cut off of 37U/L. FTIR spectroscopy was able to distinguish between cancer and non-cancer specimens with an accuracy of ≥90%.

Conclusions

Multi-analyte markers can be used to increase the diagnostic accuracy of Ca19-9 in diagnosing pancreatic cancer. FTIR spectroscopy has shown promise and is undergoing further evaluation.

4.2   CHANGES TO THE INITIAL STUDY PROTOCOL

Whilst we had initially planned to collect bile samples from patients to analyse, it became apparent this would not be possible/technically feasible in a large number of the participants. Given the small number of bile samples, we were able to collect, it was decided not to undertake analysis on bile as part of this investigation.

Whilst trying to optimise the DuoSet ELISA protocol for Glypican-1, we were unable to demonstrate linearity in our initial results with different dilution factors. Given the need to dilute the samples for them to fall on the standard curve and the fact we had been successful in optimising the other 2 DuoSet biomarkers (THBS2 and YKL-40), we opted not to proceed with GPC-1 analysis.

4.3 STATISTICAL ANALYSIS

As the biomarker concentrations represent a continuous dataset of variables, median values are presented and compared between groups using the Mann-Whitney U test. Categorical variables are shown as frequencies and compared using Pearson's chi-square test. A p-value of <0.05 was deemed statistically significant.

Statistical analysis and the construction of relevant figures and graphs were undertaken using IBM SPSS statistics for Windows, Version 28.0 (IBM Corp, Armonk, NY).

4.4 CHARACTERISTICS OF THE STUDY POPULATION

As discussed in the preceding chapter, the hope for this study was to obtain blood and urine samples from 100 participants within a 12-month period – 40 of which were to have a diagnosis of PDAC (20 early cancer and 20 advanced Cancer) and 60 without PDAC (30 benign pancreatic pathology and 30 control). Due to the COVID-19 pandemic which began to spread across the UK in early 2020, recruitment of participants into the study was temporarily suspended by the health board to ensure the safety of the potential participants and the research team. By the time research was suspended, 88 participants had been successfully recruited, with the first participant being recruited on 5th June 2019, and the final participant being recruited on 10th March 2020. Once non-COVID research was reinstated within the health board, the remaining 12 participants were recruited between 6th April 2021 and 26th January 2022.

Thirty patients were initially thought to have resectable pancreatic cancer and were therefore enrolled into group 1. Unfortunately, 9 of these patients initially recruited to group 1 were subsequently found to have advanced disease (either on up-to-date pre-operative imaging or at the time of attempted resectional surgery). As a result, they were subsequently moved to group 2. One patient recruited to group 1, underwent resectional surgery, with the resultant histology revealing a benign inflammatory biliary stricture. This patient was therefore moved to group 4. Eleven participants had upfront advanced PDAC at the time of recruitment so were enrolled into group 2. Groups 3 and 4 each had the full 30 participants enrolled (including the one participant mentioned above, who was moved from group 1 to group 4 when benign histology was revealed). The breakdown of underlying pathology amongst the group 3 participants was as follows: 15 patients with acute pancreatitis, 7 patients with chronic pancreatitis, and 8 patients with pancreatic cysts.

The characteristics of the participants in each group are summarised below in Table 4.1

Table 4.1   Participant Characteristics

	Early PDAC	Advanced PDAC	"Cancer" (combined early and advanced)	Benign	Control	"Non-Cancer (combined benign and control)
Median Age	71 (50-82)	72 (50-82)	71 (50-82)	64 (34-86)	57 (21-83)	60 (21-86)
Gender **Male** **Female**	19 1	11 9	30 10	11 19	8 22	19 41

As can be seen in the table above, there was significant heterogeneity between cancer and non-cancer participants in particular with regard to age. The difference in median age between cancer and non-cancer groups was statistically significant ($p=<0.001$) and will have to be taken into account when analysing the experimental data. Whilst it must be taken into account, it is not surprising, given we know pancreatic cancer predominantly affects an older population, and the majority of participants in the control groups were otherwise healthy and undergoing elective, non-cancer-related procedures.

The difference in gender distribution in the four groups may also have an impact on the interpretation of the data.

4.5   OPTIMISATION OF DUOSET ELISA PROTOCOLS FOR PLASMA AND URINE

The provided protocol for undertaking an ELISA using the DuoSet kits (R&D systems), had been developed using cell culture spent media in which pancreatic cancer cell lines were cultured. Therefore, further optimisation of the protocol was recommended by the manufacturer for the use with plasma samples. The provided reagent diluent that came in the Ancillary Reagent Kit (R&D systems) was a 10% solution of BSA which was diluted to a 1% solution using deionized water on the day of assay. This diluent was used to block the wells following the washing out of the capture antibody. It was also used to reconstitute the detection antibodies and standards, as well as dilute them to the appropriate working concentration.

Preliminary experiments aimed to establish the technique at the different stages and ensure that the pipetting technique and wash technique were adequate and repeatable.

It was decided to start by optimising the Thrombospondin-2 ELISA. Initially, a variety of samples from different participant groups were run without dilution to see if the neat plasma samples would fall within the standard curve of 156pg/ml - 10,000pg/ml. None of the initial samples from any of the participant groups fell within this range and it was therefore clear that the samples would need to be diluted to achieve values on the standard curve. Samples were subsequently diluted to 1:2 and 1:5 dilutions using the 1% BSA solution, and then the 1:5 solution underwent serial 2-fold dilutions to create 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions. Using this method, the values produced from the samples were found to lie on the standard curve. These calculated concentrations were then multiplied by the dilution factor to give a surrogate value of a 1:1 solution. To ensure that these surrogate 1:1 values were as accurate as possible, the linearity of the serial dilutions was assessed. Using the 1% BSA provided in the ancillary reagent kits did not provide satisfactory linearity when used to dilute samples to measure concentrations of THBS2 and YKL40. Therefore, we sought to try different reagents to dilute the plasma samples with. We used a mixture of PBS + 0.05% Tween20® to dilute a selection of samples with and assayed at different dilutions in parallel to samples which had been diluted in the 1% BSA provided. These tests revealed improved linearity when PBS/Tween20® was used as the diluent compared to BSA, therefore the PBS/Tween20® mix was used for subsequent assays with both THBS2 and YKL40. Once linearity had been established, we set about to determine

the dilution factor required for each of the assays. This was done by going back to the linearity assessments and finding which dilution factors produced the most stable results (a plateau). The lowest dilution factor which produced linear results was then used for further experiments. In the case of THBS2, this was a 1:20 dilution. The YKL-40 assay required further dilutions due to the high concentrations achieved with the lower dilutions, therefore a further 2-fold dilution of the 1:180 samples was used to create a 1:360 dilution. The lowest dilution factor which had good linearity was 1:80.

Despite trying a variety of different dilution factors and diluents with the GPC-1 kits, linearity could not be comprehensively demonstrated, therefore it was decided not to proceed further with determining plasma or urine concentrations of GPC-1.

Once the protocol had been optimised for plasma samples, attention was turned to optimising the ELISA protocol for the urinary samples. As we had decided not to pursue the analysis of GPC-1 in the plasma samples, we felt that there was no role in analysing urinary GPC-1 concentrations. As with the plasma samples, we sought to initially run a selection of samples without dilution. Interestingly, all of the neat urine samples which were analysed for THBS2 concentrations fell within the standard curve, therefore we decided not to use any dilutions for future analysis and just use neat urine. When it came to measuring YKL-40 concentrations within the urine samples, initial samples of undiluted urine revealed several of the samples to have concentrations just higher than the upper limit of the standard curve. We, therefore, undertook serial dilutions of x5, x10, x20 and x50 using the supplied BSA in the ancillary reagent kits. Linearity was shown and all samples were found to lie on the standard curve when using the x5 dilution, therefore this was chosen as our optimised dilution factor. Given satisfactory linearity had been achieved using the supplied BSA as the reagent sample diluent, we did not run the samples using the PBS/tween mix which was used on the blood samples.

Once the protocols were optimised, we standardised the way we would set out the samples and standards in out 96-well plates. A schematic of the layout is shown in Figure 4.1 and a photo of a THBS2 ELISA being run just prior to the application of the stop solution is shown in Figure 4.2.

B	B	Sa1	Sa1	Sa9	Sa9	Sa17	Sa17	Sa25	Sa25	Sa33	Sa33
St7	St7	Sa2	Sa2	Sa10	Sa10	Sa18	Sa18	Sa26	Sa26	Sa34	Sa34
St6	St6	Sa3	Sa3	Sa11	Sa11	Sa19	Sa19	Sa27	Sa27	Sa35	Sa35
St5	St5	Sa4	Sa4	Sa12	Sa12	Sa20	Sa20	Sa28	Sa28	Sa36	Sa36
St4	St4	Sa5	Sa5	Sa13	Sa13	Sa21	Sa21	Sa29	Sa29	Sa37	Sa37
St3	St3	Sa6	Sa6	Sa14	Sa14	Sa22	Sa22	Sa30	Sa30	Sa38	Sa38
St2	St2	Sa7	Sa7	Sa15	Sa15	Sa23	Sa23	Sa31	Sa31	Sa39	Sa39
St1	St1	Sa8	Sa8	Sa16	Sa16	Sa24	Sa24	Sa32	Sa32	Sa40	Sa40

Figure 4.1 Example of plate layout for DuoSet ELISA (St = standard, B = blank, Sa = sample).
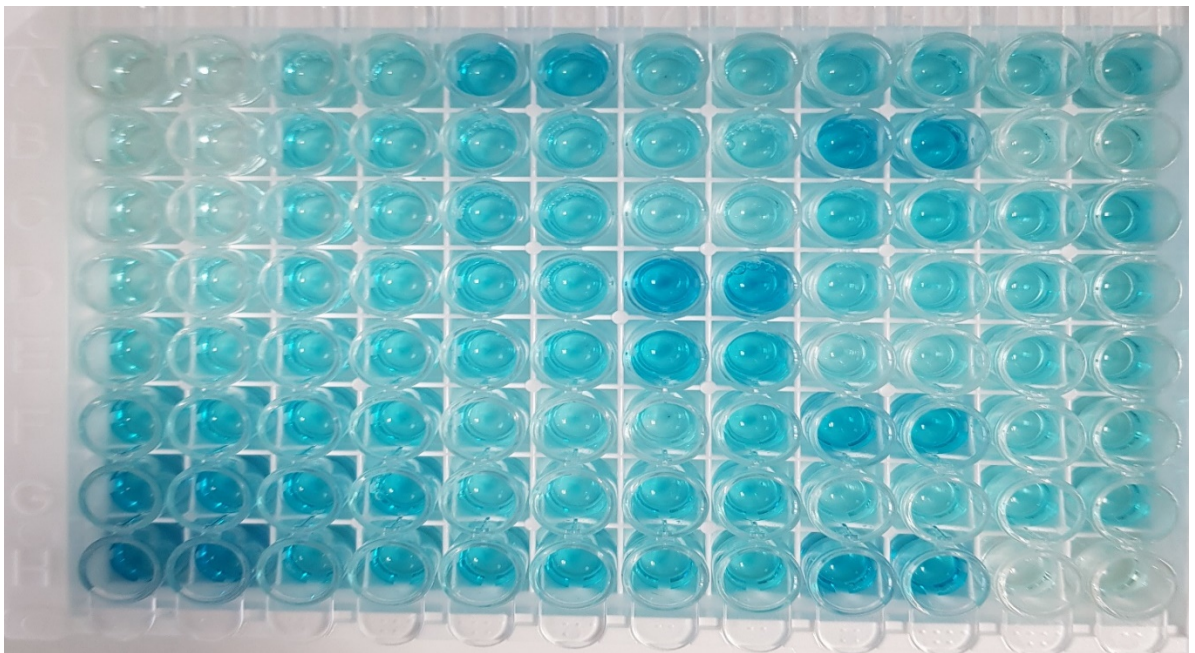

Figure 4.2 Photograph of THBS2 ELISA assay after the addition of Colour Reagents (Hydrogen Peroxide and Tetramethylbenzidine) prior to the addition of stop solution (2N Sulfuric Acid).

The plate is set out as shown in Figure 4.1.

4.6 QUANTIFYING CONCENTRATIONS OF SELECTED BIOMARKERS USING ELISA

4.6.1 Plasma samples

Blood samples were obtained and processed from all 100 recruited participants and underwent ELISA using the methods previously described.

4.6.1.1 Ca19-9

As the current gold standard biomarker for pancreatic cancer, and the only one currently used in routine clinical practice, we opted to start our investigation by quantifying plasma concentrations of Ca19-9 in our collected samples using the Bio-Techne Novus biologicals Colorimetric Ca19-9 ELISA kit (Bio-Techne, Abingdon, UK). The supplied kit/protocol was not optimised as all reagents came as standard as part of the kit.

The median plasma Ca19-9 concentration for all patients was 40.60 U/L (4.00U/L – 220.81U/L).

Plasma Ca19-9 concentration was analysed by participant gender and age. The median concentration in male participants was 72.55U/L compared to 28.84U/L in women. Plasma concentrations of Ca19-9 based on gender and age are shown in Figures 4.3 and 4.4 respectively.

Analysis of the samples into 2 broad groups of "Cancer" (groups 1 and 2) and "Non-Cancer" (groups 3 and 4) revealed a statistically significant difference in the plasma concentration of Ca19-9 between these two groups. The "cancer" group had a median concentration of 111.30U/L, compared to 20.95U/L in the non-cancer group (p=<0.001). The results are represented in Figure 4.5.

Ca19-9 concentrations were then compared between the 4 participant groups. The median plasma concentration of Ca19-9 was highest in the advanced PDAC group at 119.85 U/L, followed by the early PDAC group which had a median plasma concentration of 109.00U/L. Benign pancreatic pathology patients had a median plasma concentration of 34.26 U/L and control patients had a median concentration of 16.41U/L. These results are shown in Figure 4.6.

In clinical practice, Ca19-9 is used as part of pre-operative staging and post-treatment surveillance. Assuming a patient is a Ca19-9 secreter, a higher plasma concentration of Ca19-9 is likely to represent a more advanced disease. Comparing the difference in median concentration in our early and advanced PDAC groups did not show statistical significance (p=0.261). However, the difference in concentration between early PDAC and both the benign and control groups was significant (p=<0.001).

A Receiver Operating Characteristic (ROC) curve was created to assess the acceptability of Ca19-9 to be used to differentiate between cancer and non-cancer samples (Figure 4.7). The Area Under the Curve (AUC) was calculated to be 0.885, which is considered to be an excellent diagnostic test.

A summary of the obtained Ca19-9 plasma concentration results is shown in Table 4.2
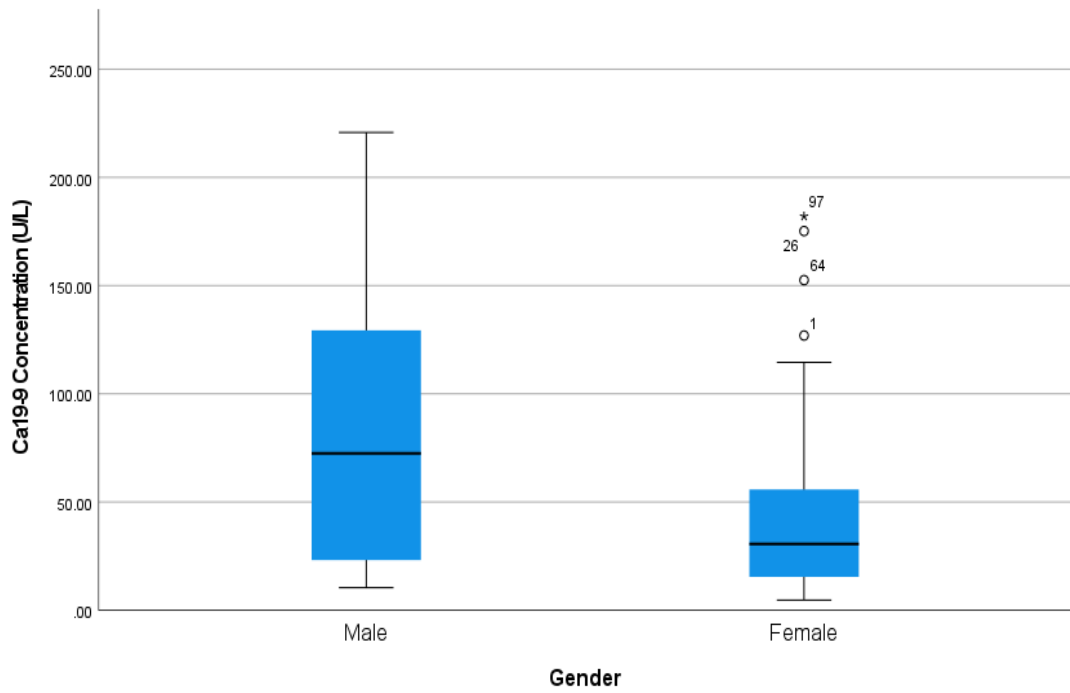
Figure 4.3 **Boxplot showing plasma concentration of Ca19-9 dependent on participant gender.**

Median Ca19-9 concentration is seen to be higher in male participants than in female participants. There is a greater range of Ca19-9 concentrations in the male participants compared to the female participants as demonstrated by the greater interquartile range.
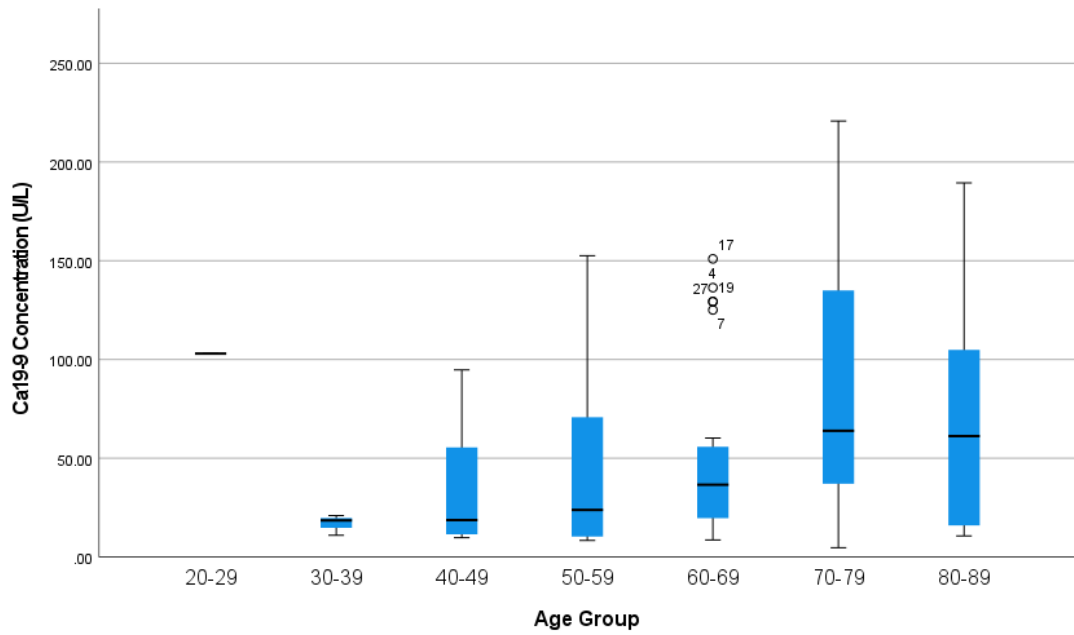
Figure 4.4   Boxplot showing plasma concentration of Ca19-9 dependent on participant age.

The median concentration of Ca19-9 is seen to go up with increasing age. It should be noted, as mentioned previously, the cancer patients were more likely to be older than the non-cancer patients so this increase may not be purely due to age.
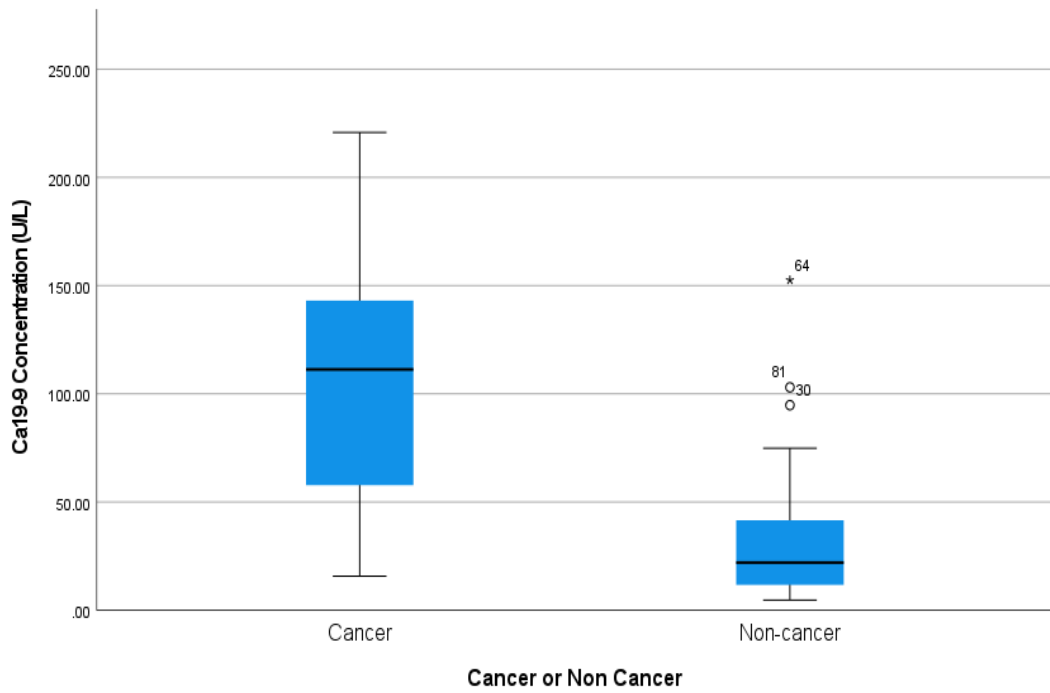
Figure 4.5   Boxplot showing plasma concentration of Ca19-9 in cancer and non-cancer specimens.

A significant difference is demonstrated in the median Ca19-9 concentration amongst the cancer cohort, compared to the non- cancer cohort.
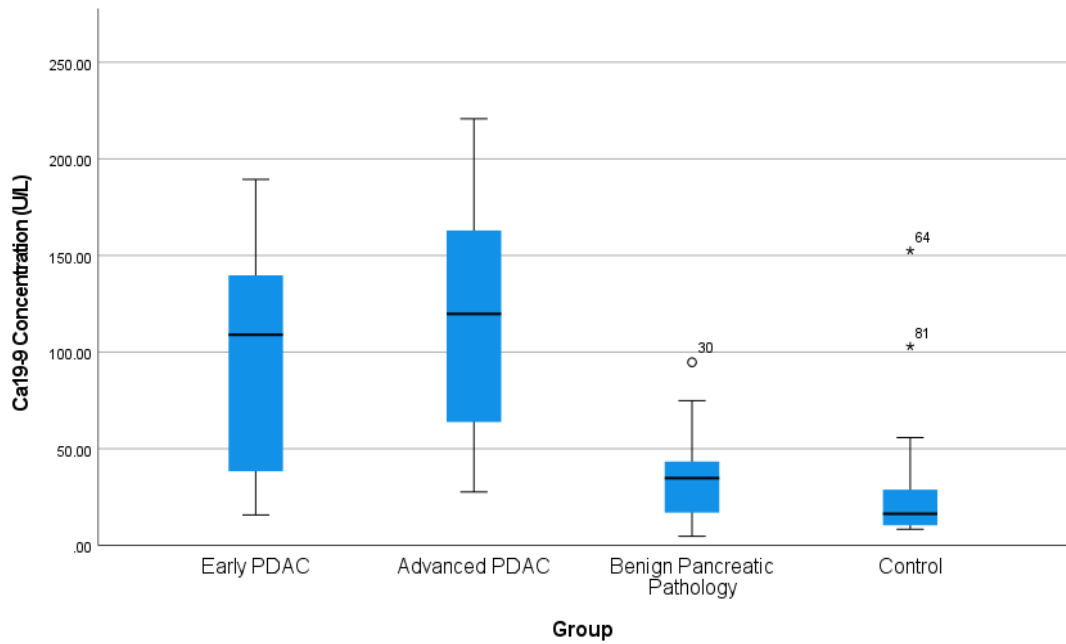
Figure 4.6   Boxplot showing plasma concentration of Ca19-9 in all 4 participant groups.

This clearly shows Ca19-9 to be significantly higher in both early and advanced pancreatic cancer compared to benign pancreatic pathology and control participants. It can also be seen that the median concentration is higher in advanced PDAC compared to early PDAC, demonstrating its utility in monitoring disease progression in patients with confirmed PDAC (in those who are shown to be Ca19-9 secretors).

Figure 4.7 **RoC Curve showing the diagnostic ability of plasma Ca19-9 to differentiate between cancer and non-cancer samples (AUC = 0.885).**

The sensitivity and specificity of different concentrations of Ca19-9 at differentiating PDAC from non-PDAC in our cohort are calculated. For each concentration of Ca19-9, the calculated sensitivity is plotted a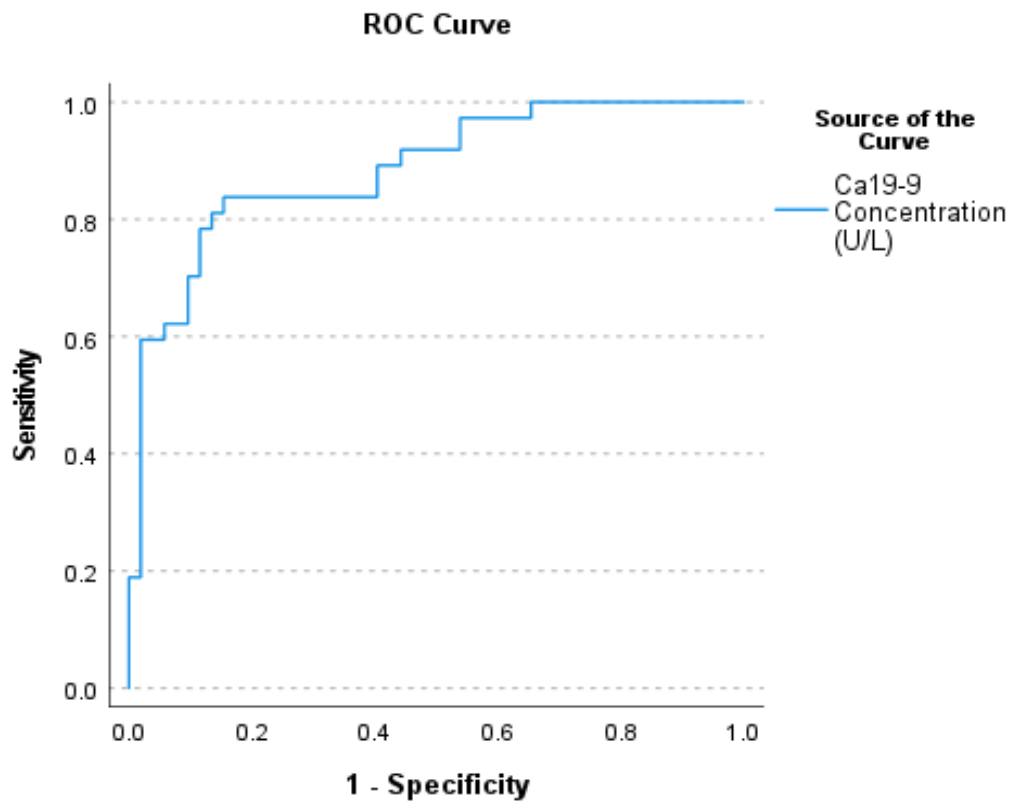gainst 1-specificity to form the curve, therefore whilst the curve itself doesn't plot individual concentrations of Ca19-9, it does demonstrate how sensitivity and specificity of Ca19-9 are inversely proportionate when different concentrations are used as cut-offs to discriminate cancer from non-cancer samples. The point of the curve closest to the upper left corner represents the Ca19-9 concentration cut-off with the highest accuracy for differentiating cancer from non-cancer (corresponding to a concentration of 45.02U/L in this case). The Area Under the Curve value of 0.885 is deemed to be excellent for distinguishing PDAC from non-PDAC.

Table 4.2   Summary of Results of Plasma Concentrations of Ca19-9 determined by ELISA (U/L).

	Early PDAC	Advanced PDAC	"Cancer"	Benign	Control	"Non-Cancer
Mean	95.05	115.96	105.22	34.19	28.65	31.53
Median	109.00	119.85	111.30	34.85	16.40	22.02
Minimum	15.76	27.68	15.76	4.70	8.40	4.70
Maximum	189.45	220.81	220.81	94.77	152.56	152.56

4.6.1.2   Thrombospondin-2 (THBS2)

Once the ELISA had been optimised as previously described, plasma from each participant was run using the optimised protocol.

The median plasma concentration of THBS2 for all participants was 28.96ng/ml (8.37ng/ml-171.03ng/ml).

To ensure that any observed differences in our study groups were not confounded by patient factors, we analysed the impact of age and gender on plasma concentrations of THBS2. The median concentration of THBS2 in male participants was 33.71ng/ml compared to 26.19ng/ml in the female cohort. This difference was not significant (p=0.054). Multivariate analysis showed that patient age did not have a significant impact on THBS2 concentrations (p=0.895). Figures 4.8 and 4.9 show the impact of gender and age on THBS2 concentration respectively. As it was apparent that host factors did not significantly impact the levels of circulating THBS2, we proceeded to analyse the samples based on the study group/pathology.

Analysis of the samples into the "Cancer" and "Non-Cancer" groups revealed a statistically significant difference in the median plasma concentration of THBS2 between these two groups, with a median concentration of 37.16ng/ml in the cancer group, compared to 24.31ng/ml in the non-cancer group (p=<0.001). The results are represented in Figure 4.10.

Further sub-analysis to compare THBS2 concentrations between all four participant groups revealed that the advanced PDAC group had the highest median concentration of THBS2 at 37.33ng/ml, followed closely by the early PDAC group with a median concentration of 36.91ng/ml. The benign group had a median THBS2 concentration of 27.93ng/ml and the control group had a median concentration of 20.67ng/ml.

In order to prove its utility as a potential screening test in asymptomatic patients, we sought to compare the median concentration of THBS2 in the early PDAC group against those of the benign pancreatic pathology and the control group. When comparing the early PDAC samples against the control group samples, there was a statistically significant difference noted (p=<0.001). Unfortunately, however, when the median of the early PDAC was compared to the benign pancreatic pathology group, the difference was not significant (p=0.218).

If THBS2 could differentiate between early and advanced PDAC, with higher concentrations representing more advanced disease, then it might be able to play a potential role in pre-operative staging and post-treatment surveillance. Unfortunately, the difference in THBS2 concentrations between the early and advanced PDAC groups was not statistically significant (p=0.629).

Given the statistically significant difference in plasma concentration of THBS2 between cancer and non-cancer groups, a Receiver Operating Characteristic (ROC) curve was constructed (Figure 4.7). The area under the curve (AUC) was 0.705, meaning that it can be deemed an acceptable discriminator between specimens from patients with and without pancreatic cancer.

A summary of the Thrombospondin-2 results is shown in Table 4.3.
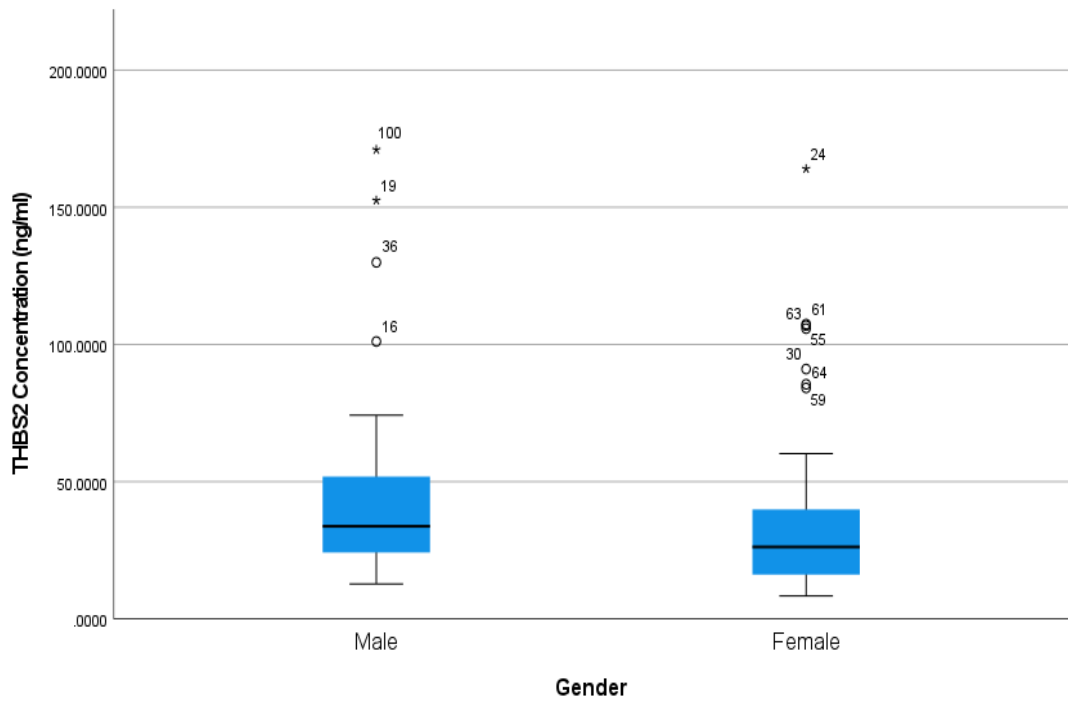
Figure 4.8 **Boxplot showing plasma concentration of THBS2 dependent on participant gender.**

The similar median concentrations and interquartile ranges of THBS2 seen in male and female participants suggest that gender does not obviously effect THBS2 concentration.
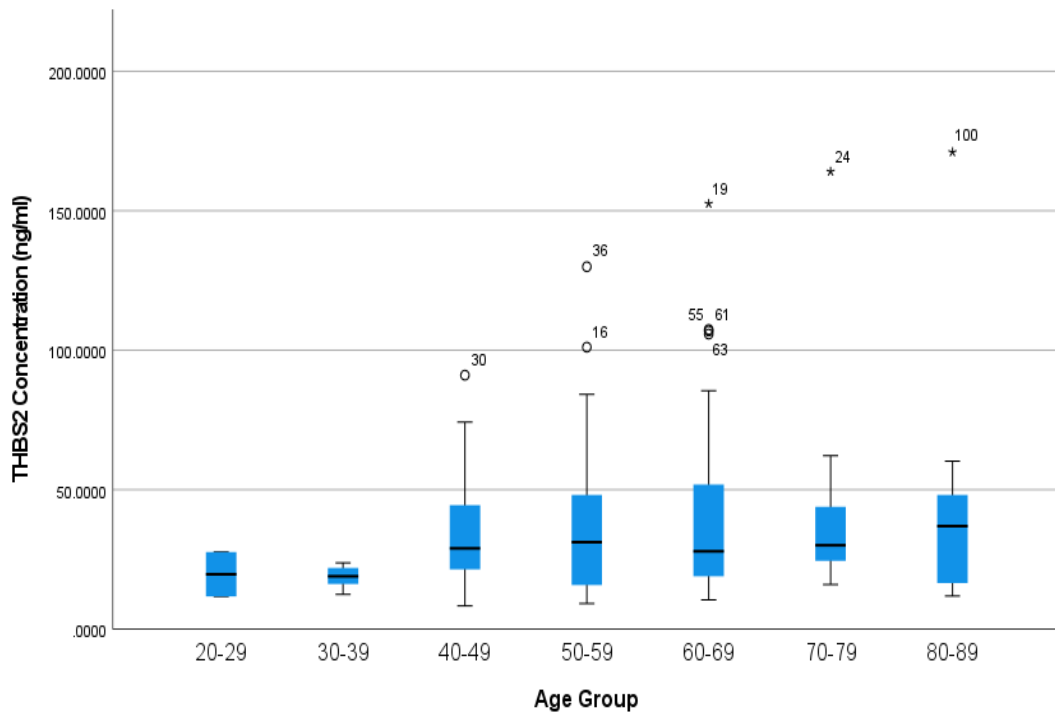
Figure 4.9 **Boxplot showing plasma concentration of THBS2 dependent on participant age.**

There is a minimal trend toward increasing median THBS2 concentration with increasing age. However, this does not appear to be significant and it can be seen that there is much variation in the interquartile ranges and number of outliers in each age bracket.
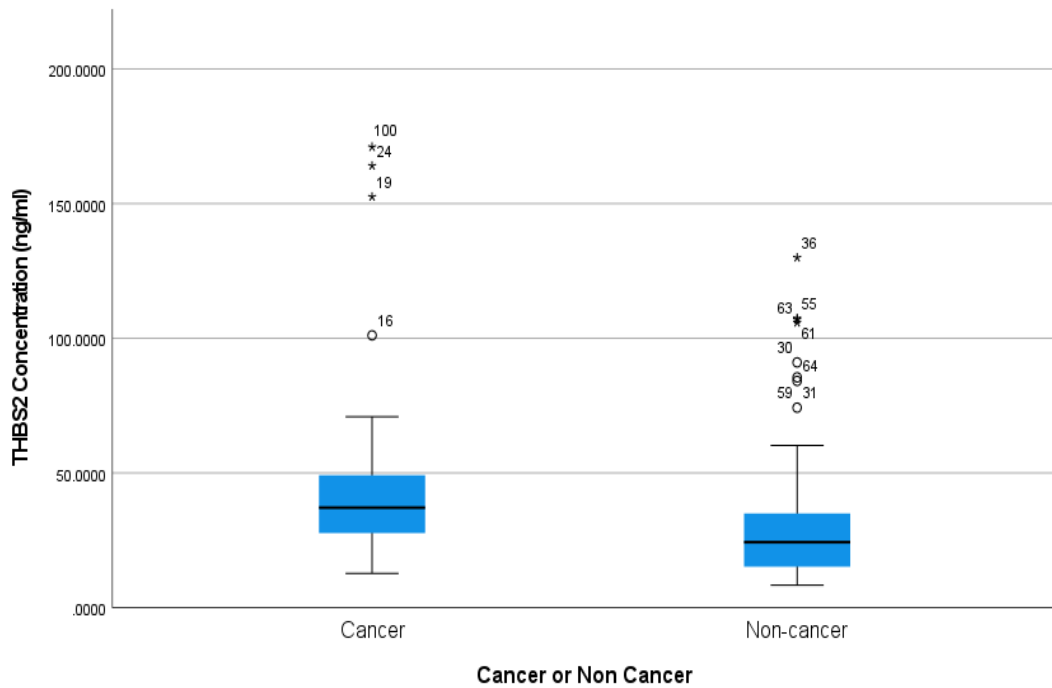
Figure 4.10   Boxplot showing plasma concentration of THBS2 in cancer and non-cancer specimens.

The median plasma concentration of THBS2 is higher in patients with cancer compared to those without cancer. This difference was found to be statistically significant.
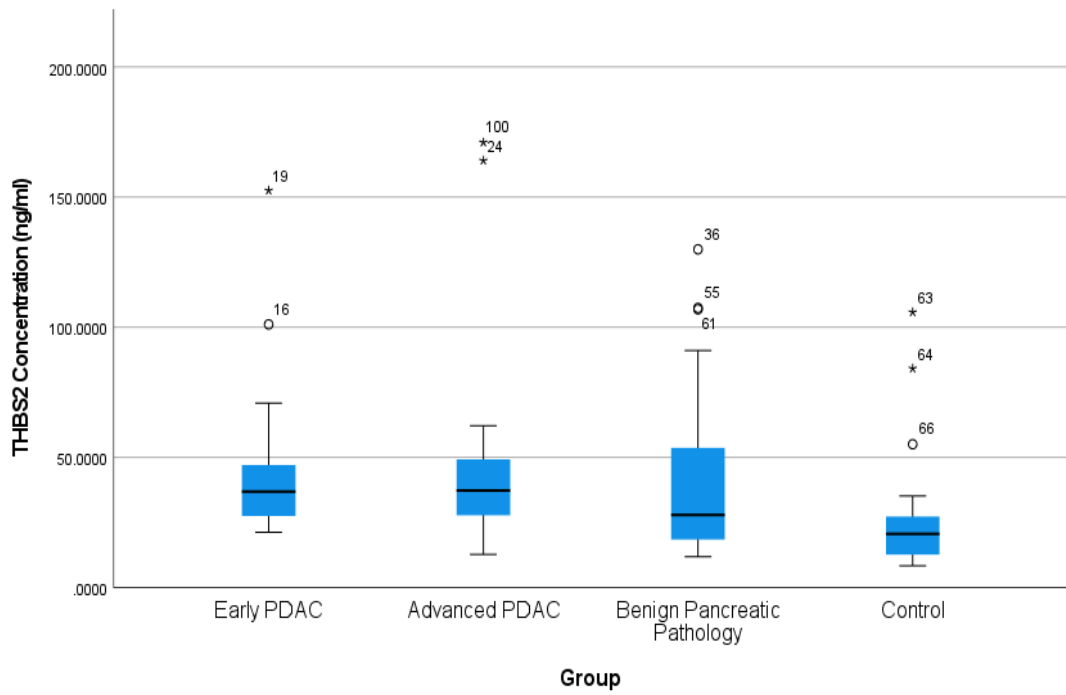
Figure 4.11   Boxplot showing plasma concentration of THBS2 in all 4 participant groups.

Median concentration is seen to be highest in the advanced PDAC group, followed by the early PDAC group, followed by benign and then control groups.
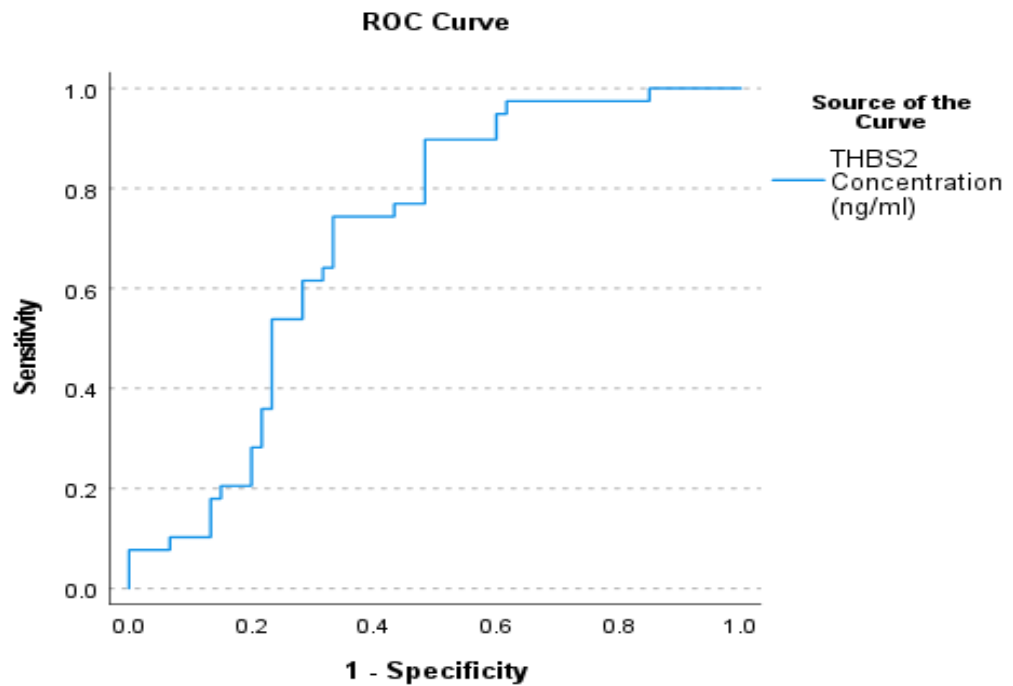
Figure 4.12   RoC Curve showing the diagnostic ability of plasma THBS2 to differentiate between cancer and non-cancer samples (AUC = 0.705).

Using a THBS2 concentration of 24.50ng/ml as a cut-off for distinguishing PDAC from non-PDAC plasma samples gave the greatest overall diagnostic accuracy, with a sensitivity of 91.7% and a specificity of 56.2%. The AUC of 0.705, shows plasma THBS2 is an "acceptable" test for distinguishing PDAC from non-PDAC samples.

Table 4.3   Summary   of   Results   of   Plasma   Concentrations   of
Thrombospondin-2 determined by ELISA (ng/ml)

	Early PDAC	Advanced PDAC	"Cancer"	Benign	Control	"Non-Cancer
Mean	46.76	49.37	49.10	42.45	25.65	34.05
Median	36.91	37.33	37.16	27.93	20.67	24.31
Minimum	21.22	12.74	12.74	11.90	8.37	8.37
Maximum	152.56	171.03	171.03	129.95	105.80	129.95

4.6.1.3 Human Chitinase 3-Like 1 (YKL-40)

We next quantified plasma concentrations of YKL-40 in the plasma samples, using the optimised protocol.

The median value of the plasma concentration of YKL-40 for all samples was 105.98ng/ml (19.86ng/ml – 289.74ng/ml).

Again, we first sought to identify if the concentrations were affected by patient gender and age. The median plasma concentration of YKL-40 amongst male patients was 110.79ng/ml, compared to 100.29ng/ml in female patients. This difference was not statistically significant (p=0.578). The impact of age on plasma levels of YKL-40 was not shown to be significant when we undertook an ANOVA analysis (p=0.155). However, when multivariate analysis was undertaken to analyse the impact of age and cancer on the biomarker levels, patient age was found to significantly affect levels of YKL-40 (p=0.010). The correlation between gender and age on YKL-40 concentrations is shown in Figures 4.13 and 4.14. It is certainly apparent that advancing age seems to correlate with an increase in the median concentration of YKL-40.

Analysis of the "Cancer" and "Non-Cancer" showed a median plasma concentration of 115.62ng/ml in the cancer group compared to 96.43ng/ml in the non-cancer group. However, this difference was not found to be statistically significant (p= 0.587). Figure 4.15 demonstrates the plasma concentrations of YKL-40 within these 2 groups.

A comparison of YKL-40 plasma concentrations between all four participant groups was undertaken. Interestingly, the benign pancreatic pathology participants had the greatest median concentration of YKL-40. The median concentration in this group was 167.73ng/ml, compared to 122.49ng/ml in the early PDAC group, 100.29ng/ml in the advanced PDAC group, and just 55.69ng/ml in the control group. This is demonstrated in Figure 4.16.

When YKL-40 plasma concentrations were compared between the early PDAC and control groups, as with the THBS2 levels, the difference was found to be statistically significant (p=0.010). However, yet again the difference in concentration between the early PDAC and benign pancreatic pathology was not statistically significant (p=0.191).

A ROC curve was constructed with the plasma concentrations of YKL-40, with the differentiator being the presence of PDAC (Figure 4.17). The area under the curve was calculated, which was found to be just 0.533. Given the above findings that on the whole, plasma YKL-40 concentrations could not differentiate between cancer and non-cancer, it is clear that it cannot be used in isolation as a biomarker to detect pancreatic cancer.

A summary of the quantified plasma concentrations of YKL-40 is shown in Table 4.4.
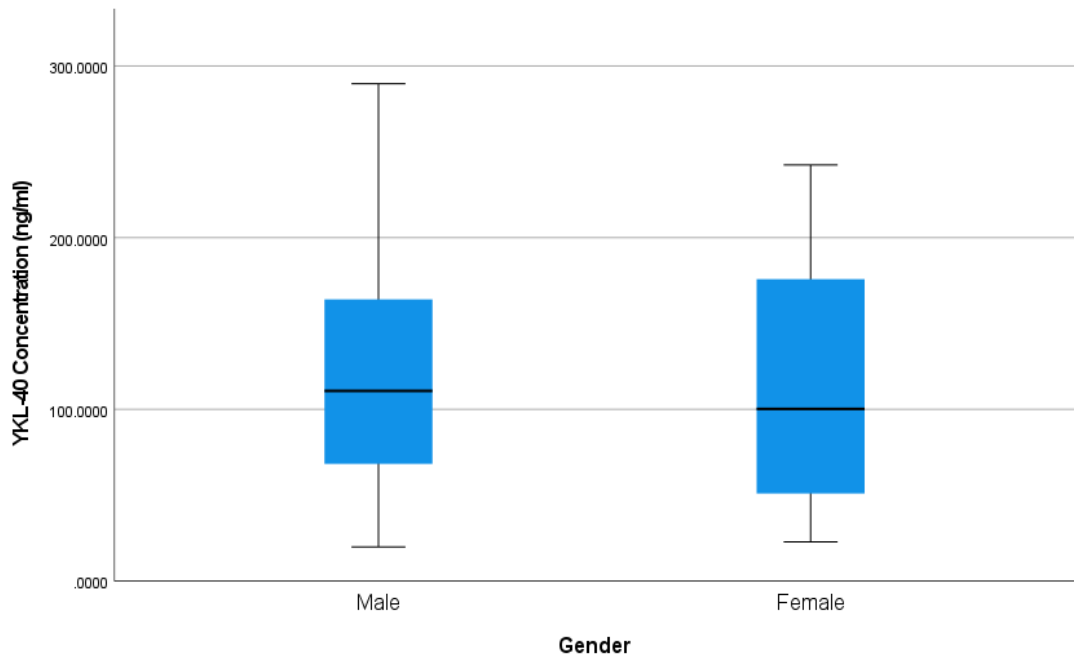
Figure 4.13   Boxplot showing plasma concentration of YKL-40 dependent on participant age.

The median concentration of YKL-40 is similar in both male and female participants showing that gender is not likely to have an impact on YKL-40's ability to distinguish cancer from non-cancer plasma samples.
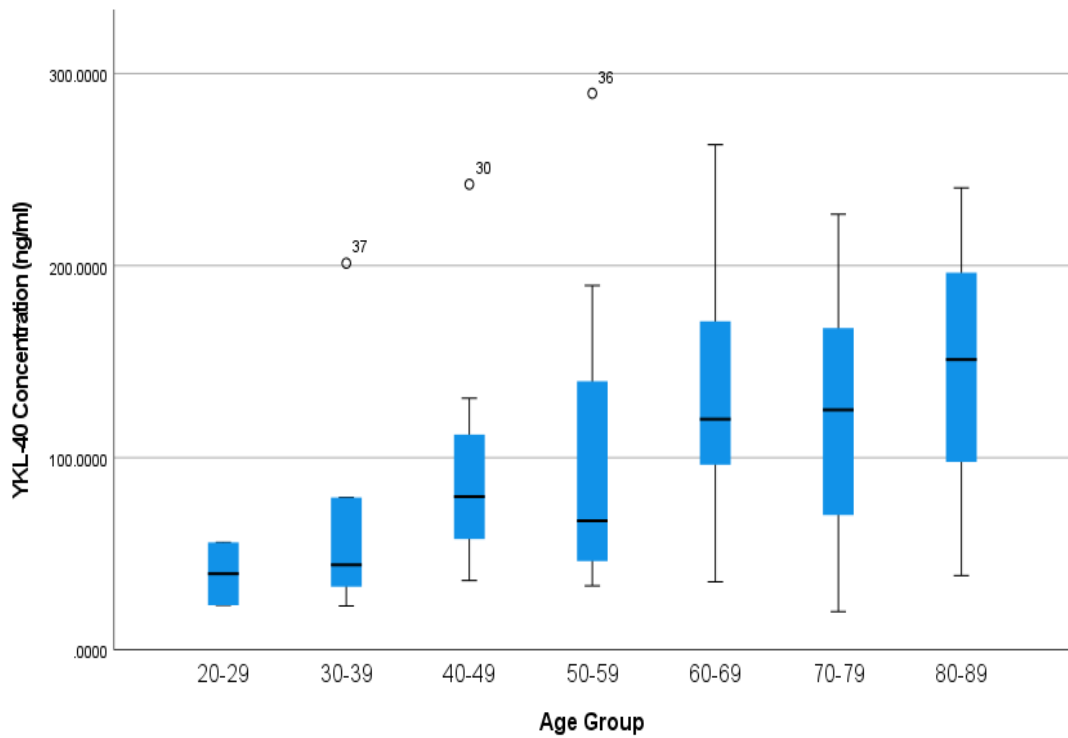
Figure 4.14   Boxplot showing plasma concentration of YKL-40 dependent on participant age.

This graph clearly demonstrates the correlation between advancing age YKL-40 concentration. The variation in YKL-40 concentrations in each age group appears to generally increase with increasing age. As with Ca19-9, it should be noted that the cancer cohort was significantly older than the non-cancer cohort, so based on this graph alone, it is permissible that the increase in YKL-40 with increasing age may in some part be attributed to underlying PDAC.
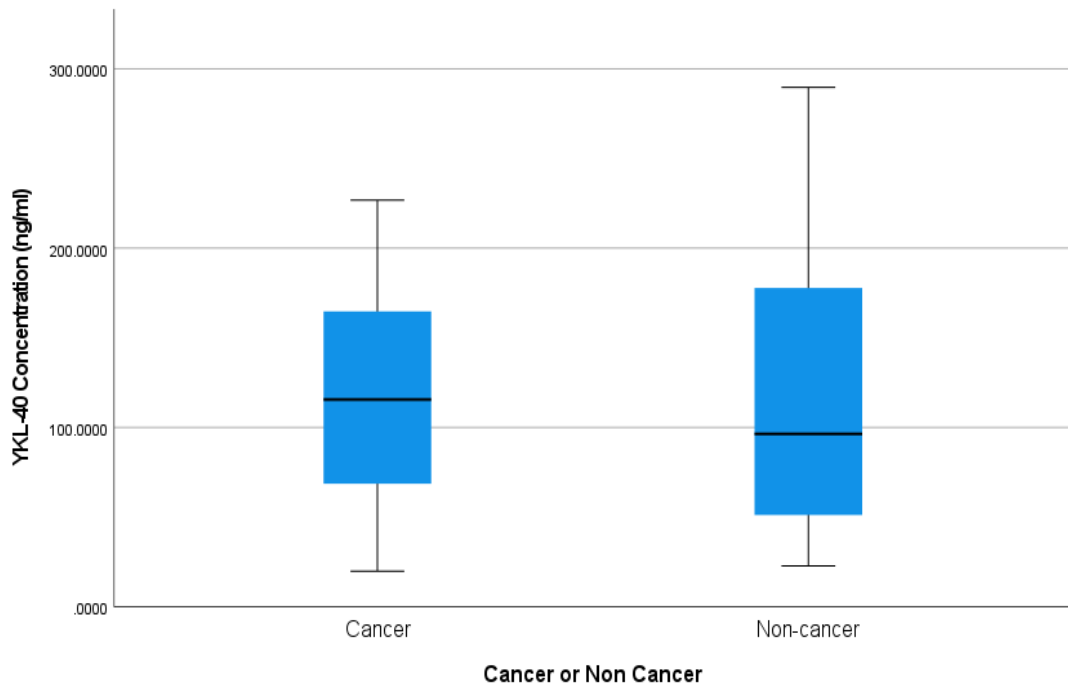
Figure 4.15   Boxplot showing plasma concentration of YKL-40 in cancer and non-cancer samples.

This graph shows that the median concentration of YKL-40 in cancer and non-cancer participants is similar. The wide variation of YKL-40 concentrations in the non-cancer group is noted and further explored in Figure 4.16 below.
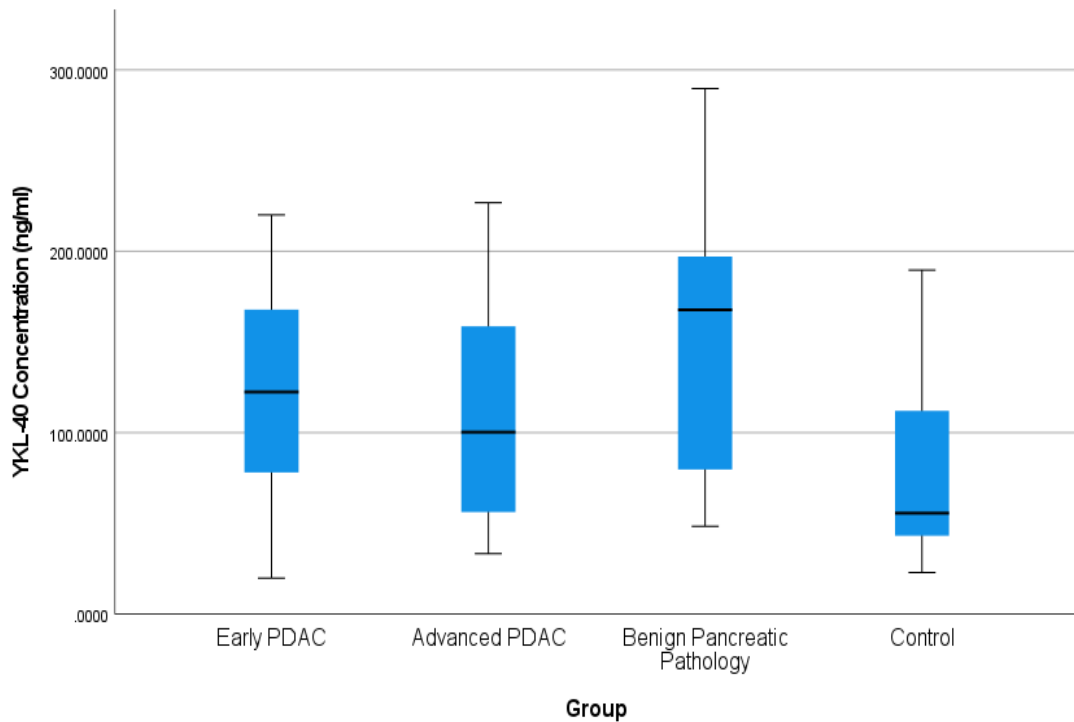
Figure 4.16 **Boxplot showing plasma concentration of YKL-40 in all four participant groups.**

The median concentration of YKL-40 was found to be highest in the benign pancreatic pathology cohort, whilst the lowest median concentration was seen within the control group. This demonstrates that YKL-40 cannot be used to differentiate cancer from benign pancreatic pathology but may have some utility in detecting PDAC in the asymptomatic population without underlying benign pancreatic pathology.
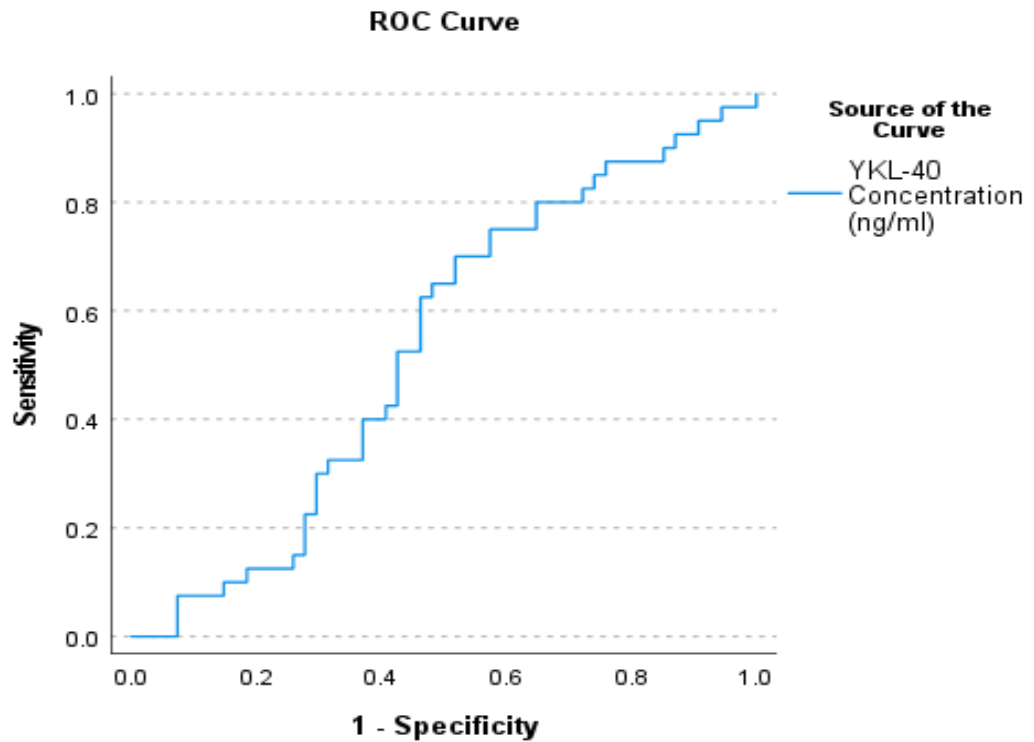
Figure 4.17 RoC Curve showing the diagnostic ability of plasma YKL-40 to differentiate between cancer and non-cancer samples (AUC = 0.533).

This RoC curve demonstrates the lack of ability for plasma YKL-40 to differentiate between cancer and non-cancer samples. The curve lies close to the diagonal line drawn between the x and y axes, and this is reflected in the AUC value of 0.533 which is deemed not to represent a good test.

Table 4.4   Summary of Results of Plasma Concentrations of YKL-40 (ng/ml), quantified by ELISA

	Early PDAC	Advanced PDAC	"Cancer"	Benign	Control	"Non-Cancer
Mean	124.13	109.66	116.90	149.33	80.73	113.76
Median	122.49	100.29	115.62	167.73	55.70	96.43
Minimum	19.86	33.35	19.86	48.39	22.87	22.87
Maximum	220.10	226.81	226.81	289.74	189.71	289.74

4.6.2 Urine Samples

Whilst it was hoped that urine samples would be collected from all participants, unfortunately, it became apparent that this would always be not possible, and it was not uncommon that participants were unable to provide a urine sample. Of the 100 participants, 87 were able to provide urine samples for analysis. Unfortunately, 12 of the 13 participants unable to supply urine samples were from either the early or advanced pancreatic cancer group, meaning only 27 "cancer" urine samples were available for analysis.

4.6.2.1 Ca19-9

Urinary concentrations of Ca19-9 were quantified using the same Bio-Techne colorimetric ELISA that was used for the plasma samples. The median urinary Ca19-9 concentration for all participants was 122.41U/L (7.73U/L – 217.55U/L).

Much to our surprise, the median urinary concentration of Ca19-9 was 117.561U/L in the cancer group compared to 123.24U/L in the non-cancer group. This difference was not statistically significant (p=0.819). Given the urinary Ca19-9 result differed vastly from the plasma results it was clear that urinary Ca19-9 levels could not be a surrogate for plasma levels, so no further analysis of urinary Ca19-9 was undertaken.

4.6.2.2 Thrombospondin-2 (THBS2)

Once the ELISA protocol for quantification of urinary THBS2 concentration had been established as described above, we analysed all the collected urine samples.

The median urinary concentration of THBS2 in all samples was 1.23ng/ml. As with the plasma specimens, males had a higher median concentration of urinary THBS2, measured at 1.36ng/ml compared to 1.17ng/ml in the female cohort, however, once again this was not statistically significant (p=0.095). Whilst there did seem to be an association of increasing age with increasing urinary THBS2 concentration, multivariate analysis showed this not to be significant (p=0.896). Figure 4.18 demonstrates the concentration of urinary THBS2 in different age groups.

When divided into cancer and non-cancer specimens, the median concentration of urinary THBS2 was higher in the cancer group, measuring 1.76ng/ml, compared to 1.06ng/ml in the non-cancer group (Figure 4.19). This difference was statistically significant (p=<0.001).

Further analysis by the group revealed the highest median urinary concentration of THBS2 to be found in the early PDAC group (1.76ng/ml), followed by the advanced PDAC group (1.75ng/ml), then the benign group (1.30ng/ml) and finally the control group (0.84ng/ml). Figure 14.20 demonstrates these results.

Further analysis of the ability to distinguish early PDAC from the other 3 groups failed to show statistical significance when compared with the advanced PDAC group (P=0.635) and benign pancreatic pathology group (p=0.217). However, the difference in urinary concentration of THBS2 between early PDAC patients and control participants was significant (p=<0.01).

A ROC curve was constructed, as with the plasma THBS2 concentrations. Interestingly, the Area under the curve for urinary THBS2 was higher than that seen with plasma THBS2 (0.734 vs 0.705), suggesting urinary THBS2 may be a more reliable test than plasma THBS2 for detecting PDAC.

A summary of the results for urinary concentrations of THBS2 is shown in Table 4.5.
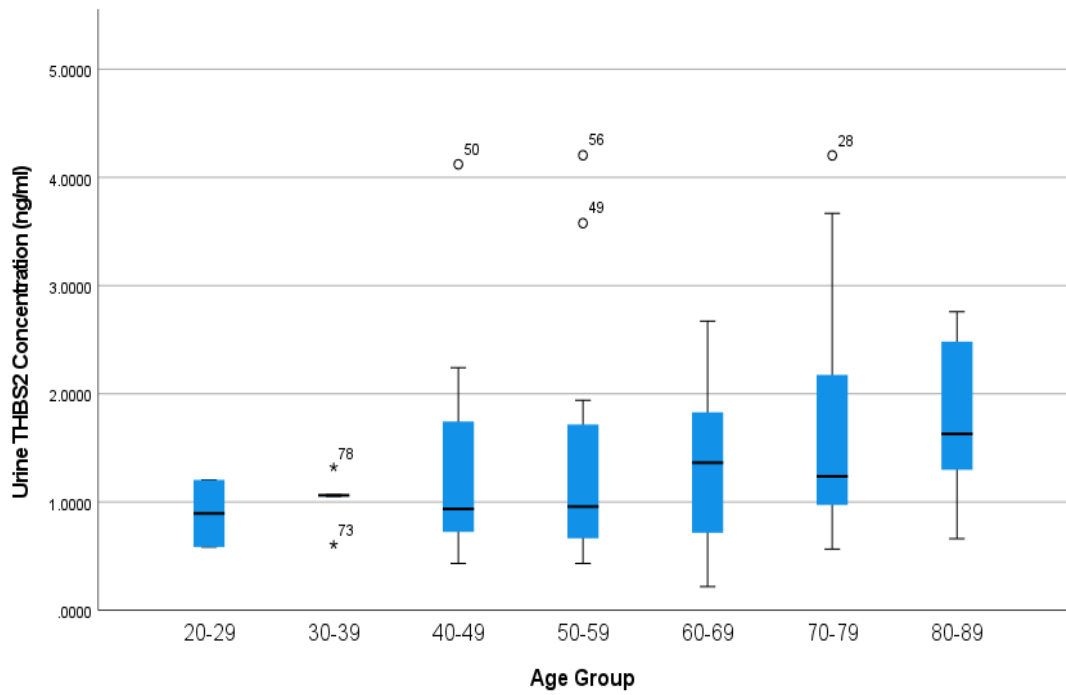
Figure 4.18   Boxplot showing the urinary concentration of THBS2 dependent on age group.

Median urinary concentration of THBS2 is higher in the oldest age group compared to the younger ones, however, there is not a definite correlation between increasing age and urinary THBS2 concentration.
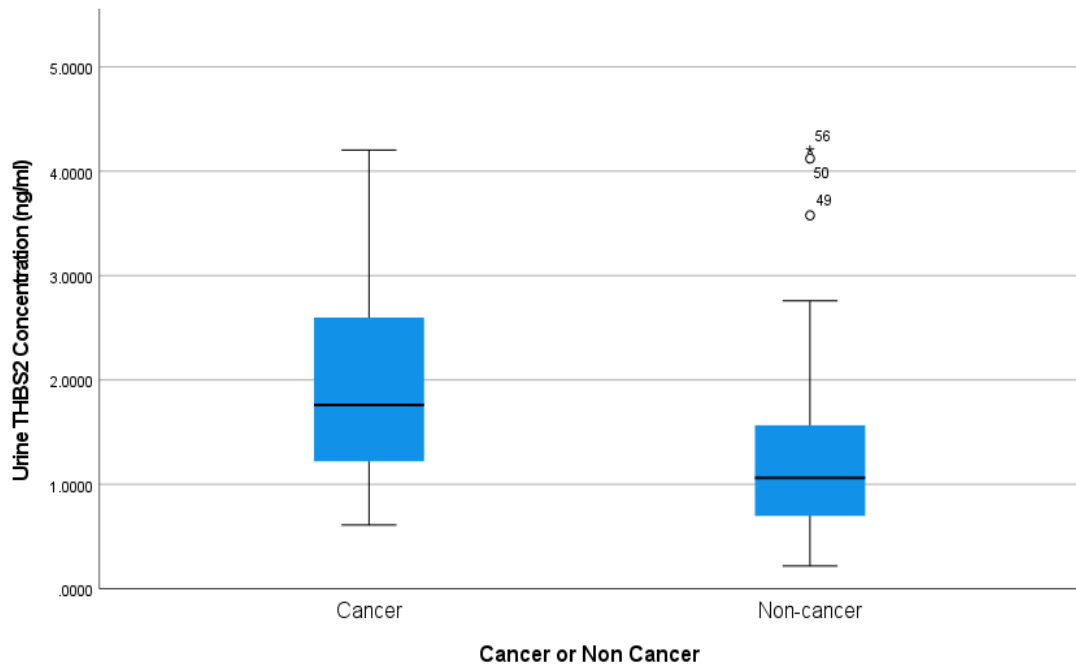
Figure 4.19   Boxplot showing urinary concentrations of THBS2 dependent on the presence of pancreatic cancer.

Median urinary concentration of THBS is seen to be significantly higher in the cancer cohort, compared to the non-cancer cohort.
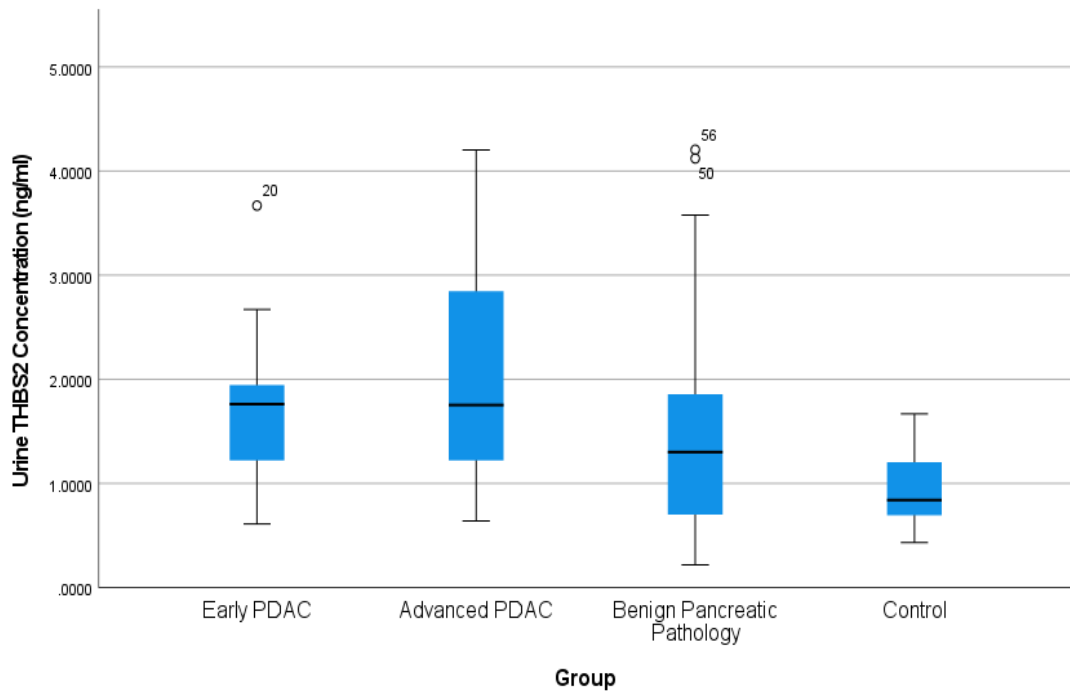
Figure 4.20   Boxplot showing urinary concentrations of THBS2 in all four participant groups.

Median Urinary THBS2 concentrations can be seen to be higher in both early and advanced PDAC compared to the benign and control groups.
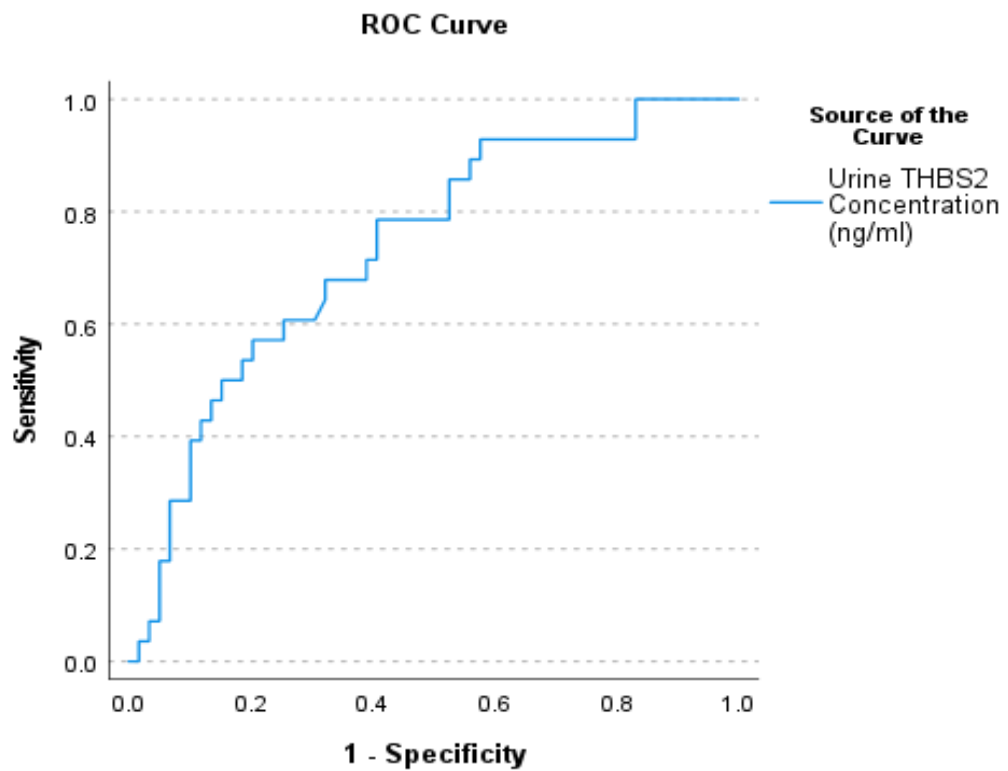
Figure 4.21   RoC Curve showing the diagnostic ability of urinary THBS2 to differentiate between cancer and non-cancer samples (AUC=0.734).

Similarly to the ROC curve for plasma THBS2, it can be seen that the ability of urinary THBS2 to discriminate between PDAC and non-PDAC samples is acceptable. The urinary THBS2 concentration cut off with the greatest accuracy was a cut-off of 1.21ng/ml (sensitivity 78.57%, specificity 57.63%).

**Table 4.5   Summary   of   Results   of   Urinary   Concentrations   of
Thrombospondin-2 determined by ELISA (ng/ml)**

	Early PDAC	Advanced PDAC	"Cancer"	Benign	Control	"Non-Cancer
Mean	1.76	1.98	1.87	1.55	0.95	1.25
Median	1.76	1.75	1.76	1.30	0.84	1.06
Minimum	0.61	0.64	0.61	0.22	0.43	0.22
Maximum	3.67	4.20	4.20	4.21	1.67	4.21

4.6.2.3 Human Chitinase 3-Like 1 (YKL-40)

Collected urine samples underwent ELISA to quantify concentrations of YKL-40 once the protocol had been optimised as described above.

Median urinary concentration of was 1.37ng/ml (0.00ng/ml – 11.82ng/ml). The male cohort of participants had a higher median concentration of urinary YKL-40 (1.42ng/ml vs 1.32ng/ml). However, this was not statistically significant (p=0.739). Multivariate analysis did not show age to have a significant effect on urinary YKL-40 concentration (p=0.486). Urinary YKL-40 concentration based on age group is shown in Figure 4.22.

The median concentration amongst participants with PDAC was 1.54ng/ml, compared to 1.21ng/ml in the non-cancer group. This observed difference was not statistically significant (p=0.317). Figure 4.23 shows urinary YKL-40 concentrations in cancer and non-cancer groups.

Similarly to the plasma results, the median concentration amongst the benign group was closer to the 2 cancer groups than the control group and was actually higher than the early PDAC group. The greatest median urinary concentration of YKL-40 was seen in the advanced PDAC group at 2.62ng/ml, followed by the benign group (2.04ng/ml), the early PDAC group (1.43ng/ml) and then the control group (0.71ng/ml). The results are shown in Figure 4.24.

Unfortunately, unlike the plasma samples, the differences observed between urinary concentration in the early PDAC group were not significant when compared to each of the other 3 groups (advanced PDAC, p= 0.291; benign, p=0.450; control, p=0.213).

A ROC curve was constructed and is shown in Figure 4.25. The area under the curve was calculated to be 0.567, which is below the acceptable level for a diagnostic test but is slightly higher than the AUC calculated for the plasma concentrations of YKL-40.

Table 4.6 summarises the results obtained from analysing urinary concentrations of YKL-40.
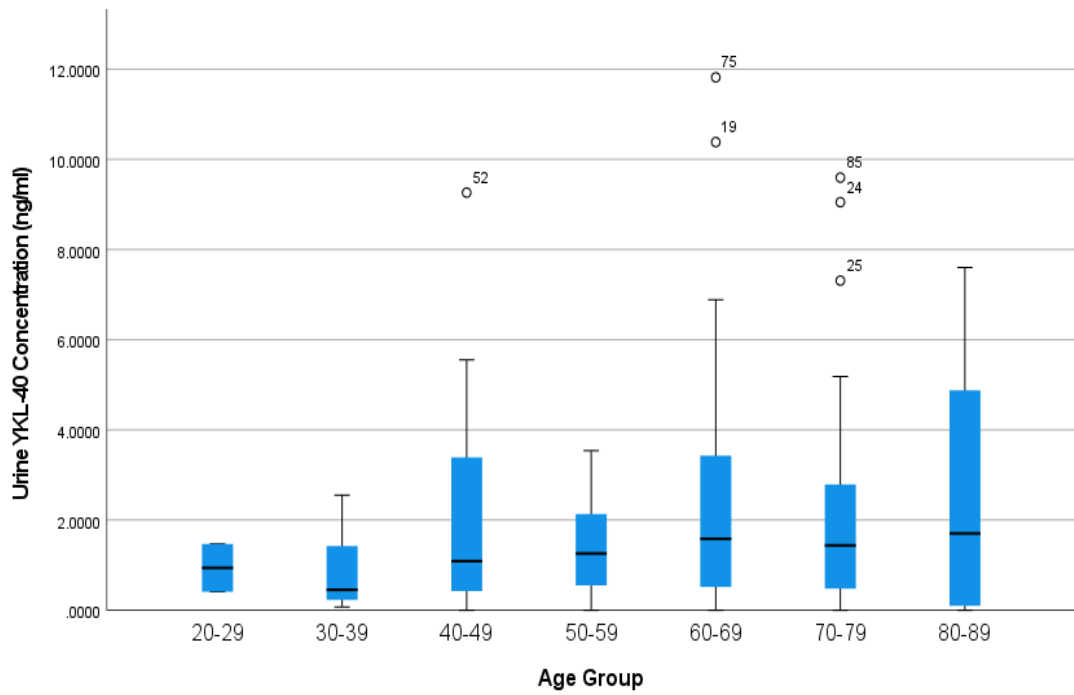
Figure 4.22   Boxplot showing urinary concentrations of YKL-40 dependent on age group.

There is no clear correlation between advancing age and increasing urinary YKL-40 concentrations (in contrast to that seen in plasma YKL-40 concentrations and increasing age).
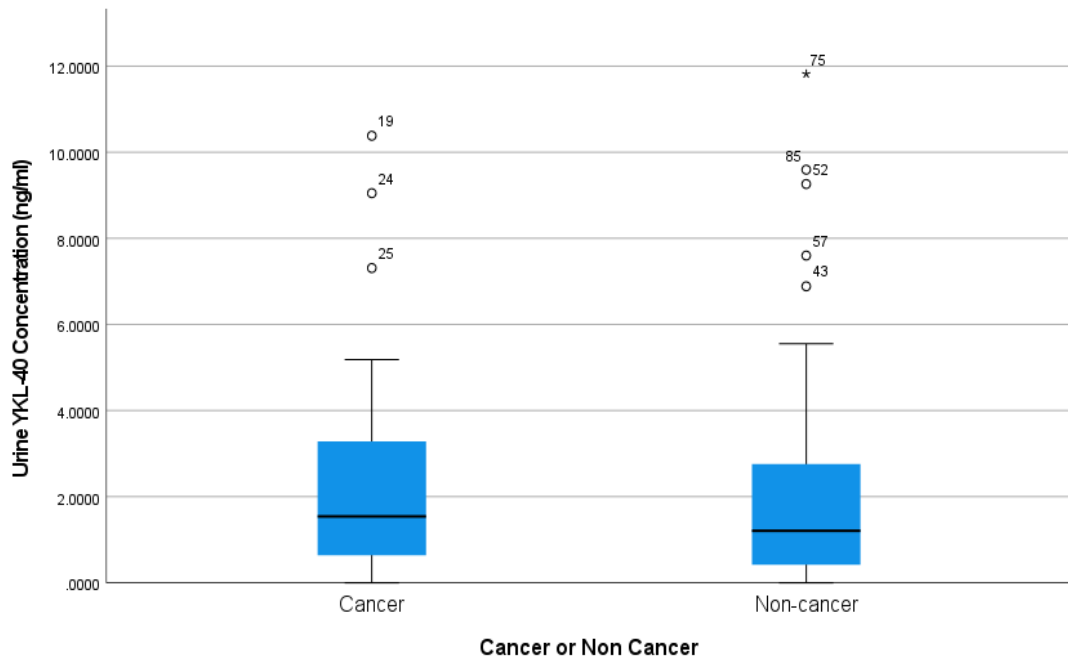
Figure 4.23   Boxplot showing urinary concentrations of YKL-40 dependent on the presence of pancreatic cancer.

The similar median concentrations of urinary YKL-40 seen in the cancer and non-cancer cohorts show its lack of utility as a diagnostic marker for PDAC.
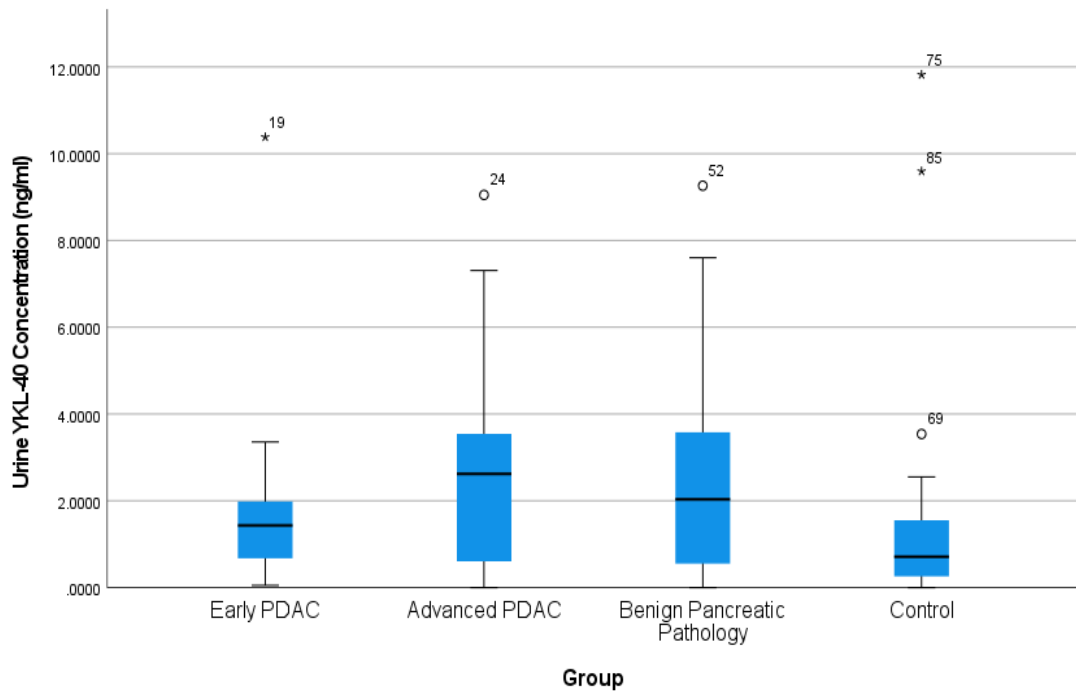
Figure 4.24   Boxplot showing urinary concentrations of THBS2 in all four participant groups.

The similar median concentrations of urinary YKL-40 seen in the advanced PDAC and benign pancreatic pathology cohorts, coupled with the fact that median concentration is higher in the benign group compared to the early cancer group again demonstrates the lack of utility of urinary YKL-40 to be a diagnostic biomarker for PDAC.
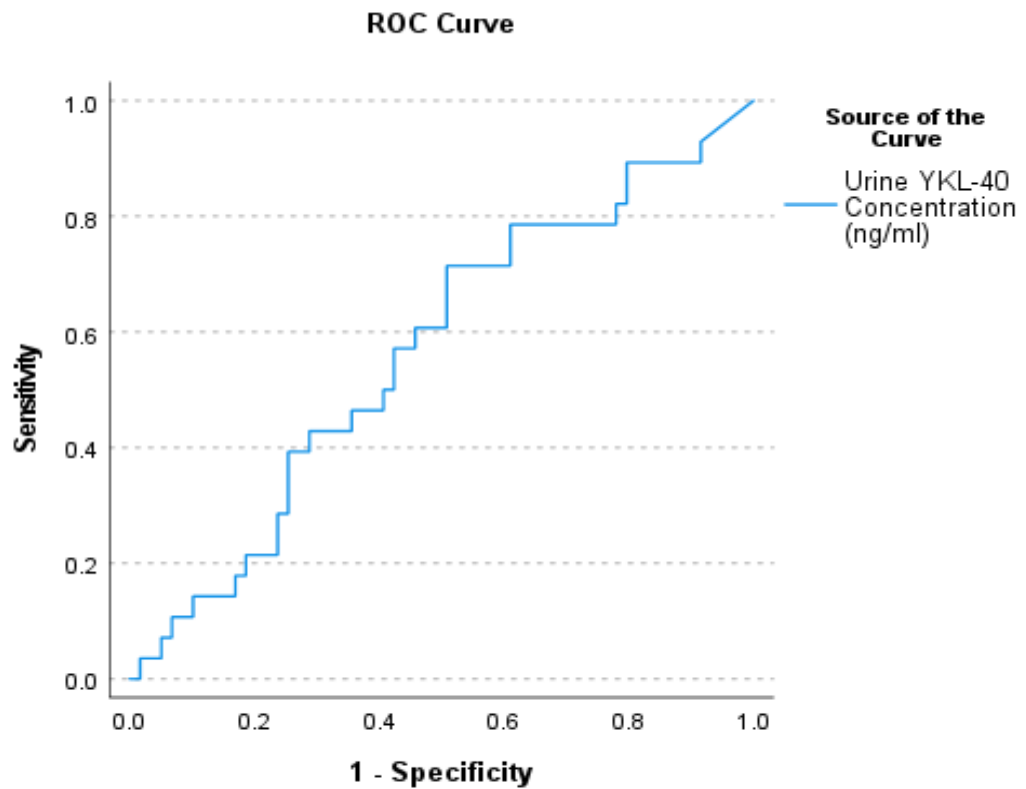
Figure 4.25 RoC Curve showing the diagnostic ability of urinary YKL-40 to differentiate between cancer and non-cancer samples (AUC=0.567).

As is seen with plasma YKL-40, the low AUC for the urinary concentration of YKL-40 shows it is not a good test to differentiate between PDAC and non-PDAC patients.

Table 4.6   Summary of results obtained quantifying urinary concentrations of YKL-40 (ng/ml)

	Early PDAC	Advanced PDAC	"Cancer"	Benign	Control	"Non-Cancer
Mean	2.02	2.95	2.49	2.49	1.61	2.06
Median	1.43	2.26	1.54	2.43	0.71	1.21
Minimum	0.05	0.00	0.00	0.00	0.00	0.00
Maximum	10.38	9.05	10.38	9.26	11.82	11.82

4.6.3 Conclusions about biomarkers quantified using ELISA

4.6.3.1 Individual Biomarkers

Quantification of biomarker levels within urine samples obtained has shown mixed results. Whilst Ca19-9 is the gold standard plasma biomarker, hence its wide use in clinical practice, it was surprising to see that there was no apparent correlation with urine levels. Spearman's correlation coefficient was 0.003 which was not deemed significant (p=0.983). Unfortunately, it is not possible to ascertain from these experiments whether this lack of correlation between plasma and urinary levels of Ca19-9 is due to a physiological factor such as a lack of consistent renal re-absorption of Ca19-9 or a problem with the assay. Unfortunately, due to the Ca19-9 ELISA kit being proprietary, we were unable to optimise it in the way we were with the duo set kits. The high AUC calculated from the RoC curve for plasma Ca19-9 which was higher than the other biomarkers confirms that Ca19-9 remains the best test out of the individual biomarkers analysed in this study.

As previously discussed in Chapter 2, there is increasing interest in THBS-2 as a biomarker for PDAC. An initial review of our results suggests that plasma THBS-2 concentrations were significantly different between cancer and non-cancer cohorts, and indeed Mann Whitney U test of these results provides a p-value of <0.001. However, when multivariate analysis was undertaken, taking participant age and gender into account, the difference in THBS2 plasma concentrations between cancer and non-cancer groups did not reach statistical significance (p=0.123). A summary of median biomarker concentrations and their statistical significance are shown in Table 4.7. As mentioned previously, the significant difference in age and gender composition of the 4 participant groups may have affected the data. Whilst plasma THBS2 concentrations would appear to be affected by these patient factors, the difference seen in the concentration of urinary THBS2 between the 2 cohorts is seen to be statistically significant when undergoing both Mann Whitney U testing and multivariate analysis (p=<0.001, p=0.038). Spearman's correlation coefficient was calculated to assess the correlation between paired plasma and urine concentrations of THBS2. This was calculated to be 0.362, which was statistically significant with a p= <0.001, suggesting that renal excretion/re-absorption of THBS2 is fairly consistent.

Paired plasma and urine samples showed a good correlation between concentrations of YKL-40 with a Spearman's correlation coefficient of 0.250 which was statistically significant (p=0.024). Whilst previous work in our lab looking at the expression of YKL-40 in pancreatic cancer tissues by immunohistochemistry had raised hopes for its utility as a biomarker, unfortunately, the results obtained here did not support this, as differences in neither plasma nor urinary concentrations of YKL-40 were significantly different between cancer and non-cancer cohorts. Its relatively high concentrations within the benign pathology cohort are perhaps not surprising given elevated concentrations have previously been identified in several inflammatory conditions.

Table 4.7 **Summary of patient demographics and median biomarker concentrations dependent on the presence of PDAC, with calculated p-values**

	Cancer	Non-Cancer	Significance	Multi-variate
Age	69	57	**<0.001**	
Gender				
Male	30	19	**<0.001***	
Female	10	41		
Ca19-9 (U/L)				
Plasma	105.22	31.53	**<0.001**	**<0.001**
Urine	117.56	123.24	0.819	**0.260**
THBS2 (ng/ml)				
Plasma	49.10	34.05	**<0.001**	0.123
Urine	1.87	1.25	**<0.001**	**0.038**
YKL-40 (ng/ml)				
Plasma	116.90	113.76	0.587	0.925
Urine	2.49	2.06	0.317	0.266

*Pearson Chi-square test

4.6.3.2 A Multi-Analyte Biomarker Panel

As discussed above, of the 3 biomarkers quantified using ELISA, Ca19-9 showed the greatest differentiation between cancer and non-cancer samples. Whilst multi-variate analysis showed plasma concentrations of THBS2 in these 2 groups not to be a significant difference, the AUC on our ROC curve did show that it could differentiate between cancer and non-cancer plasma samples. We combined the 3 ROC Curves to identify potential cut-off concentrations (Figure 4.26). In the first instance, values from the curves which were found to lie closest to the left upper corner of the grid were chosen as those with the highest sensitivity and specificity. This gave us panel A which consisted of a cut-off value of 45.02U/L for Ca19-9, 24.50ng/ml for THBS2 and 70.65ng/ml for YKL-40. Panel B was constructed with the sample values with a greater focus on sensitivity at the cost of specificity (i.e., towards the top right corner of the grid). Cut-off values of 15.87U/L for Ca19-9, 12.75ng/ml for THBS2 and 37.37ng/ml for YKL-40 were selected. Panel C was constructed with high specificity, sacrificing sensitivity. Cut-offs were 103.97U/L for Ca19-9, 96.01ng/ml for THBS2 and 205.70ng/ml for YKL. These panels are summarised below in Table 4.8. Given the low AUC of the YKL ROC curve, a further 3 panels were used, labelled A2-C2 containing just the respective values of Ca19-9 and THBS2 without including YKL-40. Assuming each panel to be true to detect cancer, the panels were compared to whether the participant had cancer or not the individual to calculate sensitivity, specificity, positive predictive value and negative predictive value. In clinical practice, a Ca19-9 cut-off value of 37U/L is often used to differentiate between cancer and non-cancer. We, therefore, used this value as the gold standard and compared the accuracy of the multi-analyte panels to this. Samples had to have detectable values of all biomarkers in order to be included in the analysis. Panel C1 (highly specific) was excluded from the analysis as it did not detect any cancer samples. The accuracy of each panel was determined by combining the number of true positives and true negatives and dividing by the total number of samples analysed. The results are summarised in Table 4.9
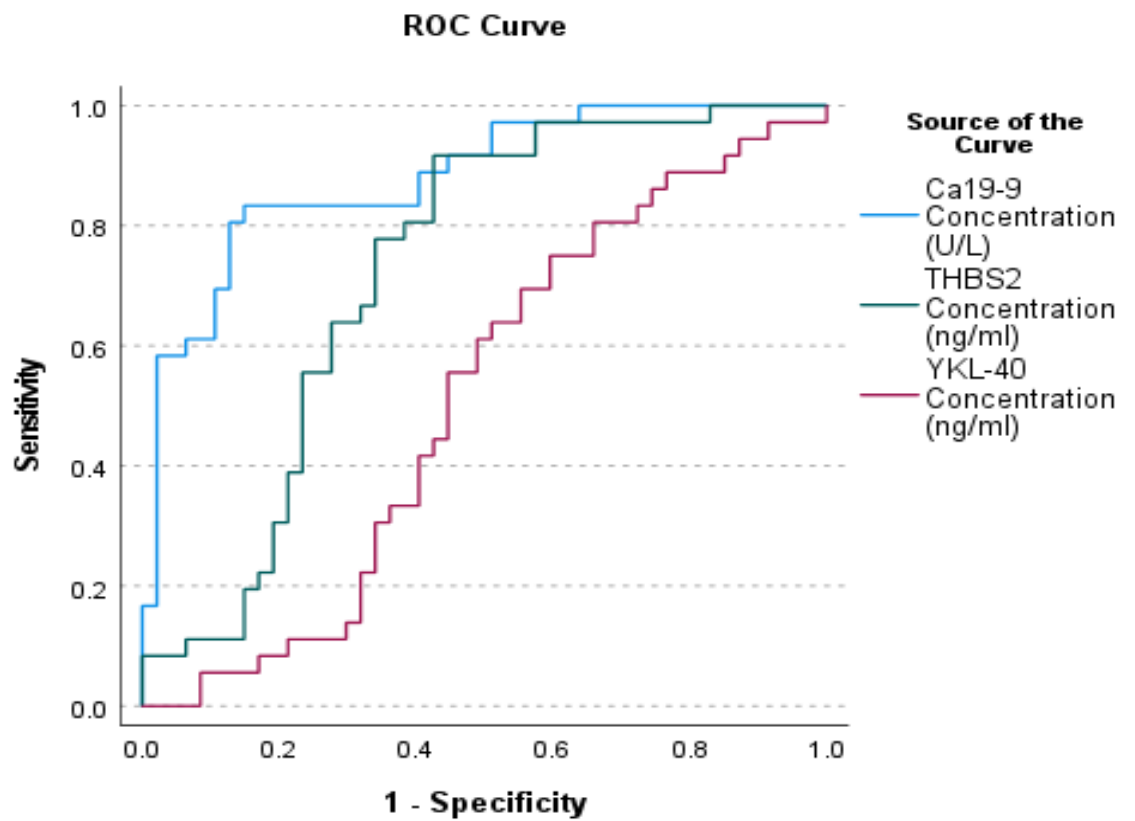
Figure 4.26   Comparison of ROC curves of the 3 plasma biomarkers analysed using ELISA.

The diagnostic superiority of plasma Ca19-9 compared to the other 2 analysed plasma biomarkers is demonstrated.

Table 4.8   Initial multi-analyte panels with respective sensitivity and specificity of the individual biomarkers at their concentration cut-off

	A1	B1	C1
Ca19-9 (U/L)	**45.02** Sens = 83.3% Spec = 85.4%	**15.87** Sens = 97.2% Spec = 37.5%	**103.97** Sens = 58.3% Spec = 97.9%
THBS2 (ng/ml)	**24.50** Sens = 91.7% Spec = 56.2%	**12.75** Sens = 97.2% Spec1 = 6.7%	**96.01** Sens = 11.1% Spec = 93.7%
YKL-40 (ng/ml)	**70.65** Sens = 75.0% Spec = 39.8%	**37.37** Sens = 97.2% Spec 8.3%	**205.70** Sens = 5.6% Spec = 91.7%

Table 4.9 Ability of initial multi-analyte biomarker panels to correctly identify plasma specimens from participants with pancreatic cancer compared to the gold standard Ca19-9 of 37U/L.

	Ca19-9 (37U/L)	A1	A2	B1	B2	C1	C2
Sens	83.78%	58.33%	77.78%	91.67%	94.4%		5.56%
Spec	71.70%	87.50%	86.80	45.83%	41.51%		100%
PPV	67.39%	77.78%	80.00%	55.93%	52.31%		100%
NPV	86.36%	73.68%	85.19%	88.00%	91.67%		60.91%
Accuracy	76.6%	75.00%	**83.14%**	65.48%	61.54%		61.80%

As can be seen from Table 4.9, panel A2 (Ca19-9 >45.02U/L, THBS2 > 24.50ng/ml) was the best-performing biomarker panel, being able to correctly distinguish between cancer and non-cancer specimens with an accuracy of 83.14%. This panel was more accurate than the current gold standard of a Ca19-9 >37U/L.

As it had been previously shown that the concentration of urinary THBS2 was significantly different between cancer and non-cancer cohorts, we determined a cut-off point using the ROC curve we had previously constructed (See Figure 4.25). The value with the greatest sensitivity and specificity was found to be a cut-off of 1.21ng/ml. This cut-off was then applied to the collected samples, initially as a stand-alone marker, and then combined to the multi-analyte panel A2 (making panel A3) to see how the accuracy compared against the gold standard of a Ca19-9 of >37 U/L as well as our new panel A2. These results are summarised in Table 4.10

Urinary THBS2 with a cut-off of 1.21ng/ml had an accuracy of 64.37% at distinguishing between pancreatic cancer and non-cancer samples, which was lower than that seen with plasma Ca19-9 with a cut-off of 37U/L (76.6%) and panel A2 (83.14%). However, when it was added to the components of panel A2, there was a marginal increase in the accuracy which was found to be 83.33%.

Table 4.10 Ability of urinary THBS2 to correctly identify specimens from participants with pancreatic cancer compared as a stand-alone marker and in combination with our previously developed panel, compared to the gold standard of plasma Ca19-9 with a cut-off of 37U/L.

	Urinary THBS2 (1.21ng/ml)	Plasma Ca19-9 (37U/L)	A2	A3
Sens	78.57%	83.78%	77.78%	64.00%
Spec	57.63%	71.70%	86.80	92.45%
PPV	46.81%	67.39%	80.00%	80.00%
NPV	85.00%	86.36%	85.19%	84.48%
Accuracy	64.37%	76.6%	83.14%	**83.33%**

4.7 FTIR SPECTROSCOPY

FTIR spectroscopy work was undertaken on samples obtained from this study within the chemistry department at Swansea University by Dr Ed Duckworth (PhD candidate), supervised by Dr Deb Roy (Senior Lecturer in Chemistry). The results of these have formed part of Dr Duckworth's PhD thesis (2023), but a summary of the findings will be detailed here with his permission.

The same plasma and urine samples which were used in the ELISA analyses above were transported to the Department of Chemistry. The samples were filtered based on molecular weight as described in the previous chapter. Initial results showed urine not to be as accurate in detecting cancer as plasma samples, therefore urine was not further analysed in the whole cohort of samples. For the final analysis, <10kDa plasma filtrate and unfiltered plasma were used. Diagnostic modelling was undertaken with a combination of Support Vector Machine (SVM) classifiers with and without Principal Component Analysis (PCA). Results were validated with complete Leave-One-Out (LOO) cross-validation. An example of the spectra obtained from FTIR is shown in Figure 4.27. The accuracy of the model was calculated as it was in the ELISA samples by looking at the proportion of samples which were correctly identified by the model (i.e. true positive and true negative).

Results are summarised in Table 4.11. As can be seen, when <10kDa filtrate is used, the accuracy of the model increases. The model is ≥90.0% accurate in distinguishing advanced cancer from benign pancreatic pathology and control samples and is also able to distinguish between early and advanced pancreatic cancer.
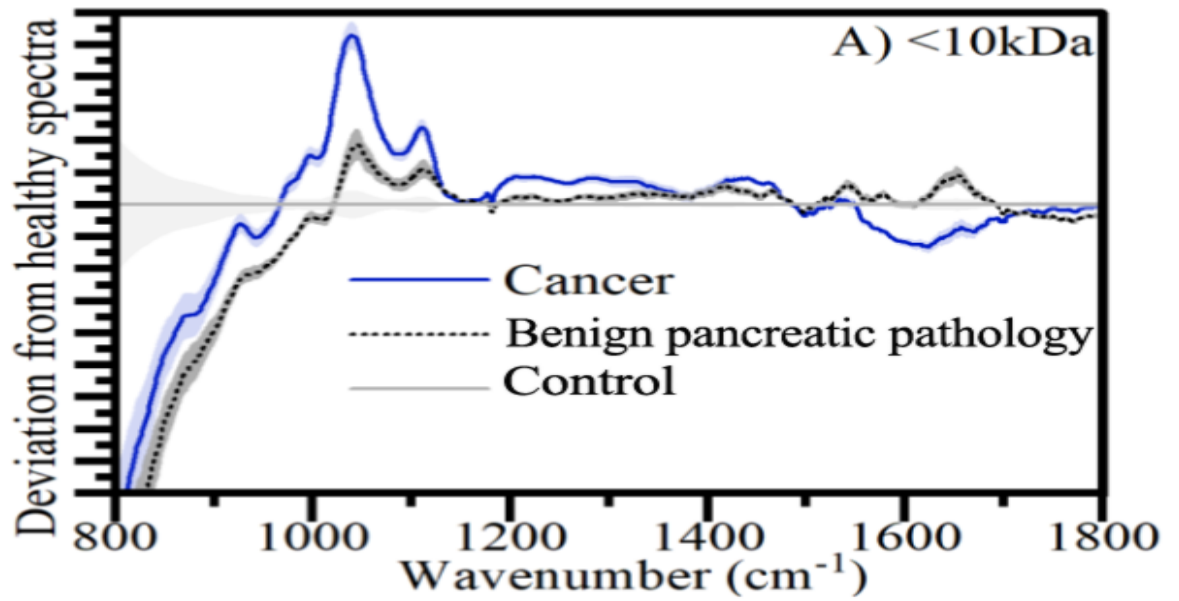
Figure 4.27 FTIR spectra obtained for <10kDa filtrate from the plasma of cancer, benign pancreatic pathology and control participants.

The spectra from the benign and control groups can be seen to be closely matched, however, the spectra obtained using the samples from PDAC patients can be seen to be clearly different from the other 2 groups.

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Table 4.11 **Comparison of diagnostic accuracy in distinguishing different patient populations obtained from the FTIR model (AC = advanced cancer, EC = early cancer, B = benign pancreatic pathology, C = control)**

Fraction	Subsets compared	Sens. (%)	Spec. (%)	PCA-SVM Accuracy (%)	95% Confidence interval(%)	SVM only Acc. (%)
Whole serum	AC v B	74	90	**84.3**	77.3-90.4	75.3
	AC v C	100	100	**100.0**	95.6-100.0	92.4
	AC+EC v C+B	75	86	**81.3**	75.4-86.2	75.7
	AC v EC	88	78	**83.5**	74.4-90.4	76.9
<10kDa window	AC v B	90	90	**90.0**	84.5-95.1	86.5
	AC v C	97	93	**95.3**	87.8-99.0	87.2
	AC+EC v C+B	90	91	**90.6**	85.7-94.3	89.2
	AC v EC	90	90	**90.0**	80.6-95.8	58.6

4.8 DISCUSSION

Both the ELISA work and FTIR spectroscopy analysis have shown promising results with regard to proof of principle and feasibility in the development of novel biomarkers. Whilst plasma measurements of Ca19-9 continue to be the gold standard when trying to diagnose pancreatic cancer, it is reassuring to see that there are emerging tests with potentially improved diagnostic accuracy which may one day replace stand-alone plasma Ca19-9 levels, allowing more people to be diagnosed with earlier disease amenable to curative treatment.

The lack of ability for all but plasma Ca19-9 to differentiate pancreatic cancer from benign pancreatic conditions is not necessarily problematic. As discussed in Chapter 1, chronic pancreatitis and some pancreatic cysts are known to be premalignant and these patients will require regular surveillance cross-sectional imaging anyway, so whilst they may be seen as false positives, despite the lack of cancer, it is still important they are brought to the attention of the pancreatic multi-disciplinary team (MDT).

Despite these encouraging results, it is important to recognise the limitations of this study. The marked heterogeneity in age and gender between cancer and non-cancer cohorts will potentially have affected the results. Whilst these don't necessarily matter in a feasibility study like this, going forward to further validate these results there will need to be more equivalence of age and gender between the 2 groups.

The lack of urine samples in comparison to blood samples may have affected the urinary work. Given the majority of participants who were unable to provide urine samples were from the cancer cohort this may affect the significance of the results obtained.

The lack of correlation between plasma and urinary Ca19-9 concentrations, coupled with the fact that the overall median concentration of urinary Ca19-9 was higher than plasma Ca19-9 is somewhat confusing, especially given the observed correlation between plasma and urinary concentrations of THBS2 and YKL-40. As mentioned above it is not clear the cause of this discrepancy. However, it is apparent that urinary Ca19-9 cannot be used as a diagnostic marker of PDAC using the Novus Biologicals kit (Bio-Techne, Abingdon, UK) that we used for our analysis. This kit was proprietary and therefore we were unable to optimise it as we did with the DuoSet kits. We did not

specifically take note of participants underlying renal function, but this may be worth exploring in future work.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1   CONCLUSIONS FROM THIS THESIS

5.1.1   Contemporary approaches to the diagnosis and management of Pancreatic Ductal Adenocarcinoma (PDAC)

It is clear from the review of the contemporary literature that pancreatic cancer remains a devastating disease with poor outcomes. The combination of late presentation due to the vagueness/lack of symptoms in the early stages, coupled with aggressive tumour biology which rapidly progresses makes for a lethal combination. Whilst surgery is still seen as the only form of treatment which can potentially offer a long-term cure, it is fraught with danger and has massive implications on the patient's quality of life, even when things go well. This being said, there have been vast developments in the oncological management of pancreatic cancer with newer chemotherapy regimens being trialled with improved survival outcomes, perhaps offering a glimmer of hope that the poor outcomes of this disease which have remained static for many decades, may finally begin to improve. The move towards personalised medicine, with large, international tissue profiling studies such as PRECISIONPanc (13) and the associated offshoot PRIMUS studies aims to offer tailored treatment regimens for patients diagnosed with this terrible disease. It may not offer a cure, but it certainly offers prolonged survival, meaning there may come a time when major, life-changing surgery is no longer needed for these patients.

Whilst surgical and oncological developments aim to deal with the aggressive biology of PDAC, it is clear from the literature that there is a desire to find new ways to identify PDAC at an earlier stage. Whilst the EUROPAC studies from Liverpool offer some hope and reassurance to those individuals with a family history of PDAC or a relevant genetic predisposition, there remains no screening programme/protocol for the asymptomatic general population. However, there have been several investigations into novel techniques and markers to detect PDAC at an earlier stage in asymptomatic people, showing that it is clear that an isolated Ca19-9 level is not adequate to diagnose cancer. Increasing interest in proteomics and metabolomics will hopefully identify more products of metabolism specific to pancreatic cancer which can be quantified from a variety of bodily fluids. The ability to detect pancreatic cancer in urine samples, as is being pushed forward by the St Mary's group who have developed the PancRISK score (129), may prove to be more acceptable to patients than blood tests and proves to be a promising area of future research.

5.1.2 Evaluation of novel biomarkers for PDAC, both as individual markers and as part of a multi-analyte panel

The results from the experimental work previously described, have shown that it is possible to analyse a selection of biomarkers in plasma and urine samples using commercially available reagents. Within the limitations of this small, feasibility study, the initial results obtained seem to offer some hope in the utilisation of other markers on top of the currently widely used Ca19-9. The development of a multi-analyte panel using a selection of biomarkers can offer more diagnostic certainty than a single marker alone. Our plasma work has echoed those results shown by Kim and colleagues, regarding the increased diagnostic accuracy of combining Ca19-9 and THBS2 levels compared to Ca19-9 on its own.

Whilst none of our single urinary biomarkers performed better than the plasma markers/panels, the fact that the inclusion of urinary THBS2 in a panel with plasma THBS2 and Ca19-9 led to the increased diagnostic accuracy of the panel is promising that urinary markers may play a future role in the diagnosis of pancreatic cancer.

The FTIR work kindly undertaken by Dr Deb Roy's group at the chemistry department at Swansea University is certainly promising concerning the use of spectroscopy and machine learning in the diagnosis of pancreatic cancer. The excellent accuracy ($\geq$90%) the model has shown for differentiating between the different groups of the studies, is impressive and we hope to further validate this as more samples are collected and analysed.

5.2 POTENTIAL FUTURE WORK TO BE DEVELOPED FROM THIS THESIS

5.2.1 Validation of preliminary results obtained from ELISA

As we have mentioned before, this was a small, feasibility study with notable heterogeneity in the age and gender of the study groups. Further validation is required to ensure that the observed results are not down to other patient factors.

An amendment has been made to the study protocol, allowing a further 100 participants to be recruited, taking the study population up to 200 in total (50, early PDAC, 50 advanced PDAC, 50, benign and 50 control). With the additional participants, we should aim to increase our recruitment of females with cancer, whilst simultaneously increasing the proportion of males recruited into the benign and control groups. It would be prudent to only recruit participants over the age of 50 in the benign and control groups to try and reduce the influence that age and gender have had on our results.

From these new participants, a random selection from each group should be selected and the results of the biomarkers should be run against Panel A3 to ensure the calculated accuracy. Obviously, the results can subsequently be integrated with the existing results, and the cut-off values of the biomarkers adjusted as appropriate if it appears that curacy would be improved.

Participants in the benign and control groups who are shown to be positive by the multi-analyte panel could then undergo Computed Tomography (CT) scanning of the abdomen to assess the pancreas.

5.2.2 Development of spectroscopy, combined with machine learning and integration of radiology

There is increasing focus on the utility of machine learning in cancer diagnostics. The results obtained from our FTIR spectroscopy echo those seen in the literature, though it must be noted there is limited literature specifically related to pancreatic cancer. We are currently looking to develop a machine-learning model integrating cross-sectional imaging with plasma spectroscopy. The primary aim of this would be to use radiology and spectroscopy to differentiate between cancer and benign conditions, in particular

chronic pancreatitis. On conventional cross-sectional imaging alone it can be difficult to differentiate between PDAC and chronic pancreatitis, and as a result, patients will sometimes undergo major surgery for what ultimately comes back as a benign disease. The hope is that by comparing spectra of patients with cancer and chronic pancreatitis etc (which has been shown to have accuracy on the current FTIR spectroscopy model), whilst at the same time comparing and analysing their scans, it will become easier to differentiate between the 2 disease entities, reducing the number of patients undergoing unnecessary treatments.

5.2.3 Assessment of K-Ras mutations and inclusion of these into multi-analyte biomarker panels

Our study used ELISA to quantify levels of specific biomarkers and FTIR spectroscopy to look for recognisable, reproducible spectra to differentiate between cancer and non-cancer specimens. We had hoped initially to complement these 2 methods with Polymerase Chain Reaction (PCR) to isolate circulating tumour DNA (ctDNA) and analyse the K-Ras status. However, this did not materialise due to pressure on time and resources. As previously described, K-Ras mutation occurs frequently in the early stages of the carcinogenesis process of pancreatic cancer. The ability to detect K-Ras mutations in the blood of participants with early PDAC would add to the strength of the panel.

Impact from this body of work

I. Published articles and collaborations associated with this research

   a. Duckworth E, *Mortimer M*, Al-Sarireh B, Kanamarlapudi V, Roy D. Frontline Clinical Diagnosis – FTIR on Pancreatic Cancer. Cancer Medicine, 2023; 12(**16**): 17340-17345

   b. *Mortimer MCM*, Ovens JL. Pancreatic cancer. InnovAiT. 2019;12(9):492-496. doi:10.1177/1755738019855101

   c. Attard JA, Al-Sarireh B, Bhogal RH, Farrugia A, Fusai G, Harper S, Hidalgo-Salinas C, Jah A, Marangoni G, *Mortimer M*, Pizanias M, Prachialias A, Roberts KJ, Sew Hee C, Soggiu F, Srinivasan P, Chatzizacharias NA. Short-term outcomes after pancreatoduodenectomy in octogenarians: multicentre case-control study. Br J Surg. 2021 Dec 17;109(1):89-95. doi: 10.1093/bjs/znab374. PMID: 34750618.

   d. Moekotte AL, Malleo G, van Roessel S, Bonds M, Halimi A, Zarantonello L, Napoli N, Dreyer SB, Wellner UF, Bolm L, Mavroeidis VK, Robinson S, Khalil K, Ferraro D, *Mortimer MC*, Harris S, Al-Sarireh B, Fusai GK, Roberts KJ, Fontana M, White S, Soonawalla Z, Jamieson NB, Boggi U, Alseidi A, Shablak A, Wilmink JW, Primrose JN, Salvia R, Bassi C, Besselink MG, Abu Hilal M. Gemcitabine-based adjuvant chemotherapy in subtypes of ampullary adenocarcinoma: international propensity score-matched study. Br J Surg. 2020 Aug; 107(9): 1171-1182. doi: 10.1002/bjs.11555

II. Presentations to learned societies

   a. *M Mortimer*, A Kambal, G Shingler, W Walker, V Kanamarlapudi, B Al-Sarireh. Developing a multi-analyte biomarker panel to detect pancreatic adenocarcinoma. 14th Congress of the EAHPBA, Bilbao, Spain, September 2021, Oral poster presentation

   b. *M Mortimer*, A Kambal, G Shingler, B Al-Sarireh. The burden of Pancreatic Cystic Neoplasms on a regional MDT. 14th Congress of the EAHPBA, Bilbao, Spain, September 2021, Oral poster presentation

APPENDIX 2

Confirmation of ethical approval for study
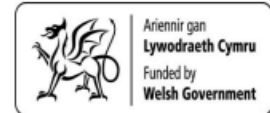
Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

Professor Bilal Al-Sarireh
Consultant HPB and General Surgeon
ABMU/Swansea University
Department of General Surgery
Morriston Hospital
Swansea
SA26 6NL

4 March 2019

Dear Professor Al-Sarireh

Study title:	**Feasibility study to identify the potential role of Raman spectroscopy and ELISA analysis in identifying biomarkers to reliably detect early pancreatic cancer**
REC reference:	19/WA/0064
IRAS project ID:	252525

Thank you for your letter of 01 March 2019, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

APPENDIX 3

Most recent study protocol synopsis (V6.3, 20/05/22)

TITLE	Feasibility study to identify the potential role of spectroscopic techniques and ELISA analysis in identifying biomarkers to reliably detect early pancreatic cancer
SHORT TITLE	Developing a reliable biomarker to detect early pancreatic cancer
Protocol Version Number and Date	Version 6.3 20.05.2022
Methodology	Feasibility study
Study Duration	Initially 12 months (Extended due to COVID-related disruptions) Extended to end date of 31/03/2023 (NSA08)
Study Centre	Morriston Hospital, Swansea Swansea University, Singleton Park Campus
Objectives	To analyse plasma, bile and urine samples collected from patients with and without pancreatic cancer using a combination of spectroscopy and ELISA to determine whether it is possible to detect a sensitive and specific biomarker to allow the earlier detection of pancreatic cancer.
Number of Subjects/Patients	50 patients with early (resectable) pancreatic cancer 50 patients with advanced (locally advanced or metastatic) pancreatic cancer 50 patients with pancreatitis or pancreatic cysts 50 controls who do not fall into the other groups
Main Inclusion Criteria	• All participants to be aged 18 years and over with the capacity to provide informed consent. • Radiological suspicion of Pancreatic cancer with the potential for histological confirmation (biopsy or surgical resection) • Radiological or clinical diagnosis of acute or chronic pancreatitis or of pancreatic cyst • Controls will be patients who do not fit into the other categories, and have no history of/are not currently under investigation for another malignancy
Statistical Methodology and Analysis	Principal Components Analysis (PCA) to measure accuracy, sensitivity and specificity measures for each patient group when analysing the Raman spectroscopy results as per colorectal Raman study. We will potentially explore Support Vector Machine (SVM), Random Forest and Linear Discriminant Analysis (LDA). Descriptive statistics and RoC curve analysis will be used to determine sensitivity and specificity of ELISA biomarker results

APPENDIX 4

Example of most recent Case Report File (V4, 24.10.19)

<u>**ELISA and Spectroscopy in the Early Detection of Pancreatic Cancer**</u>

CRF1 (Baseline visit)

Patient initials _____   Date of Birth   _____

Age   _____   Male/Female   _____

Hospital number/NHS number   _____

Unique Patient Identifier   _____

Date of blood sample   _____   Time of blood sample   _____

Blood sample taken by _____

Diagnosis	*Ca Pancreas*	*Acute Panc*	*Chronic Panc*	*Cyst*	*Control*
Cancer stage	*Resectable*	*Borderline*	*Locally Advanced*		*Metastatic*
Confirmed histology	*Yes*	*No*	*Awaited*		

Other medical diagnoses (diabetes, cirrhosis, etc)

_____

_____

_____

Jaundiced at time of sample   *Yes*   *No*

If yes, Bilirubin level   _____

APPENDIX 5

Example of most recent Informed Consent Form (V5, 11.03.21)

Participant Consent Form (Version 5; 11.03.21)

Title: Developing a reliable biomarker to detect early pancreatic cancer

Chief Investigator: Professor Bilal Al-Sarireh
MD Student: Mr Matthew Mortimer
IRAS Project ID: 252525
Research Ethics Committee (REC) Reference: 19/WAL/0064

Initials

1. I confirm that I have read and understood the relevant Participant Information Sheet (v5 11.03.21) for the above study and have had the opportunity to ask questions and have these answered.

2. I confirm that I have had sufficient time to consider whether or not I wish to be included in the study.

3. I understand that my participation is voluntary and that I am free to withdraw at anytime, without giving any reason, without my medical care or legal rights being affected.

4. I understand this study involves the analysis of bodily fluids including the potential for DNA analysis

5. I agree to the study sponsor and clinical care team accessing my medicals records for reasons related to the study.

6. I agree to my obtained anonymised samples being used for further related studies in the future without being contacted further.

7. I agree to take part in the above study.

_____   _____   _____
Name of participant   Date   Signature

_____   _____   _____
Name of researcher   Date   Signature

If you would like to be informed of the study results by letter, please tick box ☐

3 copies total Original to be kept in site file, 1 copy to participant, 1 copy to be filed in notes

Pancreatic Cancer ELISA and Spectroscopy Consent Form   V5 11.03.21

GLOSSARY

Adjuvant Therapy   Treatment that is given alongside/after the main treatment modality. In cancer surgery, this usually refers to chemotherapy and or radiotherapy given to a patient after resectional surgery to reduce the chance of disease recurrence

Anastomosis   A surgically created join between at least 2 structures

Biomarker   A naturally occurring molecule, gene or characteristic by which a particular pathological process or disease can be identified

Cancer   A disease characterised by abnormal, unregulated cell growth which affects adjacent and distant cells/organs

Chemotherapy   A broad term used to describe a range of cytotoxic treatments which target cancer cells

ELISA   An immunological assay which can be used to quantify the concentration of a variety of molecules including proteins, antigens and antibodies in biological samples

Endocrine   A group of cells/organ which secrete active substances into blood to have an effect on a distant organ

Exocrine	A group of cells/organ which secrete active substances onto an epithelial surface
FTIR Spectoscopy	A form of Infrared spectroscopy which can be used to analyse the absorption or emission of light from a specimen
Histopathology	Medical specialty which undertakes microscopic examination of resected/biopsied tissue
Laparoscopic Surgery	A form of minimally invasive abdominal surgery which utilizes distension of the peritoneal cavity with carbon dioxide and small incisions to allow the use of cameras and instruments. Commonly referred to as "keyhole surgery." Often results in a faster recovery than standard open surgery
Metastasis	A deposit of cancer cells in a tissue/organ distant from the primary site of the cancer. Often a representation of "incurable" cancer, particularly in the instance of pancreatic cancer
Neoadjuvant Therapy	A treatment that is given prior to to the main treatment modality which aims to locally control/reduce disease burden. In pancreatic cancer, this is usually chemotherapy +/- chemoradiotherapy for localised disease which is not amenable to upfront surgery
Oncology	The medical specialty responsible for non-surgical treatments of cancer through the provision of chemotherapy, radiotherapy, immunotherapy etc.

Palliation	A form of treatment in which the primary aim is to treat a patient's symptoms and improve quality of life without necessarily treating/curing the underlying cause
Radiotherapy	The use of x-rays/ionising radiation to treat to treat a disease, usually cancer
Staging	The classification of how advanced a cancer is. The stage of cancer will have implications on treatment options and disease prognosis

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