The identification and evaluation of predictive and prognostic biomarkers in order to personalise treatment pathways for patients diagnosed with pancreatic cancer.

A thesis submitted to fulfil requirements for the degree of Doctor of Philosophy

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Statement of originality

This is to certify that to the best of my knowledge the intellectual content of this thesis is my own. This thesis has not been submitted for any other degree or purposes.

I certify that all the assistance received, and sources have been acknowledged.

Sarah Maloney
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Authorship attribution statement

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Chapter 4: I helped collect some of the surgical specimens and assisted with interpretation of proteomic results. I performed TGM2/OSMR immunohistochemistry experiments and was one of the two assessors that scored the staining. I performed the cell culture viability and siRNA knockdown but not the western blot experiments. I collected corresponding patient data, analysed the results, and performed statistical and data analysis.

Chapter 5: I co-designed the study, helped collect patient specimens and conducted the immunohistochemistry experiments, including optimisations, for all three proteins. I was one of two assessors that scored the slides, and I collected matched patient demographics. I analysed the results, wrote the prospective protocol (appendix).

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Sarah Maloney

26 th September 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Prof. Nick Pavlakis
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Other publications during candidature

Sahni S, Nahm C, Ahadi MS, Sioson L, Byeon S, Chou A, **Maloney S,** Moon E et al. Gene expression profiling of pancreatic ductal adenocarcinomas in response to neoadjuvant chemotherapy. Cancer Med. 2023; 00: 1-12. doi:10.1002/cam4.6411

Sahni S, Nahm C, Krisp C, Molloy M, **Maloney S**, Itchins M et al. Discovery Proteomic Analysis of Pancreatic Tumor Tissue to Identify Biomarkers for the Prediction of Response to Neoadjuvant Chemotherapy. HPB. 2021; 23. S53. 10.1016/j.hpb.2020.11.127.

Nahm C, Sahni S, Pandya A, Hadden W, **Maloney S,** Cook et al. A Unique Urinary Metabolomic Signature for the Detection of Pancreatic Ductal Adenocarcinoma. Int. J. Cancer. 2021; 148: 1508–1518. https://doi.org/10.1002/ijc.33368

Sahni S, Krisp C, Molloy MP, Nahm C, **Maloney S,** Gillson J et al. PSMD11, PTPRM and PTPRB as novel biomarkers of pancreatic cancer progression. Biochim Biophys Acta Gen Subj. 2020 Nov;1864(11):129682. doi: 10.1016/j.bbagen.2020.129682. Epub 2020 Jul 12. PMID: 32663515.

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Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variation
APS	Ammonium persulfate
ATM	Ataxia Telangiectasia
AUROC	Area under the receiver operating characteristic
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CA	Coeliac axis
CA19.9	Carbohydrate antigen 19.9
CAFs	Cancer associated fibroblasts
CAP	Chest abdomen pelvis
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CEA	Carcinogenic embryonic antigen
CHA	Common hepatic artery
CCI	Charleson comorbidity index
CI	Confidence interval
CRP	C-reactive protein
CT	Computerised tomography
CTC	Circulating tumour cells
DIA	Data independent acquisition
dMMR	Mismatch repair deficient
DMSO	Di-methyl sulfoxide
DTT	Dithiothreitol
ECOG	Eastern co-operative oncology group
EMT	Epithelial-mesenchymal transition
ERCP	Endoscopic retrograde cholangiopancreatography
EUS	Endoscopic ultrasound
FA	Formic acid
FDG-PET	Fluorodeoxyglucose- positron emission tomography
FDR	False discovery rate
FNA	Fine needle aspiration
FNB	Fine needle biopsy
FOLFIRINOX	5- fluorouracil, irinotecan and oxaliplatin
G-CSF	Granulocyte-colony stimulating factor
GPS	Glasgow prognostic score
HCC	Hepatocellular carcinoma
hENT1	Human equilibrative nucleoside transporter 1
HIER	Heat induced epitope antigen retrieval method
HR	Hazard ratio
IAA	2-iodoacetamide
IHC	Immunohistochemistry
IPA	Ingenuity pathway analysis

IPMN	Intraductal papillary mucinous neoplasm
IVC	Inferior vena cava
KRAS	Kirsten ras sarcoma virus
LC-MS/MS	Liquid chromatography mass spectrometry/ mass spectrometer
LMR	Lymphocyte monocyte ratio
MAPK	Mitogen activated protein kinase
MCN	Mucinous cystic neoplasm
MDACC	MD Anderson cancer centre
MDT	Multidisciplinary team
mGPS	modified Glasgow prognostic score
miRNA	microRNA
MLH1	MutL protein homolog 1
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSH2	Muts homolog 2
MSH6	Muts homolog 6
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NAC	Neoadjuvant chemotherapy
NC	Normal control
NCCN	National comprehensive cancer network
NLR	Neutrophil lymphocyte ratio
OS	Overall survival
OSM	Oncostatin M
OSMR	Oncostatin M receptor
PALB2	Partner and localizer of BRCA2
PanIN	Pancreatic intraepithelial neoplasia
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PLR	Platelet lymphocyte ratio
PLS	Partial least squares
pMMR	Mismatch repair proficient
PMS2	Postmeiotic segregation increased 2
PV	Portal vein
PVDF	Polyvinylidene fluoride
QM- PDA	Quasi-mesenchymal
RCT	Randomised controlled trial
RFS	Recurrence free survival
SDS	Sodium dodecyl sulfate-polyacrylamide
SMA	Superior mesenteric artery
SMAD4	Mothers against decapentaplegic homolog 4

SMV	Superior mesenteric vein
TBS	Tris-buffered saline
TEMED	Tetramethyl ethylene diamine
TGM2	Transglutaminase 2
TNF	Tumour necrosis factor
TP53	Tumour protein 53
UFS	Upfront surgery
VIP	Variable importance in projection
5-FU	5- Fluorouracil

Abstract

Pancreatic cancer survival has stagnated in recent years, with only 12% of patients alive after five years. The majority of patients present with either locally advanced or metastatic disease, that is not amenable to surgery. For patients with locally advanced disease, treatment involves neoadjuvant chemotherapy (NAC) with the view to shrink the tumour away from vital vasculature to allow for surgery to occur. The success of this approach, combined with poor survival outcomes has seen the introduction of NAC in patients with earlier (upfront or borderline resectable) disease. Whether this approach of NAC leads to a survival advantage over upfront surgery in this cohort of early pancreatic cancer patients, is debatable.

A retrospective cohort analysis was conducted to help answer this question. This identified that for all patients there was no survival difference between neoadjuvant chemotherapy and upfront surgery, however, in patients with early disease (radiological stage 1a), treatment with upfront surgery resulted in longer survival than NAC.

To ascertain whether any biomarkers can assist with selecting the ideal upfront modality for each patient, a series of discovery experiments were conducted in both tumour tissue and blood to identify any novel prognostic or predictive biomarkers. In tissue, TGM2 (Transglutaminase 2) and OSMR (Oncostatin M receptor) were highlighted as a potential pathway involved in chemoresistance and CA125 expression on biopsy and surgical samples were found to be poor prognostic biomarkers. In blood, low levels of TNF-α (Tumour necrosis factor-α) in patients were predictive of chemoresistance whereas higher levels of neutrophil lymphocyte ratio and eotaxin predicted for shorter overall survival.

Whilst these results are promising, validation of these findings in a larger cohort of patients is needed, to enable use of these biomarkers as prognostic and predictive tools to guide treatment that may result in more meaningful survival gains.

CHAPTER 1:

Literature review

The majority of this chapter has been published as: *Maloney, S., Clarke, S.J., Sahni, S. et al. The role of diagnostic, prognostic, and predictive biomarkers in the management of early pancreatic cancer. J Cancer Res Clin Oncol (2023).* https://doi.org/10.1007/s00432-023-05149-4.

1.1 Introduction

Pancreatic cancer continues to have poor overall survival, with 1 in 8 patients alive 5 years after diagnosis[1]. These outcomes have changed little in recent years despite more aggressive chemotherapy regimens and advanced surgical techniques. As the success of immunotherapy and tyrosine kinase inhibitors has seen the five-year survival rates of melanoma and lung cancer rapidly rise, pancreatic cancer survival remains on a plateau at just 12%[1].

1.1.1 Pathophysiology

Pancreatic cancer can arise from precursor lesions which include pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN) [2]. Of these PanIN occurs the most frequently and the pathogenic mutations which transform these lesions into an invasive adenocarcinoma are now well established. The most common oncogene mutations include Kristen rat sarcoma virus *(KRAS)*, tumour protein-53 (*TP53*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and Mothers against decapentaplegic homolog 4 (*SMAD4*) (Figure 1.1)[3]. *KRAS* mutations occur early in low grade (PanIN-1) lesions, whereas *CDKN2A* mutations occur as an intermediate event (PanIN-2), with *SMAD4* and *TP53* mutations occurring in late lesions (PanIN-3)[4].

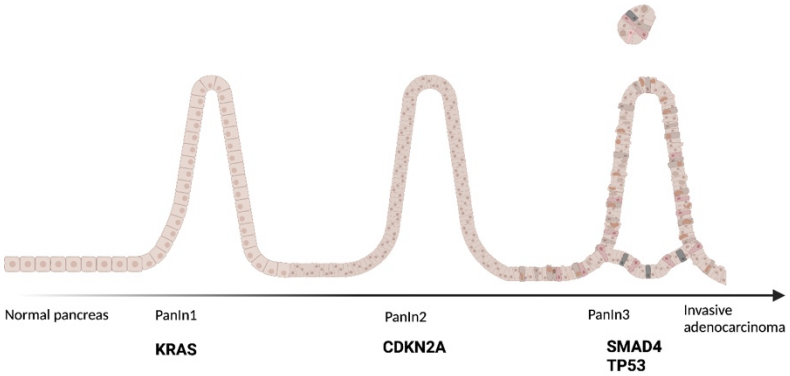

Figure 1.1 Oncogenes in the progression of pancreatic adenocarcinoma from pancreatic intraepithelial neoplasia (PanIN).

1.1.2 Molecular subtypes of pancreatic cancer

Whilst the majority of pancreatic tumours are of adenocarcinoma origin, there are a small subset (1-4%) that have adenosquamous differentiation. These tumours are associated with a worse prognosis[5]. Recent years has seen a greater appreciation of the molecular subtypes of pancreatic tumours. Collison et al first defined three subtypes of pancreatic cancer based on messenger RNA (mRNA) expression of treatment naive tumour specimens: classical, quasi-mesenchymal (QM-PDA) and exocrine like[6]. In later years, Moffitt et all identified two subtypes using RNA sequencing (basal-like and classical) as did Bailey et al using mRNA hybridization (squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine)[7]. Of these there is a strong correlation between the RNA expression in squamous, basal like and QM-PDA subtypes, all of which are associated with adenosquamous histology and hence worse prognosis[7]. Whilst these subtypes have not proven to be clinically practice changing, they do lend insight into the biology of pancreatic cancer and highlight differences that may prove useful as potential targets in future.

1.1.3 Stroma

Pancreatic tumour is surrounded by an abundant dense matrix of connective tissue termed 'stroma' which consists of a number of cells including stellate, endothelial and immune cells as well as extracellular matrix proteins[8]. This stroma acts as a barrier for drug penetration and hence promotes chemoresistance to chemotherapeutic agents[9]. These cells also interplay with tumour cells to create an immunosuppressive microenvironment and recruit a range of pro and anti-inflammatory cytokines to the tumoral site[9]. At present there are a number of therapeutic clinical trials targeting stromal tissue that are either ongoing or reported, however none so far, have demonstrated survival advantage in the phase 3 trial setting[10].

1.1.4 Presentation

Due to the anatomic location of the pancreas, patients can remain asymptomatic until later stage disease. These symptoms are dependent on the location of the primary tumour. The majority of tumours develop in the head of the pancreas (70%) and patients with these tumours can present with either epigastric or right upper quadrant pain, obstructive jaundice or vomiting (in patients that develop gastric outlet obstruction)[11]. For patients with body, tail, or multifocal disease (30%) symptoms can include back pain and systemic signs such as anorexia, weight loss and new or worsening control of diabetes. The non-specific nature of these symptoms mean that these tumours often go unnoticed and this delay to diagnosis is often sufficient to render these tumours incurable. The advanced nature of presentation combined with the loss

of weight, makes treatment of advanced disease challenging due to the poor performance status of these patients.

1.1.5 Diagnosis

A diagnosis of pancreatic cancer requires radiological evidence of a pancreatic lesion in combination with a histological confirmation, which due to the location of the pancreas involves either an endoscopic ultrasound biopsy fine needle aspiration (EUS-FNA) of the lesion, or via common bile duct brushings from an endoscopic retrograde cholangiopancreatography (ERCP)[12]. Recent years have seen the improvement in endoscopic ultrasound (EUS) techniques and a reduction in associated risks. A systematic review conducted by Wang et al demonstrated a low incidence of morbidity <1% and mortality 0.02% with EUS[13]. Whilst there is a lack of literature demonstrating an increased risk of morbidity with an increase in the number of passes in EUS, clinicians are often reluctant to perform them, despite these passes increasing the sensitivity of identifying a pancreatic malignancy[13-15].

Recent years have seen the introduction of fine needle biopsy (FNB) for obtaining core biopsies of pancreatic lesions. Whilst fine needle aspiration (FNA) can obtain scant cells, FNB allows a core sample to be obtained. This introduction has seen improvements in diagnostic accuracy and histological yield over FNA[15]. In addition, the opportunity to obtain more biological material will allow tissue for research and hence has the potential to further our understanding of this disease.

1.1.6 Staging

Adequate staging is required in the diagnosis of pancreatic cancer to necessitate appropriate treatment. Minimum staging investigations involve computerised tomography of chest abdomen and pelvis (CT CAP) and may include magnetic resonance imaging (MRI) of the pancreas or positron emission tomography (PET) scanning[16]. At diagnosis, pancreatic cancer presents as either localised or metastatic disease. Localised pancreatic cancer falls into three categories (upfront resectable, borderline resectable or locally advanced) and is determined by the tumour's location to local vasculature as determined by pancreas focused CT. Whilst there are subtle differences in criteria in different trials/research teams, this thesis uses the criteria outlined in the national comprehensive cancer network (NCCN) guidelines(Table 1.1)[16, 17].

Table 1.1 Resectability status of pancreatic tumours as per NCCN guidelines[16]

Resectability	Arterial	Venous
Resectable	No arterial tumour contact	≤180° contact with superior mesenteric vein (SMV) or portal vein (PV) without contour abnormality
Borderline	**Head** Any contact of tumour with common hepatic artery (CHA) that does not extend to coeliac axis (CA) or hepatic artery bifurcation. ≤180° contact with superior mesenteric artery (SMA) **Body/Tail** ≤180° with CA	>180° SMV or PV or ≤180° with contour abnormality Contact with inferior vena cava IVC
Locally advanced	**Head** >180° with SMA or CA **Tail** >180° with SMA or CA or Contact with CA and aorta	Un-reconstructable SMV/PV due to tumour or thrombus

1.1.7 Treatment

1.1.7.1 Metastatic disease

The majority of patients are diagnosed with metastatic disease at diagnosis[1]. For these patients' treatment consists of palliative chemotherapy. In Australia, two chemotherapy regimens are commonly used: gemcitabine plus nab-paclitaxel (abraxane) or FOLFIRINOX (5-fluorouracil, irinotecan and oxaliplatin)[18, 19]. After progression with these drugs, there is no standard of care and patients may be referred to early phase clinical trials. Targetable mutations in pancreatic cancer are rare but a select few may predict for response to a particular therapy. The IMPACT trial highlighted the difficulty in personalising treatment in advanced pancreatic cancer[20]. In addition to the low rate of targetable mutations it also demonstrated the difficulty in obtaining high quality biological material to perform genomic sequencing and the challenge of returning results in a reasonable timeframe[20]. In addition to chemotherapy or clinical trial, early refer to palliative care team is thought to improve quality of life in patients with pancreatic cancer[21].

1.1.7.2 Locally advanced disease

As surgery is not an initial option for patients with locally advanced disease, treatment algorithms often use neoadjuvant chemotherapy, aiming to downsize the tumour and separate it away from the adjacent blood vessels. The introduction of FOLFIRINOX in the neoadjuvant setting resulted in more patients proceeding to resection and, allowing once incurable patients potentially curative surgery[22].

1.1.7.3 Upfront or borderline resectable disease

For the few patients who have early (upfront or borderline resectable) disease at the time of diagnosis, treatment traditionally involved surgery followed by adjuvant chemotherapy; however, up to 30% of patients did not receive chemotherapy due to the morbidity associated with surgery[23]. This is of particular concern as patients who receive adjuvant chemotherapy have a significant improvement in 5-year survival, compared to those who do not receive chemotherapy (21 vs 8%)[24]. Low rates of adjuvant chemotherapy administration in combination with the success of neoadjuvant chemotherapy in the locally advanced setting led to an increased interest in neoadjuvant chemotherapy in patients with early (upfront or borderline resectable) disease. The most common chemotherapy regimens are either gemcitabine based (gemcitabine plus a fluoropyrimidine or gemcitabine plus nab-paclitaxel) or FOLFIRINOX. The efficacy of gemcitabine plus abraxane versus FOLFIRINOX in the neoadjuvant setting in patients with resectable disease was compared in the SWOG S1505 trial, with similar two year survival in both groups [25]. The role of gemcitabine plus nab-paclitaxel in the adjuvant setting has not been established, due to lack of evidence in this cohort and is currently not PBS listed for this indication in Australia.

1.1.8 Tumour viability

Whilst upfront resectability status is determined using imaging, histopathological staging is formally assessed using TNM staging system on surgically resected tumour. In patients who receive neoadjuvant chemotherapy, the histopathological response to chemotherapy is also assessed[26]. At present there are up to thirteen different scoring systems utilised to measure this response, with no consensus as to the optimal system[27]. The amount of remaining viable tumour is often recorded as a percentage, and tumours with a higher viability are indicative of more ‘chemoresistant’ tumour. Due to the discrepancies between scoring system, there is no exact cut-off for what constitutes a ‘chemosensitive’ or ‘chemoresistant’ tumour[27].

1.2 Neoadjuvant chemotherapy versus upfront surgery in early pancreatic cancer

At present it is unknown whether the neoadjuvant therapy approach leads to superior outcomes compared with upfront surgery in early-stage patients. Four randomised controlled trials (RCTs) have been conducted to address this question, with conflicting results. The PREOPANC trial, a phase III multicentre RCT which randomised patients with upfront or borderline resectable disease to receive either neoadjuvant treatment (gemcitabine based

chemoradiotherapy) or upfront surgery. This study of 246 patients revealed an improvement in median overall survival in patients who received neoadjuvant chemoradiation (15.7 months) compared to the upfront surgical group (14.4 months)[28]. In addition, patients in the neoadjuvant group were more likely to have a negative surgical margin, and there was no difference in surgical complication rates in the two groups[28]. The PREP 02-JSAP 05 trial, a phase II/III RCT randomised 362 patients with resectable pancreatic cancer to neoadjuvant therapy consisting of gemcitabine + S1 or upfront surgery[29]. In this study, patients treated with neoadjuvant chemotherapy had a significant increase in median survival at 36.7 months compared to only 26.6 months in the upfront surgical group[29]. In this cohort the resection rate, negative surgical margin rate and complication rates were comparable between the two groups. ESPAC-5F, a phase II RCT randomised 90 patients to upfront surgery or neoadjuvant treatment with either gemcitabine + capecitabine, FOLFIRINOX or chemoradiation in patients with borderline resectable pancreatic cancer. Overall survival at one year was superior in patients treated with either neoadjuvant gemcitabine plus capecitabine (78%) or FOLFIRINOX (84%) compared to upfront surgery (39%)[30]. There was no difference in surgical complication rates, surgical margin or rates of resection between patients treated with upfront resection versus neoadjuvant chemotherapy or chemoradiotherapy[30]. Finally, the NorPACT-1 trial, a randomized controlled trial, which evaluated four cycles of neoadjuvant FOLFIRINOX compared to upfront resection in 140 patients with resectable pancreatic cancer, revealed a non-statistically significant improvement in median overall survival in patients treated with upfront surgery at 38.5 months compared to 25.1 months (p=0.096)[31].

Despite these promising results each of these studies had their own limitations. The median overall survival difference in the PREOPANC study was clinically modest with only 1.3 months between the two groups, although the hazard ratio did favour neoadjuvant chemoradiotherapy (0.73, 95% confidence interval (CI) 0.56-0.96, p = 0.025). This study also utilised chemoradiation with gemcitabine, a modality yet to show survival benefit over chemotherapy alone in early pancreatic cancer. Whilst the 5-year overall survival favouring the neoadjuvant chemoradiotherapy arm was clinically meaningful (20.5 % v 6.5%), the 6.5% of patients alive in the upfront surgical arm was substantially lower than what was observed in the CONKO-001 trial of adjuvant gemcitabine (20.7%)[32]. In the PREOPANC trial, patients with T1(<20mm) resectable tumours were excluded, which may account for the poor five-year survival in this cohort. The PREP 02/JSAP05 study used gemcitabine and S1 chemotherapy, a regimen not commonly used outside Japan. The ESPAC-5F trial was a small study of only 90

patients. Finally, the NorPACT-1, the most clinically relevant to our patient cohort in Australia, utilised only four cycles of neoadjuvant chemotherapy, and although there was no significant difference between the two cohorts the longer median survival in the upfront surgery arm, raises doubt of the superiority of the neoadjuvant approach in this cohort[31].

In the absence of a consensus supporting one treatment pathway over another the question remains open - should patients with upfront resectable and/or borderline resectable pancreatic cancer receive either neoadjuvant chemotherapy or upfront surgery?

A biomarker driven approach to assist with this decision is long overdue.

Diagnostic biomarker

The ideal diagnostic biomarker should be able to distinguish pancreatic cancer from benign pathologies such as chronic pancreatitis or precursor lesions such as IPMN or PanIN, with high sensitivity and specificity and be minimally invasive. A high-quality diagnostic marker may prevent unnecessary surgeries in patients with benign disease and may be able to detect pancreatic tumours earlier, in patients with risk factors.

Prognostic biomarker

Treatment for early pancreatic cancer consists of both chemotherapy and surgical resection, which can have significant toxicity. The ability to differentiate which patients may have more aggressive disease, would assist clinicians to determine the optimal treatment pathway for each patient, in addition it may inform clinicians about which patients should receive more intensive therapies. At present, prognosis is determined by stage based on imaging in the initial diagnosis and histopathology on surgical resection[16].

Predictive biomarker

Due to the equipoise between neoadjuvant chemotherapy versus upfront surgery in early pancreatic cancer, biomarkers that may predict for response to chemotherapy may assist clinicians in determining which of the two clinical pathways is more appropriate for the patient in their clinic. In patients with an inherently chemoresistant tumour, early intervention with surgery may be more appropriate whereas in patients with a chemosensitive tumour early exposure to neoadjuvant chemotherapy may produce the best survival outcomes.

The challenge presented in the search for useful biomarkers in pancreatic cancer is that tissue obtained via biopsy at diagnosis is often scant, therefore an ideal biomarker must require small amounts of tissue, or be obtained via less invasive measures such as peripheral blood. In addition, biomarkers must be cost effective and accurate with an emphasis on a rapid turnaround time so that the results can be quickly factored into patients' treatment. This is particularly important in early disease with the aim to start treatment soon after diagnosis.

1.3 Biomarkers in early (upfront and borderline resectable) pancreatic cancer

CA 19.9 (Serum)

Diagnostic, Prognostic, Predictive Biomarker

Serum carbohydrate antigen (CA19.9), also known as sialyl-lewis A, is the most common tumour marker utilised in the diagnosis and management of pancreatic cancer (Table 1.2, Table 1.3). It is commonly secreted in pancreatic malignancies as well as benign conditions of the biliary system including pancreatitis or pre-malignant lesions such as intraductal pancreatic mucinous neoplasm (IPMN)[33]. It is related to the Lewis blood group and 10% of the population are Lewis antigen negative and do not secrete CA 19.9[33].

Despite the high sensitivity and specificity of serum CA 19.9 in symptomatic patients ranging from 79-81% and 82-90% respectfully, the low prevalence of pancreatic cancer, makes it a poor screening and diagnostic test with a positive predictive value of less than 1%[33]. Whilst its use as a single diagnostic biomarker is limited, the positive predictive value for diagnosis of pancreatic cancer is higher when combined with other clinical factors. Ritts et al discovered that an elevated CA 19.9 combined with an image identified pancreatic mass, increased the positive predictive value to 94%[34]. Whereas, Tessler et al demonstrated that the positive predictive value of CA 19.9>37 U/mL increased the sensitivity to close to 100% when associated with clinical and biochemical features including weight loss of more than 10kg and hyperbilirubinemia (>3mg/dL)[35].

CA 19.9 has also been assessed as a marker to assess stage, resectability status and hence prognosis in patients with a confirmed histological diagnosis of pancreatic cancer. Although the data is exclusively retrospective, these studies do demonstrate promise, with higher levels of CA 19.9 associated with more advanced disease at diagnosis and hence worse prognosis[33]. One such study by Humphris et al analysed patients with early disease that underwent curative resection and discovered that patients with a pre operative CA19.9 <120U/ml had a

significantly longer disease free survival compared to those with levels ≥120U/ml at 36 versus 17 months respectively[36]. Post-operative CA19.9 has also been investigated as a prognostic marker, with elevated CA19.9>37 U/mL associated with a poor prognosis and shorter overall survival then patients with a CA 19-9 ≤37U/ml (12 versus 22 months)[36].

Despite the abundance of retrospective reviews illustrating the potential of CA19.9 as a prognostic biomarker there are limitations to its use. At present optimal cut offs for CA19.9 vary between studies. Furthermore, the association between biliary obstruction and raised CA19.9 makes results difficult to interpret, as does the absence of this antigen in the 10% of patients with a Lewis antigen negative phenotype.

Preliminary studies analysing the role of CA19.9 have demonstrated its potential as a predictive biomarker in early disease. Humphris' study of early pancreatic cancer patients, identified the importance of post operative CA19.9 levels in determining benefit of further therapy[36]. In patients with a CA19.9 <90 U/ml administration of adjuvant chemotherapy prolonged survival by nine months, whereas no survival benefit to adjuvant chemotherapy was observed in patients with CA19.9>90 U/ml[36]. Whilst extrapolation of CA19.9 as a biomarker to predict for neoadjuvant chemotherapy response based on this result is inappropriate, it is a hypothesis worth exploring in future prospective studies.

CA19.9 is the most versatile of biomarkers in treatment of pancreatic cancer, despite its lack of validation in all contexts. For patients with upfront or borderline disease, a disproportionately high CA 19.9 (in the presence of a normal bilirubin) may indicate the presence of micro-metastatic disease and hence treatment with systemic therapy early for these patients through the form of neoadjuvant chemotherapy may be more beneficial than upfront surgery.

Table 1.2 Diagnostic, prognostic, predictive biomarkers in early pancreatic cancer

Diagnostic	Prognostic	Predictive
CA19.9	CA19.9	CA19.9
KRAS	KRAS	KRAS
TP53	TP53	CDK2NA
SMAD4	SMAD4	BRCA
CDK2NA	CDK2NA	MMR
Cytokines (e.g., TGF-β, IL-1, IL-6 or IL-10)	NLR, PLR, LMR	mGPS- Albumin, CRP
	mGPS- Albumin, CRP	hENT1
		GATA6
		MicroRNA21

CEA (Serum)

Diagnostic, Prognostic

Carcinogenic embryonic antigen (CEA) is a glycoprotein that is elevated in 30-60% of pancreatic cancer patients[37]. As a diagnostic biomarker for pancreatic cancer, it has limitations with a sensitivity of only 43% and specificity of 82% in early or advanced disease[37, 38]. Despite attempts at increasing sensitivity and specificity using various CEA based panels, the rates remain low, making it an unreliable diagnostic biomarker.

A meta-analysis discovered that for all pancreatic cancer patients (regardless if early or advanced) a higher baseline CEA was associated with a worse prognosis[37]. Two retrospective reviews also demonstrated the use of CEA as a prognostic biomarker to discern between early or more advanced disease after standard staging. Both Van Manen and Fujioka et al discovered that preoperative CEA was significantly higher in patients that had non curative and unresectable tumours at time of operation compared to those that had resectable, margin negative disease[39, 40]. Although, validation in a prospective cohort is needed, the use of CEA as a prognostic biomarker in certain populations is promising.

There is minimal evidence that baseline CEA levels can predict for chemotherapy response in early pancreatic cancer patients. One retrospective review in patients with advanced disease

demonstrated that patients that elevated CEA at baseline predicted for a worse response to palliative chemotherapy. Extrapolation of these results in patients with early disease is challenging as CEA is less likely to be elevated in that cohort[41].

Like CA19.9, higher CEA levels at baseline may indicate more advanced disease, even in patients with radiological determined upfront or borderline resectable pancreatic cancer. As such, for patients with an elevated CEA at presentation, neoadjuvant chemotherapy, may be more appropriate than upfront surgery.

Oncogenes

KRAS (Cytology, Histology, Plasma)

Diagnostic, Prognostic

The *KRAS* gene encodes for the KRAS protein which is a crucial cell membrane protein involved in cell signalling, growth and differentiation[42]. When activated it promotes downstream signalling which leads to activation of the mitogen activated protein kinase (MAPK) pathway[43-45]. A mutation of the KRAS protein results in this pathway being constitutively active, which leads to uncontrolled cell growth[3, 43]. Up to 95% of pancreatic tumours contain a KRAS mutation, the majority of which involve a mutation in codon 12 [43, 46, 47]. The most common KRAS mutation in pancreatic cancer is *KRAS* p.G12D, which is thought to infer a worse prognosis than patient with KRAS wildtype and may be useful biomarker to identify patients that may benefit from early exposure to cytotoxics through neoadjuvant chemotherapy[48-50].

The role of KRAS in tissue in the diagnosis of pancreatic cancer has been debated. It often occurs early in carcinogenesis and is a common mutation in preneoplastic disease such as PanIN or IPMN[2, 3] (Figure 1.1). It may be useful to discriminate between either invasive carcinoma or benign conditions including autoimmune pancreatitis [2]. One prospective study demonstrated that the addition of KRAS testing on EUS-FNA material increased the sensitivity, negative predictive value and accuracy of the cytopathology used to diagnosis pancreatic adenocarcinoma[51] . In this study KRAS mutations were identified in almost all samples (98%), thus making it a feasible addition to the cytopathology from the EUS[51]. At present, use of KRAS mutation testing on EUS-FNA material is not part of routine clinical practice, in part due to lack of standardization of sampling and often insufficient tumour tissue obtained with this approach[51].

In addition to its role as a potential diagnostic marker, many studies have examined the role of KRAS as a potential prognostic biomarker. Despite the discordant results, the majority of studies in patients with localised disease suggest the presence of a KRAS mutation in histological specimens correlates with a worse prognosis[3, 43].

KRAS as predictive marker (to targeted therapy) in pancreatic cancer is limited to the advanced or metastatic setting. Although present in only 1-2% of pancreatic adenocarcinoma, *KRAS* p.G12C has recently been identified as a druggable target, with the introduction of a novel tyrosine kinase inhibitor, sotorasib[52]. In the CodeBreaK trial, patients of various malignancies with a *KRAS* p.G12C mutation were treated with sotorasib. In the 38 advanced or metastatic pancreatic cancer patients enrolled, 21% had a partial response[52]. As such, KRAS may be useful as a predictive biomarker, specifically in the setting of KRAS targeting tyrosine kinase inhibitors, however this is limited to the advanced or metastatic setting. At present there is no role of KRAS in prediction of chemotherapy response and as such there is no role in identifying which patients may be more suitable for neoadjuvant chemotherapy or upfront surgery.

Due to the fact that insufficient tumour tissue for molecular testing is common in pancreatic cancer in addition to the high prevalence of KRAS mutations in pancreatic tumour tissues (>90%), KRAS mutational status on circulating tumour DNA has also been assessed as a diagnostic and prognostic tool with varying levels of success[3, 49, 53]. Early studies used polymerase chain reaction (PCR) methods performed on patient blood samples to assess for the presence of KRAS mutation in plasma of patients with confirmed pancreatic adenocarcinoma and in healthy controls[53]. In these studies, KRAS mutations in patients with confirmed adenocarcinoma plasma ranged from 27-35% [53-55], compared with up to 72% of histological specimens. Although demonstrating early promise, the low sensitivity makes it an unreliable early diagnostic biomarker[55]. Studies are conflicting regarding the role of KRAS in plasma as a prognostic biomarker[53, 54, 56]. In part this conflict is due to variability of detection assays used to measure KRAS mutations in blood.

Validation studies are needed, however KRAS mutation testing may have a role in assisting in diagnosis in patients with early pancreatic cancer with sufficient tumour tissue on endoscopic ultrasound. In addition, the role of plasma KRAS has the potential as a non-invasive prognostic biomarker, once more sensitive assays are developed.

Table 1.3 Genes, proteins, and RNA biomarkers

Genes	Proteins	RNA
	CA19.9	
KRAS	KRAS	
TP53	P53	
SMAD4	SMAD4	
CDKN2A	p16INK4A	
MLH1, MSH2, MSH6, PMS2	MLH1, MSH2, MSH6, PMS2	
	mGPS- Albumin, CRP	
	hENT1	
GATA6	GATA6	
		MicroRNA21
	Cytokines (e.g., TGF-β, IL-1, IL-6 or IL-10)	

TP53 gene (Histology)

Diagnostic, Prognostic

TP53 is the gene that codes for the p53 tumour suppressor protein and is mutated in approximately 50-75% of pancreatic cancers[57-59]. It inhibits cell proliferation and regulates programmed cell death[60]. Mutations in this gene can lead to uncontrolled cell growth and it is found later in pancreatic cancer pathogenesis (Figure 1.1) [58, 61]. It is infrequently mutated in benign conditions such as chronic pancreatitis however its low sensitivity for pancreatic adenocarcinoma makes it a poor diagnostic marker in early disease[58]. Its use as a prognostic biomarker has also been assessed in small retrospective reviews. Of these only two identified a relationship between positive staining for p53 and a poor prognosis, however, only one study was in patients with early pancreatic cancer.[62, 63] The majority of studies identified no role as a prognostic biomarker in patients with early pancreatic cancer[57, 59, 62, 63]. *In vitro* studies have demonstrated that the presence of a *TP53* mutation in cell culture resulted in chemotherapy (gemcitabine) resistance, however further *in vivo* studies are needed to validate these findings before it can be used as a biomarker to predict chemotherapy response[64, 65].

SMAD4 gene (Cytology, Histology)

Diagnostic, Prognostic

The *SMAD4* gene encodes for the SMAD4 protein, a transcription factor protein involved in the TGF-β pathway, which controls signal transduction from cell membrane to nucleus[66]. TGF-

β has biphasic effects on tumour cell function. Initially it is involved in inhibition of tumour cell production by inducing cell apoptosis, however later in tumorigenesis it promotes epithelial to mesenchymal transition (EMT) which results in transition of the tumour cell into a more aggressive phenotype[66-68]. Due to its role as a tumour suppressor, mutations in SMAD4 result in reduction of cell apoptosis and increased cell proliferation.

SMAD4 is mutated in up to 55-60% of pancreatic adenocarcinomas and occurs later in tumorigenesis (Figure1.1)[60, 66]. The most common mutation being gene deletion[69]. Mutations in SMAD4 are uncommon in benign conditions and immunohistochemical testing of SMAD4 on biopsy material, may prove to be a useful adjunct diagnostic marker, with loss of staining more likely to indicate invasive adenocarcinoma [69, 70].

The role of SMAD4 as a prognostic biomarker in patients with early disease is controversial. Two small studies demonstrate conflicting results with regards to SMAD4 mutations. A study conducted of 114 patients that underwent surgical resection demonstrated that patients with a mutated *SMAD4* gene had a worse median survival compared to those with wildtype *SMAD4*[71]. Conversely, in a smaller study of 45 patients that underwent resection, a mutation in the *SMAD4* gene resulted in an improved overall survival[60]. To resolve this conflict, a meta-analysis involving patients with both early and advanced disease was conducted, in this analysis, patients with early disease, the presence of a *SMAD4* mutation was associated with worse prognosis and a hazard ratio of 1.5, however this was not evident in patients with more advanced disease [72].

Preliminary cell culture studies, have revealed that SMAD4 deficient cell lines have an increase sensitivity to cisplatin and irinotecan, thus offering a very early insight into SMAD4 mutations as a biomarker to predict for chemosensitivity[73]. In addition, both *in vitro* and mouse studies have demonstrated that presence of SMAD4 mutation, may predict for radiotherapy resistance [74]. These studies have yet to be validated in humans.

<u>CDKN2A gene (Histology)</u>

Diagnostic, Prognostic, Predictive

The *CDKN2A* gene encodes for two proteins $p16^{INK4A}$ (p16) and $p14^{ARF}$ with the former, the more common in pancreatic cancer[75]. The p16 protein prohibits cell progression from the G1 to the S phase, hence arresting cell growth[76, 77]. Whilst KRAS mutations occur early in the pathogenesis of pancreatic cancer and *TP53* and *SMAD4* mutations occur later, *CDKN2A*

mutations occur as an intermediate event[76-78] (Figure 1.1). *CDKN2A* mutations occur in 30-50% of pancreatic cancer cases[2, 79, 80].

A metanalysis was conducted to assess the role of CDKN2A mutations (in either tissue, blood, or pancreatic juices) in pancreatic cancer. In the 14 studies analysed, *CDKN2A* was significantly higher in patients with pancreatic cancer then in healthy controls (OR 17.2,p<0.00001), however the low sensitivity (41%) and positive predictive value (58%) make it an unsuitable diagnostic biomarker[81].

Chen et al assessed the potential of p16 as a prognostic biomarker in a retrospective analysis of 88 patients with early cancer treated with neoadjuvant chemoradiation[82]. In this study the presence of a p16 mutation led to shorter recurrence free survival. Whilst promising, this was in contrast with several retrospective studies including a metanalysis by Gu et al, which found with no association between mutation and a worse prognosis[83-85].

The evidence for CDKN2A as a potential predictive biomarker is limited to one retrospective review. Chen et al analysed the role of CDKN2A as a predictive biomarker in patients with early (resectable) disease receiving gemcitabine chemoradiation and found that the presence of a CDKN2A mutation did not predict for treatment response[82, 85, 86].

DNA Repair genes: Breast cancer gene 1 and breast cancer gene 2 (*BRCA1* and *BRCA2*) (Histology, Plasma)

Predictive

DNA repair in the presence of cellular insult (exogenous or endogenous) is conducted by the homologous repair genes and up to 14.5-16.5% of patients diagnosed with pancreatic cancer, are found to have either a germline or somatic mutation in one of these genes[87]. The most frequent mutations are *BRCA1* and *BRCA2* (0.9 and 3.5%), ataxia telangiectasia (*ATM* 2.2%) and partner and localizer of BRCA2 (*PALB2* 0.2%)[87]. Germline mutations are tested using a plasma test, whereas somatic mutations are tested on tumour tissue.

The presence of a mutation in one of the homologous repair genes, predicts for response to drugs that induce double or single stranded breaks in DNA, the most common being platinum chemotherapy or poly-ADP ribose polymerase (PARP) inhibitors[87]. FOLFIRINOX, one of the two main neoadjuvant regimens used in pancreatic cancer contains oxaliplatin. The use of

BRCA as a predictive biomarker in patients receiving platinum chemotherapy has been well studied in a range of different cancer types, most notably breast and gynaecological malignancies[88]. In the metastatic pancreatic cancer setting, the POLO1 trial investigated the role of a maintenance PARP inhibitor following treatment with a platinum chemotherapy in patients with a germline *BRCA1* or *BRCA2* mutation[89]. This study revealed a significant increase in progression free survival in patients treated with a PARP inhibitor vs placebo (7.4 vs 3.8months), however, did not demonstrate increase in overall survival[89-92].

Studies assessing the role of *BRCA* mutation to predict chemotherapy response in early (upfront and borderline resectable) pancreatic cancer are confined to small retrospective cohort analyses. In part this is due to a lack of testing in this population. One retrospective review of 61 patients with upfront or borderline resectable pancreatic cancer patients with known *BRCA* mutations, treated with neoadjuvant FOLFIRINOX, identified significantly higher rates of histological complete response in the patients with a germline *BRCA* mutation (44%) compared to the *BRCA* wildtype cohort (10%)[93]. Patients with *BRCA* mutations also went on to have a longer disease-free survival[93].

Although validation is needed, there is a potential role of *BRCA* as a predictive biomarker in patients with early or borderline resectable pancreatic cancer to help decide if upfront surgery versus neoadjuvant chemotherapy may be more appropriate. With an increased sensitivity to platinum chemotherapy, the presence of a BRCA mutation may make early treatment with a platinum chemotherapy (FOLFIRINOX) more appealing than upfront surgery. There are, however, limitations to its use. At present germline testing from plasma is offered only in patients with a significant family history and somatic testing on tumour tissue is limited to the advanced or metastatic setting.

<u>Mismatch repair proteins (MMR) (Histology)</u>

Predictive

The four genes and corresponding proteins that govern mismatch repair (MMR) in humans include mutL protein homolog 1 (MLH1), mutS homolog 2 (MSH2), mutS homolog 6 (MSH 6) and postmeiotic segregation increased 2 (PMS2)[94]. These genes have been extensively studied in a range of cancer types, most prominent of which is colorectal cancer[94]. Their function is to correct any DNA base mismatch that may have occurred during DNA replication[95]. Tumours with a loss of function of one or more of these proteins is labelled

MMR deficient (dMMR) and occurs in 1-2% of patients with pancreatic cancer[96-98]. Germline mutations of one of these g is seen in patients with Lynch syndrome. Unlike their MMR proficient (pMMR) counterparts, tumours that have dMMR develop hundreds of somatic mutations, which encode potential neoantigens[94]. These neoantigens serve as a potential target for treatment with immunotherapy. Testing of MMR proteins in pancreatic cancer is performed on the tumour tissue either by immunohistochemistry or polymerase chain reaction (PCR) to detect amplified microsatellite loci[99, 100]. Success of immunotherapy in targeting dMMR tumours became apparent through the results of the KEYNOTE 158 trial[94]. This phase 2 single arm- pan tumour study assessed response of patients with dMMR tumours to pembrolizumab, after failure of standard therapy, highlighting the role of dMMR as a potential biomarker to predict response to immunotherapy. This study included 22 pre-treated stage IV pancreas cancer patients with dMMR and found an objective response rate of 18% with one patient undergoing a complete response[94]. Although a single arm trial without comparator, these response rates are higher than expected in a heavily pre-treated metastatic pancreatic cancer cohort. At this stage, however, use of mismatch repair status to predict for immunotherapy response is restricted to advanced disease due to the lack of data in the early pancreatic cancer.

Preliminary studies have also been conducted assessing the role of mismatch repair status as a predictive biomarker for chemotherapy response with encouraging results[101]. Riazy et al demonstrated that in patients with early disease undergoing upfront resection the survival benefit of adjuvant therapy was dependent on the underlying mismatch repair status[102]. In patients with pMMR tumours, the addition of adjuvant chemotherapy (with a pyrimidine analogue) significantly increased survival in patients that underwent surgical resection, however this survival advantage could not be demonstrated in patients with dMMR tumours[102]. The lack of survival benefit in the dMMR cohort may reflect innate chemoresistance from dMMR tumours, a feature seen in colorectal cancer[45]. Although prospective studies are needed for validation, this early data may indicate the potential for dMMR as a useful predictive chemotherapy biomarker. For patients with early disease with dMMR staining, treatment with upfront resection may be more appropriate than treatment with neoadjuvant chemotherapy.

The literature surrounding use of mismatch repair as a prognostic biomarker is early disease is conflicting. Nakata Et al demonstrated a survival benefit in 46 pancreatic cancer patients that underwent resection and found that the 17% of patients with a mismatch repair deficient phenotype had a significantly longer survival time[103]. In contrast, Lupinacci et al analysed 445

pancreatic adenocarcinoma patients and there was no survival difference in the 1.6% with dMMR[104]. In both studies, chemotherapy data was lacking.

Inflammatory markers- neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), lymphocyte monocyte ratio (LMR) (Plasma)

Prognostic

Cancer associated inflammation is now an established hallmark of cancer[105]. Inflammation is a key risk factor in pancreatic cancer with a fivefold increase in risk of pancreatic cancer in patients with chronic pancreatitis[106]. Inflammation occurs initially through the innate immune system, as monocytes and neutrophils migrate to the damaged tissue and release proinflammatory cytokines (including tumour necrosis factor (TNF), interleukins and chemokines[46, 107]. Many of these cytokines (CXCR1,2 and 4) create a positive feedback loop and recruit additional neutrophils to the site of inflammation[108]. After the initial immune response, the cells then activate the adaptive immune system which if unchecked can lead to fibrosis and metaplasia[107]. After the tumour is established there is an ongoing dynamic relationship between the cancer and the body's immune system, with inflammation a core component in the microenvironment of most neoplastic tissues[105]. In recent years the role of neutrophils in the promotion of carcinogenesis has become more apparent. In pancreatic cancer, high neutrophil infiltration into the tumour is associated more aggressive disease[109, 110]. In one study of 102 patients, a high level of CD177 (a protein associated with neutrophil activation) expression in pancreatic surgical specimens was associated with a significantly worse prognosis[111].

In addition, NLR has been studied to assess its role as a potential prognostic biomarker[112]. A meta-analysis conducted on 1804 patients discovered higher NLR in patients with pancreatic cancer, was associated with reduction in overall survival, with a hazard ratio of 2.6 (95%CI: 1.68-4.06, $p<0.001$)[112]. This analysis had only one study of patients with early disease, and this study demonstrated the prognostic ability of NLR was attenuated in this cohort with a hazard ratio of 1.2, and just meeting significance (95% CI, 1.00-1.44, $p=0.048$). In patients with advanced disease that underwent mixed modality treatment or patients that received palliative chemotherapy alone, higher NLR predicted for significantly shorter survival with a hazard ratio of 4.4 (95% CI, 2.50-7.61, $p<0.001$) and 2.1(95% CI, 1.49-2.90, $p<0.001$) respectively indicating that NLR ratio may be of most value as a prognostic biomarker in patients with advanced disease[112]. Stotz et al, similarly identified that a NLR >5 in patients at baseline was

associated with shorter survival both in patients with early disease that underwent surgery (HR 1.6, 95% CI, 1.02-2.53, p=0.04)) and in patients with advanced disease (HR 2.5, 95% CI, 1.64-3.91, p<0.001), with the hazard higher in the latter[113]. Other inflammatory blood tests including PLR and LMR have also been studied. Two meta-analyses have found that both higher PLR and lower LMR were associated with shorter survival, in patients with upfront or borderline resectable pancreatic cancer[114, 115].

There are limitations to the use of these inflammatory tests as potential prognostic biomarkers. Firstly, there is lack of consensus as to what a 'high' versus 'low' NLR cut-off should be with considerable heterogeneity between studies[112, 113]. In addition, the levels of these inflammatory markers in the peripheral blood are often dynamic and change with other stimuli, including infection, chemotherapy, and supportive therapy (granulocyte-colony stimulating factor (G-CSF)).

Modified Glasgow prognostic scale (mGPS) (Serum)

Prognostic, Predictive

Other key mediators in the cancer-inflammatory relationship are C-reactive protein (CRP) and albumin. During an immune response, levels of CRP increase, and albumin levels decrease[116]. Forrest et al first established the Glasgow prognostic score (GPS) in 2003[117]. Patients are accorded a score from 0-2, with 1 point for an elevated CRP (>10mg/l) and 1 point for a reduced albumin (< 35g/l)[117]. This has since been updated to a modified GPS (mGPS). There have been several studies that have demonstrated that higher mGPS is associated with a poorer prognosis in patients with early disease. Yamada et al analysed mGPS in resected pancreatic cancer patients and discovered that those a higher mGPS was associated with a median overall survival of only 17 months compared to 28 months in those with a low mGPS[118]. A meta-analysis revealed that higher GPS was strongly associated with worse outcomes in patients with pancreatic cancer, however survival difference was only evident in patients with advanced disease[119].

At present, use of mGPS as a marker to predict chemotherapy response has not extensively been studied however one retrospective study of 56 patients revealed that in patients treated with neoadjuvant chemotherapy, a higher baseline mGPS was associated with a worse histological response[120].

Cytokines (Serum)

Diagnostic

Cytokines are important mediators within the pancreatic tumour microenvironment. They are produced by both cancer and stromal cells and can be pro or anti-inflammatory. Cancer associated fibroblasts (CAFs) are key cells in this microenvironment and produce a large proportion of extracellular matrix proteins. They can be activated by TGF-β, IL-1, IL-6 or IL-10. At present there is conflicting evidence regarding their role as diagnostic biomarkers, with some studies suggesting overexpression and some studies demonstrating under expression in patients with pancreatic cancer compared to controls [121, 122]. This discordance is similarly observed when individual cytokines are interrogated as prognostic biomarkers in patients diagnosed with pancreatic cancer[123].

Human equilibrative nucleoside transporter 1(hENT1) (Histology)

Predictive

Human equilibrative nucleoside transporter 1 (hENT1) is a transmembrane protein that transports nucleoside like drugs, into the cells[70]. In vitro studies have demonstrated that gemcitabine, utilises hENT1 to gain entry to cells where it has its cytotoxic effect [124]. In 2004 the first *in vivo* study was conducted to assess the relationship between the presence of hENT1 and response to chemotherapy[125]. Shortly after, Spratlin et al discovered that the absence of hENT1 was associated with poorer survival in patients with advanced pancreatic cancer[125]. Studies conducted in early pancreatic cancer including subgroup analysis from RTOG9704 and ESPAC-3 trials confirmed these findings. In the RTOG9704 trial, in patients receiving adjuvant gemcitabine, the presence of hENT1 in the surgical specimen was associated with an improved overall survival and a hazard ratio of 0.5 (95% CI, 0.29-0.73, p=0.57)[126]. For patients receiving 5 Fluorouracil (5-FU, an anti-metabolite chemotherapy), there was no difference in survival based on hENT1 expression. Similar results were appreciated in the ESPAC-3 study with a hazard ratio of 0.6 (95% CI, 0.43-0.83, p=0.002) for high hENT1 expression in patients treated with gemcitabine[127]. In contrast, one small study of 63 patients treated with neoadjuvant chemoradiation with gemcitabine, the presence of hENT1 expression in the surgical specimen was not associated with survival[128]. This lack of result may be due to testing on tumour that has already been exposed to gemcitabine, or due to the small sample size of only 63 patients.
In contrast Perera et al analysed hENT1 expression on diagnostic biopsies (rather than surgical resections) in patients with advanced disease[129]. Using RNA sequencing hENT1 high

expression was associated with an improved overall survival in patients treated with gemcitabine, compared to hENT1 low expression, at 10.6 versus 6.7 months ($p<0.001$). This survival advantage of hENT1 could not be appreciated in the patients that received FOLFIRINOX[129]. As the treatment paradigm shifts towards more neoadjuvant therapies in pancreatic cancer, the role of hENT1 in biopsy samples as a predictor of chemotherapy response in patients with early disease will have to be further investigated.

GATA6 (Histology)

Predictive

GATA 6 is a transcription factor, from the *GATA6* gene[130]. Although varying mechanisms have been proposed for the role of GATA6 in pancreatic cancer, the Collison et al classification and subsequent molecular studies have demonstrated that GATA6 inhibits de-differentiation of epithelial-mesenchymal transition (EMT), a fundamental step in metastatic spread[6, 130]. At present there are early studies demonstrating its role as a potential biomarker to predict response to 5-FU, one of the key drugs utilised in management of early-stage disease[130]. Martinelli et al demonstrated in a cohort of ESPAC-3 patients that GATA6 expression strongly predicted for survival in patients treated with adjuvant 5-FU, with a median survival of 27 months in patients with high expression versus 14 months with low expression ($p=0.018$). This survival difference in GATA expression levels was not detected in patients treated with adjuvant gemcitabine[130]. Other small retrospective cohort studies have demonstrated similar predictive capabilities of GATA6[131].

MicroRNAs (Histology)

Predictive

MicroRNAs (miRNA) are small non coding RNAs that are involved in the regulation of gene expression[132]. In recent years, the emphasis has been on the role of miRNA in oncogenesis and chemoresistance[132-134]. Of these studies, a number of miRNAs have been postulated to be involved in chemoresistance in pancreatic cancer, the most well established being miRNA21[132]. Hwang conducted a retrospective analysis of early pancreatic cancer patients and found that high levels of miRNA21 expression was associated with reduced survival (15.6 compared to 24.4 months in low levels, $p=0.006$), in patients receiving adjuvant chemotherapy (either gemcitabine or 5-FU) compared to patients with low expression[132]. This survival advantage was not evident in patients that did not receive adjuvant chemotherapy, suggesting its role in chemoresistance[132]. The potential for miRNA21 as a prognostic marker was also

identified in a metanalysis, with high expression associated with increased hazard ratio of 2.7 (95%CI, 2.06-3.43, p<0.001) [133].

1.4 Conclusion

In the absence of consensus in phase 3 studies the optimal upfront treatment in patients with upfront and borderline resectable disease remains a contentious issue. Whilst neoadjuvant chemotherapy remains an attractive option in a disease is that is widely thought of as systemic from the onset, the recent results of the NorPACT-1 trial lends doubt to this approach. Due to these conflicting results, and due to the poor overall survival in pancreatic cancer there is a need to identify biomarkers that can assist to personalise the treatment of pancreatic cancer. Biomarkers are required for both prognosis and to predict response to treatment, thus allowing clinicians to identify if upfront surgery or neoadjuvant chemotherapy is more appropriate for the given patient in the clinic.

The aims of this thesis are to

1)Evaluate if either neoadjuvant chemotherapy or upfront surgery results in increased survival in our patient cohort of upfront resectable pancreatic cancer
2) Identify potential prognostic or predictive biomarkers that could assist in the management of patients with pancreatic cancer and to determine the best method to assess these biomarkers.

CHAPTER 2:

Materials and Methods

2.1 Patient samples

All experiments conducted were approved by the NSLHD ethic committee. Written informed consent was obtained for all patient specimens (2019/ETH08639). Serum samples were obtained through tumour bank at RNSH, on the day of surgery. For this thesis, plasma samples were obtained on the day of surgery. Biopsy samples for proteomics (Chapter eight) were obtained from endoscopy and stored as fresh frozen samples. Biopsy samples for Chapter five were obtained from anatomical pathology department at RNSH. For these samples, as per the Human Tissue Act 1983, 'a waiver of consent can be granted for a human research ethics committee if tissue was removed for purpose other than research and is now to be used for research and is currently held in a tissue block or slide'. Surgical samples were obtained from pathology and were stored either as fresh frozen samples (for proteomics) or as formalin fixed paraffin embedded (immunohistochemistry).

Patients were identified from 2013 through to 2020. All patients underwent surgery at Royal North Shore hospital or North Shore Private hospital. Chemotherapy, pathological and clinical data was obtained from hospital records.

2.2 Retrospective cohort analyses (Chapter 3 and Chapter 8)

2.2.1 Patient cohort

Methods and materials are included in these chapters for to assist with interpretation of results. For both chapters data was obtained using prospectively collected chemotherapy and surgical databases on patients diagnosed with pancreatic cancer, at our centre from 2013-2020.

2.2.2 Statistic analysis

Statistical analysis was performed using IBM (SPSS Version 27). Survival was performed using cox proportional hazards or log-rank analyses. Univariate analysis using the chi-squared test was performed on categorical data and spearman's rank analysis was performed on non-parametric variables to assess correlation. One-way analysis of variation (ANOVA) was performed to assess baseline differences in continuous variables between groups.

2.3 Proteomic discovery (Chapter 4):

2.3.1 Specimen collection

Surgically resected tissue specimens were collected by our research team and stored as frozen tissue at -80°C. Proteomic discovery was performed using a liquid chromatography mass spectrometry/ mass spectrometer (LC-MS/MS) (Kolling Institute, University of Sydney).

2.3.2 Sample preparation

Surgical samples were lysed in 1% of sodium deoxycholate in 100mM of triethylammonium bicarbonate. They then underwent homogenisation using a bead-mill before incubation at 100°C for five minutes. Samples were then treated with a probe sonicator to allow further protein release (5 sonication cycles for 15 seconds). Protein concentrations were measured using BCA test (Thermofisher). Proteins were reduced and denatured with the addition of 20mM of dithiotheitol (DTT) and incubated at 56°C for 30 min, prior to alkylation with 60mM of 2- iodoacetamide (IAA). Proteolytic digestion was performed with the addition of Trypsin at a concentration of 1:100 and then underwent a 12-hour incubation at 37°C. Formic acid (FA) was added at a concentration of 1:50 prior to centrifugation at 21,000 rpm for ten minutes. The supernatant was then collected, and peptides were desalted utilising a reversed phase column (SepPak C18 cartridge, Waters), as per protocol. Following this, the peptides were lyophilized using a vacuum centrifuge.

2.3.3 Sample injection and separation

In the reverse phase column, peptides up to 1µg were eluted at 60°C. Peptides were loaded into the column at 2% buffer (80% acetonitrile (ACN) and 0.1% FA. The buffer concentration was increased to 20% at 66 min, 45% at 87min and 95% at 103min. Buffer was then reduced back to 2% for fifteen minutes at a flow rate of 300nL/min.

2.3.4 Library creation

EncylopeDIA v0.9 was used to generate a list of peptides. A charge state of 2 and normalized collision energy of 33 was utilised. A limit of 1 missing tryptic cleavage was permitted. A spectral library was created using six phase gas separations of the pooled sample, the Prosit proteomics library and FASTA database, using the following settings: the acquisition type was set to data independent acquisition (DIA) with a normal target decoy approach, the higher-

energy-c trap dissociation was used for fragmentation using y and b ions, the mass tolerance was set to 10ppm and the number of quantitative ions were set to between 3-5.

DIA was used to generate a dataset of all the ions detected. Both wide and small windows were used to allow for an increase in sensitivity and specificity. Two cycles were run through with overlapping m/z windows. 1800V was applied to allow for ionisation of the samples prior to capillary transfer (set at 250^{o}C). The library previously established was then used to identify proteins isolated in the sample.

2.3.5 Acquisition

One full scan followed by 50x25m/z DIA windows generated the data for the wide and small windows with a range of 395-1000m/a for wide windows and a split range in small windows (300-400, 500-600, 700-800...) up to six parts. Using the spectral library already obtained, DIA window data was analysed. All data was converted into Skyline files and ProteinProphet software was used to determine false discovery rate (FDR) of individual peptides.

2.3.6 Statistical Analysis

Pancreatic tumour proteins were normalised by comparing to the matched normal pancreas proteins and data was analysed using multiple t-tests in and area under the receiver operating characteristic (AUROC) in GraphPad prism (GraphPad Software, San Diego, California, 9th edition). Multivariate analysis was performed using partial least squares using JMP® 17th edition.

2.4 Ingenuity pathway analysis (IPA, Chapter 4)

Results from multiple t-tests from mass spectrometry were inputted in ingenuity pathway analysis (QIAGEN Inc., https://www.qiagenbio- informatics.com/products/ingenuity-pathway-analysis). Using IPA software, upstream or downstream regulators that may have resulted in the observed protein changes were identified. These results were then divided into highest activation z-score (a statistical measure of match between expected relationship from known literature and observed protein expression in our samples) and p-value of overlap. The p-value of overlap defined the overlap between observed dataset and regulator molecules and values <0.01 were considered significant. These regulators were then reviewed in the literature, and two were chosen for validation experiments.

2.5 Immunohistochemistry (Chapters 4 and 5)

2.5.1 Sample collection

(a) TGM2 and OSMR: Only surgical samples were used in this experiment. Samples were collected by our research team at the time of surgery. They were placed in formalin for 24 hours prior to temporary storage in 70% ethanol, before paraffin embedding.

(b) Mesothelin, CA125 and S100A4: Biopsy and matched surgical specimens of patients that were diagnosed with non-metastatic pancreatic cancer were obtained from anatomical pathology department at RNSH. Surgical and biopsy samples were fixed using formalin and were embedded into paraffin.

2.5.2 Sample preparation

Four µm slices of tissue blocks were cut using a microtome and placed onto glass slides (Trajan Series 2, adhesive microscope slides). All samples were de paraffinized and rehydrated using a sequence of xylene and ethanol solutions. For all experiments, tissue sections were incubated overnight with primary antibody in sequenza prior to addition of secondary antibody. ImmPACT Novared® was used as the chromogen for detection, the reaction was stopped with slide insertion into water.

2.5.3 Optimization

The following was performed for all optimizations. The tissue used, antigen retrieval methods and concentrations are listed in Table 2.1. CA125 was optimised with and without a linker. Dual link HRP was used when linker was added. The primary antibody for OSMR was Abcam, (Ca # ab210771, 3.6mg/ml). This was optimised but did not proceed to experimentation.

Following rehydration, a heat induced epitope antigen retrieval method (HIER) was adopted by placing samples in an antigen retrieval solution (Dako Target retrieval solution) in hot water bath (95- 100℃) for twenty minutes. Endogenous peroxidase was blocked by incubating slides in hydrogen peroxide for five minutes (chem-supply hydrogen peroxide 30% w/w). Sections were incubated at varying concentrations of primary antibody (see Table 2.1) at 4 degrees overnight (16 hours). A negative control at highest concentration was also added (see Table 2.2). Following incubation, a secondary antibody was added to samples (Table 2.1).

ImmPACT® Novared was added for fifteen minutes and then placed promptly in water. Finally slides were counterstained using haematoxylin before dehydration with sequential ethanol concentrations followed by xylene. Coverslips were added and allowed to dry.

Table 2.1 Tissues, concentrations, and conditions for optimization.

Antibody	Tissue	Concentrations	Antigen Retrieval method	Linker	Secondary Antibody
TGM2	Colon, lung and pancreatic cancer	1:50, 1:100, 1:150	HIER pH 6	No	Dako EnVision+ anti- rabbit HRP
OSMR	Brain and lung cancer	1:200, 1:400, 1:800, 1:1600	HIER pH 6 and pH 9	No	Dako EnVision+ anti- rabbit HRP
Mesothelin	Lung cancer, mesothelioma and thyroid cancer	1:20, 1:40, 1:50, 1:80, 1:100, 1:200, 1:400, 1/800	HIER pH 6	No	Dako EnVision+ anti-mouse HRP
CA125	Lung cancer, mesothelioma, and pancreatic cancer	1:100, 1:200, 1:400, 1:500, 1:750, 1:100	HIER pH 6	With and without EnVision mouse linker (SM804)	Dako EnVision HRP+ anti mouse for samples without linker, dual link Dako HRP+ anti-rabbit/mouse for samples with linker.
S100A4	Speen and pancreatic cancer.	1:500, 1:750, 1:1000	HIER pH 6	No	Dako EnVision+ anti- rabbit HRP

2.5.4 Experiment

Final dilution of antibody is listed in Table 2.2. Both biopsy and surgical specimens had the same dilution.

OSMR was not used due to lack of staining in positive controls.

Table 2.2 Antibodies used in Chapters 5 and 6.

Antibody	Company and concentration	Mouse/Rabbit Clonality	Antigen Retrieval method	Dilution	Chromogen	Negative control
TGM2	Cell signalling D11A6, 17µg/ml (cat #3557S)	Rabbit monoclonal	HIER pH 6	1:50	ImmPACT ® Novared	Rabbit (cell signalling, 2.5mg/ml, 1:50)
Mesothelin	Novocastra 5B2, 40µg/ml (Cat # MESO-L-CE	Mouse monoclonal	HIER pH 6	1:20	ImmPACT ® Novared	Mouse IgG1 (Cell signalling 0.25mg/ml, concentration at 1:20)
CA125	Dako M11, 452.5µg/ml (Cat # GA701)	Mouse monoclonal	HIER pH 9	1:200	ImmPACT ® Novared	Mouse IgG1 kappa (Abcam 0.5mg/ml, 1:100)
S100A4	Dako A5114, 1000µg/ml (Cat # 13018S)	Rabbit polyclonal	HIER pH 9	1:1000	ImmPACT ® Novared	Rabbit IgG (Dako 15mg/ml, 1:500)

2.5.5 Scoring

Scoring was performed by two independent observers, blinded to patient outcome. In the event of a discordance, a resolution was reached through discussion of the sample. The scoring system was unique to each antibody and included both distribution and intensity (Table 2.3). After review of the distribution of staining and small sample size, dichotomous scoring system was adopted (Table 2.4).

Table 2.3 Scoring system for antibodies.

Antibody	Localisation	Scoring
TGM2 surgical Tissue	Nucleus Cytoplasm Membranous Extracellular space Mitochondria	Intensity Ductal 1 Weak staining of ductal cells 2 Intense staining of ductal cells Stromal 1 Weak staining of stromal cells 2 Intense staining of stromal cells
Mesothelin Biopsy and surgical tissue	Membranous Extracellular space (secreted)	% of cells positive staining 0 Negative 1 <25% 2 25-50% 3 >50%
CA 125 Biopsy and surgical tissue	Membranous Extracellular space (secreted)	% of cells positive staining 0 Negative 1 <10% 2 10-50% 3 >50%
S100A4 Biopsy and surgical tissue	Nucleus Cytoplasm Membranous	% of cells positive staining 0 Negative 1 Cytoplasm only 2 Cytoplasm and nuclear <25% 3 Cytoplasm and nuclear >25%

Table 2.4 Revised scoring system.

Antibody	Scoring
TGM2	0 Weak staining of either ductal and stromal cells or both 1 Intense staining of both ductal and stromal cells.
Mesothelin Biopsy and surgical tissue	0 Negative 1 Any positive staining
CA 125 Biopsy and surgical tissue	0 Negative or <10% of cells staining. 2≥10% of cells staining
S100A4 Biopsy and surgical tissue	0 Negative or cytoplasm staining only. 1 Positive cytoplasm and nuclear staining

2.5.6 Statistical analysis

All statistical analysis was performed using IBM (SPSS Version 27). Log rank and cox hazards method were used to evaluate survival. Chi-squared was used to compare dichotomous groups. Cohens kappa was used to evaluate the relationship of binary data.

2.6 Cell culture (Chapter 4)

2.6.1 Cell lines

PANC-1 and MIA Paca-2 gemcitabine resistant cells were developed by exposing wildtype cells to sequential increasing concentrations of gemcitabine up to 5 or 50 μM, respectively. The concentration which 50% reduction in cellular viability was observed (IC_{50}), was established. The IC_{50} for PANC-1 wildtype was 42.9nM, versus 690μM for PANC-1 gemcitabine resistant. The IC_{50} for MIA Paca-2 was 3.22 nM for wildtype and 45.96 μM for gemcitabine resistant cells.

PANC-1 and MIA Paca-2 cells and their gemcitabine resistant counterpart cells were cultured in DMEM media which consisted of 1% foetal calf serum and 10% penicillin streptomycin. Cells were stored in a 37°C with 5% carbon dioxide (CO_2, Table 2.5).

2.6.2 MTT Proliferation assay

A cell viability assay was performed at 500, 1000, 2000, 4000 and 8000 cells each well in 150μl of DMEM. After 96 hours 50μl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was added to each well. After 2 hours incubation MTT was

removed and dimethylsulfoxide (DMSO) was added prior to being placed on the plate reader. Absorbance values were collected and plotted using GraphPad prism (GraphPad Software, San Diego, California, 9th edition). Simple linear regression was performed with 4,000-8000 cells giving an R value of >0.9. Five-thousand cells were chosen for further experiments.

2.6.3 siRNA knockdown

A 96 plate well was used for cell culture experiments. Five-thousand cells (using cell types in Table 2.5) were plated in 125μl OptiMEM media. 100μM of siRNA (TGM2, OSMR or Control, Table 2.6) in 12.5μL of OptiMEM media was added to a solution of 12.5 μl lipofectamine (19.6μl per 1000μL of OpiMEM). Outside wells had 150μl of sterile water added. Plates were then incubated at 37°C for 96 hours in an incubator. 50μl of MTT was added for two hours prior to being removed and 200 μl of DMSO was added and placed on the plate reader at 570nm wavelength. Absorbances were measured and data was analysed in GraphPad prism (GraphPad Software, San Diego, California, 9th edition). This experiment was repeated x3. TGM2 and OSMR results were normalised to control siRNA and the average of all three experiments was used for analysis.

Table 2.5 Cell types used in siRNA experiment.

Cell line	Media
PANC-1 Wildtype	450ml of DMEM media + 45ml of Foetal calf + 5ml penicillin streptomycin
PANC-1 Gemcitabine resistant	450ml of DMEM media + 45ml of Foetal calf + 5ml penicillin streptomycin
MIA Paca-2 Wildtype	450ml of DMEM media + 45ml of Foetal calf + 5ml penicillin streptomycin
MIA Paca-2 Gemcitabine resistant	450ml of DMEM media + 45ml of Foetal calf + 5ml penicillin streptomycin

Table 2.6 siRNA used in cell culture.

siRNA	Brand
TGM2	TGM2 siRNA: ID:S14087 (ThermoFisher/Ambion, Cat # 4390826)
OSMR	OSMR siRNA: ID: S17542 (ThermoFisher/Ambion, Cat # 4390824)
Standard	Negative Control siRNA: (ThermoFisher/Ambion, Cat # AM4637)

2.6.4 Statistical analysis

One way ANOVA was performed comparing both TGM2 and OSMR siRNA knockdown to control siRNA in GraphPad prism (GraphPad Software, San Diego, California, 9th edition).

2.7 Western blot (Chapter 4)

2.7.1 Sample preparation and western Blot siRNA knockdown

Cells were cultured in 6 well plates in DMEM media and incubated at 37°C at 5% CO2 for 96 hours prior to use in experiment, after siRNA knockdown. For Western blot where OSM (295-OM/CF, R&D, Cat # 295-OM-010/CFF) was used, this was added to the cells at 24 hours. Following incubation, the plate was transferred to ice and the DMEM media was removed, and phosphate buffered saline (PBS) was added and kept on ice. Following this, PBS was removed and replaced with a lysis buffer consisting of 10mM Tris HCL buffer, 150mM NaCl, 0.5% sodium dodecyl sulfate-polyacrylamide (SDS), 1% Triton x-100, 1mM EDTA, 0.04mM NaF, H20, protease inhibitor cocktail (+phosphatase inhibitor cocktail, when phosphorylated proteins were used). The lysates were transferred to an Eppendorf tube®, followed by sonication and centrifugation (14,000 rpm, 40 minutes at 4°C). The supernatant was transferred to a new tube for protein estimation using the Pierce™ BCA protein assay protocol.

2.7.2 Gel preparation and western blot

The gel was prepared using water, resolving buffer, 30% acrylamide, 10% ammonium persulfate (APS), and 10% tetramethyl ethylenediamine (TEMED). Proteins were added to the gel and a charge of 80V was applied and gradually increased. To allow transfer, filter paper and sponges were soaked in the transfer buffer. The polyvinylidene fluoride (PVDF) membrane was placed in 100% methanol for 45 seconds. The gel was removed and soaked in the transfer buffer before assembling the gel, filter papers and sponge in a stack. 30V was then run through the stack at 4°C overnight to allow for the transfer of proteins from the gel to the membrane.

The membrane was then treated with 100% methanol for ten minutes before incubation at 4°C with the primary antibody (Table 2.7). Following incubation, the membrane was washed with TBST (tris-buffered saline (TBS) and polysorbate 20/L). The secondary antibody was added, and the membrane was incubated for one hour. Following incubation, the western blot detection reagent (Immobilon forte- western HRP substrate) was added. The western blot was then imaged and analysed using the ChemiDoc MP imaging system (BioRad). All antibodies and cell lines were performed in duplicate.

Table 2.7 Antibodies used in western blot.

Antibodies	Brand
TGM2	TGM2 (D11A6) XP® Rabbit mAb (Cat #3557S)
OSMR	Anti-OSMR antibody (Cat #ab210771)- discontinued and replaced with Anti-OSMR antibody (Cat # ab282577)
STAT 3	Stat3 (124H6) Mouse mAb (Cat #83541)
MAPK	p44/42 MAPK (L34F12) Mouse mAb (Cat #4696)
p-MAPK	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb (Cat #9101)
AKT	Akt (40D4) Mouse mAb (Cat #2920)
p-AKT	Phospho-Akt (Thr308) (D25E6) XP Rabbit mAb (Cat #13038)

2.8 MicroRNA (miRNA, Chapter 6)

2.8.1 Sample collection

Plasma was obtained from patients at time of surgery after completion of chemotherapy and stored in the Bill Walsh pancreatic research laboratory. Specimens were collected and transported in an EDTA tube from theatre to the Bill Walsh Lab for immediate processing, after consent was obtained. Plasma samples were then centrifuged at 1200g for ten minutes at room temperature. Plasma supernatant was aspirated and transferred into Eppendorf® tubes and stored at -80°C.

2.8.2 RNA extraction

RNA extraction was performed using QIAGEN® minElute® virus spin and QIAmp® circulating nucleic acid kit on plasma samples. Lysis of 200μl of patient plasma was performed through addition of proteinase K and buffer ACL to inactivate DNases and RNases and allow release

of nucleic acids from bound proteins, lipids, and vesicles. Buffer ACB was added to allow binding of nucleic acids to membrane. Lysates were then transferred to a QIAmp® mini column and nucleic acids were adsorbed into the membrane through centrifugation. Samples were washed to remove residual contaminants before elution to a volume of 20μl. Following this, samples were concentrated to a volume of 10μl in a vacuum concentrator. Using Nanodrop, RNA concentrations for each of the samples were obtained. Samples obtained in Chapter 7 had a range of 160-400ng/µl RNA and were frozen after RNA estimation at -80°C prior to their use in the Nanostring nCounter® miRNA expression assay.

2.8.3 Nanostring nCounter® miRNA Expression Assay

Analysis was performed using nCounter® miRNA expression assay using the experimental protocol. 100ng of purified RNA obtained from RNA extraction was used as input material. The first step involved miRNA extraction from the RNA input through annealing oligonucleotide tags with target miRNA, followed by ligation and purification to remove un-ligated tags. Once extracted the miRNA samples were then incubated for twenty hours with capture and reporter probes which bound to the target miRNA in the samples forming a miRNA probe- complex. Each capture-reporter probe set had its own unique identifier. Samples were then loaded onto cartridges and processed using the nCounter® prep station prior to being read using the nCounter® system giving quantitative values for abundance of miRNA in each sample. Housekeeping probes included ACTB, B2M, GAPDH, RPLP0 as well as positive and negative controls.

2.8.4 Statistical analysis

Analysis of raw miRNA data was performed using Nanostring nSolver analysis software version 4.0. Data was normalised using housekeeping probes. Lower threshold values were set as 1. Further analysis was conducted using multiple t testing in GraphPad prism (GraphPad Software, San Diego, California, 9th edition) and IBM SPSS Statistics (Version 28). Both log rank and cox proportional hazards were used for survival analysis.

2.9 Cytokine panel (Chapter 6)

2.9.1 Sample collection

Serum was obtained from Kolling tumour bank for use in the cytokine assay and was taken from patients at time of surgery following completion of neoadjuvant chemotherapy.

2.9.2 Cytokine assay

Cytokine assays were performed using Bio-Plex Pro™ Human 27-Cytokine kit and using the instruction protocol. 12.5µl of serum was used, and two replicates were used for each patient sample. Samples and controls were diluted 1:4 using supplied diluent and were added to 2 x 96- well plate. Fluorescently dyed microspheres known as beads coupled to antibodies directed at individual cytokines were then added to each of the samples and controls. Following this the samples then underwent a series of washes to remove any unbound protein and then detection antibodies were added to each well. Streptavidin- phycoerythrin was added followed by further washes and then the plate was read using Bio-plex™ manager software.

2.9.3 Statistical analysis

Analysis of data was performed using multiple t testing in GraphPad prism (GraphPad Software, San Diego, California, 9th edition) and multivariate analysis was performed using partial least squares using JMP® 17th edition. Both log rank and cox proportional hazards were used for survival analysis, and were performed in IBM SPSS (version 28).

CHAPTER 3:

Neoadjuvant chemotherapy versus upfront surgery in upfront resectable pancreatic cancer

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3.1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) continues to have a poor prognosis with 9% of patients alive at five years[135]. Only 15–20% of patients at diagnosis are considered to have resectable disease and the standard treatment for these patients includes upfront surgery (UFS) followed by chemotherapy in the adjuvant setting[136]. Increases in survival rates are impeded in part due to deliverability of these chemotherapeutic agents post-surgery with up to 25% of suitable patients in Australia, not receiving their planned chemotherapy secondary to surgical morbidity[137].

These early-stage resectable patients comprise of two cohorts. Borderline resectable (arterial: contact with common hepatic artery, superior mesenteric artery or celiac axis artery ≤180 degrees; or venous; contact with superior mesenteric vein or portal vein >180° or ≤180° with contour abnormality) and upfront resectable disease (no arterial contact and tumour contact ≤180° with the superior mesenteric or portal vein) [16].

Although initially used for locally advanced pancreatic cancer with the view to downstage disease to make surgical resection possible, there has been an increase in the use of neoadjuvant chemotherapy (NAC) over UFS in borderline resectable candidates and the success of this in the borderline setting has led to national comprehensive cancer network (NCCN) guidelines recommendation that NAC should be first-line therapy in borderline resectable patients [16, 138, 139]. The evidence for NAC in the resectable cohort (excluding borderline disease), is not as well established. Randomized controlled trials (RCTs) from Casadei et al. and Golcher et al. comparing UFS with NAC were ceased prematurely due to slow accrual [140, 141]. At the time of publication of this chapter, only one RCT, had revealed preliminary results in the resectable pancreatic cancer cohort. The PREP-02/JSAP-05 demonstrated an improved median overall survival (OS) in patients receiving NAC (gemcitabine and S1) at 36.7 months compared to UFS at 26.6 months)[29]. While these results provided some optimism on the use of NAC in this population, it must be noted that the chemotherapy combination of gemcitabine and S1 has had

limited success in a Caucasian population and is infrequently used due to the success of more intensives regimens such as 5-fluorouracil, irinotecan and oxaliplatin (FOLFIRINOX) [142].

At our centre, NAC became standard of care for both borderline and resectable patients that were considered chemotherapy candidates from 2016 onwards.

The lack of completed randomised controlled trials comparing UFS to NAC in the upfront resectable setting, led to clinical equipoise. A concern from clinicians in favour of upfront resection is that the delay to surgery from neoadjuvant chemotherapy may result in disease progression beyond resectability and hence render a curable patient, incurable. Conversely, the appeal of NAC is that it provides early exposure of possible micro-metastatic cells to cytotoxins and increases the chances of a margin negative surgical resection[143].

In light of this paucity of evidence, we conducted a retrospective cohort analysis to compare outcomes in patients diagnosed with resectable pancreatic cancer who received NAC with a retrospective patient cohort in the same centre who received traditional UFS.

In recent years, interest in inherent tumour biology or "biomarkers" as an independent determinant of treatment response highlighted Chapter 1[144]. In addition, there has also been recent interest in the anatomical location of the primary tumour. Pancreatic body/tail lesions make up a small proportion of all new diagnoses, however tend to behave more aggressively than head of pancreas tumours[145]. In part, this is due to the anatomical location as tail tumours tend to result in fewer symptoms presenting with later stage disease, however, more recently it is understood location of the tumour is an independent predictor and head and body/tail tumours are biologically distinct[146].

Aims

The aims of this study were to (1) determine if our patient cohort demonstrated an improvement in recurrence-free (RFS) or overall survival (OS) for NAC compared to UFS; (2) establish if administration of NAC resulted in improved surgical outcomes; and (3) identify if biological or imaging markers commonly reported in the literature have a role in prognosis or prediction of chemotherapy response.

3.2 Materials and Methods

3.2.1 Data Acquisition and Cohort Details

From December 2013 to January 2019 patients with resectable disease were identified using prospective chemotherapy and surgical databases, imaging reports, multi-disciplinary team (MDT) discussions and surgeon review. In this cohort, only upfront resectable patients were included. Patients with borderline disease were excluded.

The decision as to upfront treatment (either upfront surgery (UFS) or neoadjuvant chemotherapy (NAC)) was based on time of diagnosis. Due to the success of NAC in the borderline and locally advanced setting, from December 2016 onwards NAC replaced upfront surgery (UFS) as standard of care at our center[147]. In our cohort patients treated prior to this date received UFS and patients after this date were treated with NAC. Patients after December 2016 that were not chemotherapy candidates received upfront surgery.

All surgeries were performed at two hospital sites (Royal North Shore Hospital and North Shore Private Hospital) as part of the same pancreatic cancer centre by two specialized pancreatic cancer surgeons (JS and AM). As this is a tertiary referral centre, patients were referred from a diversity of metropolitan and regional locations; however, the majority of chemotherapy regimens were given within the one local health district. The choice of regimen and duration of chemotherapy was based on MDT consensus recommendation in collaboration with treating clinicians. Each patient was discussed at our MDT meeting at the time of diagnosis and again during their treatment.

All patients underwent baseline investigations which included: baseline carbohydrate antigen 19-9 (CA 19-9), computerized tomography of the chest, abdomen, and pelvis (CT-CAP) as well as endoscopic ultrasound (EUS) guided biopsy for histological diagnosis. Resectability and staging were based on radiological imaging. Fluoro-deoxyglucose-positron emission tomography (FDG-PET) scans were performed as per MDT recommendation and were subject to patient preference (due to cost). Staging laparoscopy and peritoneal washings were used as an adjunct pre-operatively in UFS and pre-chemotherapy in NAC patients to rule out peritoneal or radiologically undetectable metastatic disease. For patients undergoing NAC, CA 19-9 and CT scans were repeated preoperatively. RECIST criteria was used for sequential imaging[148]. FDG-PETs were repeated preoperatively in NAC patients and a cut-off of SUVmax of 5 was used to delineate higher and lower uptake for both upfront and pre-operative scans.

Surgical complication rates, margins, venous resection rates, vascular invasion and perineural invasion were obtained. Complications were documented for type and grade of severity (1–5) using the Clavien-Dindo classification[149]. Surgical margins (microscopic) were reported for all tumours. As per guidelines, margins were considered negative if the tumour was >1 mm away from the closest surgical margin (R0) or positive if the tumour was <1 mm from the closest surgical margin (R1)[150].

Histological tumour grade and stage were measured by a gastrointestinal specific pathologist using TNM 8th system[26] . Tumor viability was reported for patients who received NAC as the percentage of the original tumor volume present in the surgical specimen and ranged from 0% (no residual tumor) to 100% (no destruction of the original tumor) viable[151]. This was analysed as continuous data, due to the lack of consensus agreement within the academic community for use of one validated grading tool.

Potential prognostic and predictive markers of interest were identified through literature, prior to analysis[152]. These markers were included and their roles in prognostication and prediction of chemotherapy response were evaluated.

3.2.2 Statistical Analysis

Patients were analysed according to the initial treatment plan (NAC vs. UFS) regardless of what treatment they received. RFS and OS were analysed using Cox proportional hazards or Kaplan-Meier (log-rank test) where appropriate. Prognostic factors were assessed using multivariate and univariate analysis by cox-hazards ratio. Univariate analysis using the chi-squared test was performed on categorical data and spearman's rank analysis was performed on non-parametric variables to assess correlation. One-way ANOVA was performed to assess baseline differences in continuous variables between groups. Statistical analysis was performed using IBM SPSS Version 27). P-values < 0.05 were considered statistically significant.

3.2.3 Institutional Ethics

Institutional ethics approval for this study was obtained (HREC/16/HAWKE/105). Written consent was obtained in select patients where possible, however, was not required as it was using historical databases[153]. This study was conducted according to the national statement on ethical conduct in human research.

3.3 Results

3.3.1 Baseline characteristics

Baseline characteristics of the patient cohorts are shown in Table 3.1. From December 2013 until January 2019, 126 patients from our centre were classified as resectable and underwent initial treatment either with neoadjuvant chemotherapy (NAC, n = 40) or upfront surgery (UFS) (n = 86) (Figure 3.1). The median age for all patients was 69 (41–90) years and 58% of patients were male. There were no significant differences in baseline characteristics in the two cohorts including patient age, gender, and stage. Staging laparoscopy and peritoneal washings were performed in 45/86 patients (52%) undergoing UFS prior to surgery and in 35/40 (88%) of patients in the NAC cohort prior to chemotherapy commencement. There were significantly more patients in the UFS surgery group with body/tail tumours 23/86 (27%) than in the NAC cohort 2/40 (5%) (chi-squared p = 0.004).

Neoadjuvant chemotherapy regimens and duration were heterogeneous and are shown in Table 3.2. As all patients were considered resectable (borderline patients were not included), routine chemoradiotherapy was not given. Thirty-eight of the 40 patients proceeded to surgery with two progressing beyond resectability. One patient in the upfront resectable cohort was found to have metastatic disease and surgery did not proceed. The percentage of patients that underwent upfront surgery versus neoadjuvant chemotherapy is listed in Table 3.3.

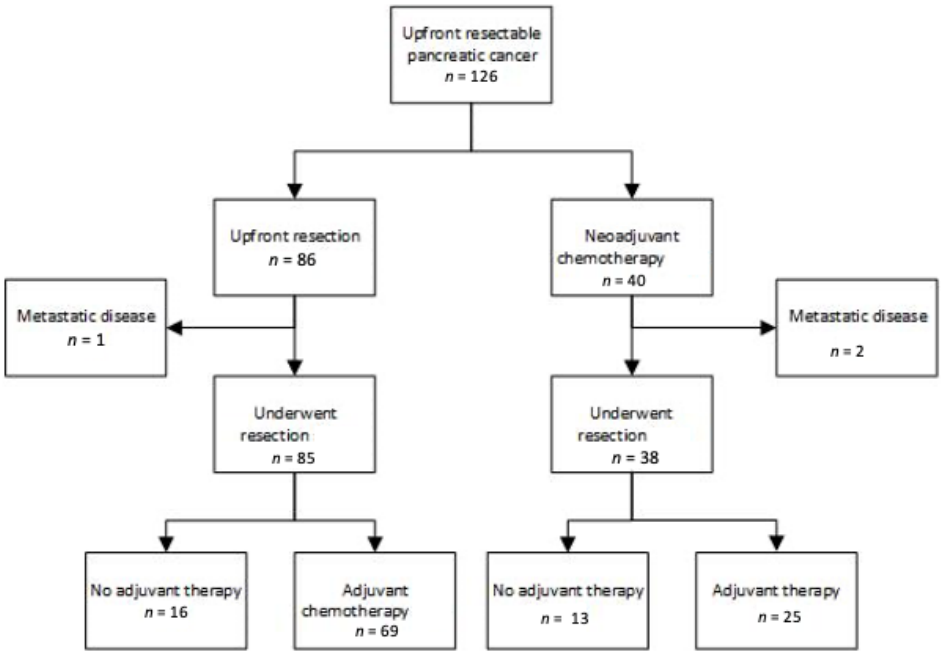


Figure 3.1 Treatment pathways for patients diagnosed with resectable pancreatic cancer.

Table 3.1 Baseline characteristics per upfront treatment modality. F- female, M- Male, a-8th addition TNM staging system [26].

	Neoadjuvant Chemotherapy	**Upfront Surgery**
Median age (years, range)	71 (42–85)	69 (41–90)
Sex		
Female	20	53
Male	20	33
Upfront radiological stage		
1a	12	32
1b	26	41
2a	2	5
2b		8
Total	40	86
Tumour location		
Head	38	63
Body/tail	2	23

Table 3.2 Type and duration of neoadjuvant and adjuvant chemotherapy

Chemotherapy	Upfront group: Neoadjuvant Chemotherapy	Upfront group: Upfront Surgery
Neoadjuvant chemotherapy FOLFIRINOX Gemcitabine + Nab-Paclitaxel Gemcitabine + capecitabine Gemcitabine alone	 12 25 1 2	 N/A
Median duration weeks (range)	8 (3–26)	N/A
Adjuvant chemotherapy FOLFIRINOX Gemcitabine + Nab-Paclitaxel Gemcitabine + capecitabine Gemcitabine alone Capecitabine alone S1 Unknown	 1 4 11 9	 2 4 24 36 1 1 1
Median duration weeks (range)	16 (3–24)	24 (4–52)

Table 3.3 Upfront treatment modality by year

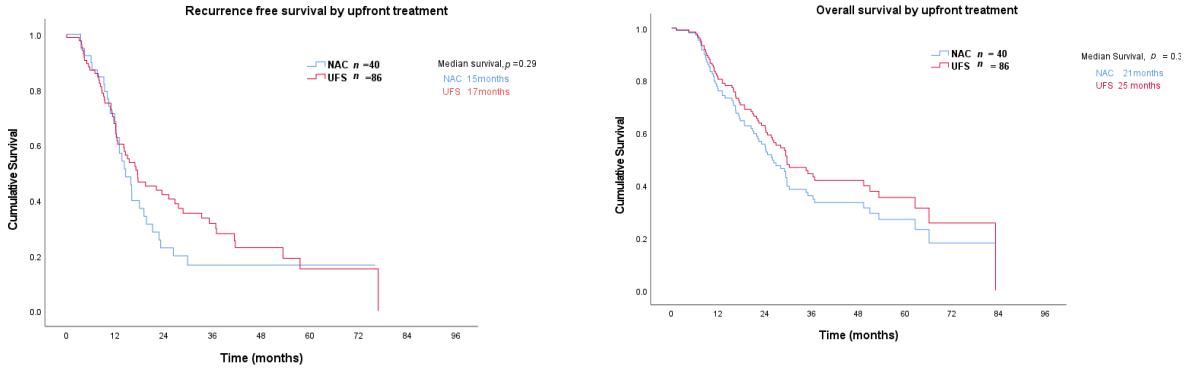
Year (Total)	Upfront surgery (% of all patients in that year)	Neoadjuvant chemotherapy (% of all patients in that year)
2013	0 (0%)	8 (100%)
2014	15 (69%)	7 (31%)
2015	30 (91%)	3 (9%)
2016	22 (81%)	5 (19%)
2017	8 (42%)	11 (58%)
2018	10 (63%)	6 (37%)
2019	1 (100%)	0 (0%)

3.3.2 Prognostic biomarkers (Survival)

3.3.2.1 Neoadjuvant Chemotherapy vs Upfront Surgery

After a median follow up 52 months, recurrence-free (RFS) and overall survival (OS) of NAC versus UFS patients were evaluated. Survival was calculated using time at diagnosis as this was closer to treatment commencement for both groups. At the time of reporting 31/40 (78%) of NAC and 60/86 (70%) of UFS had recurred and 24/40 (60%) of NAC and 49/86 (57%) of

UFS had died. There was no significant difference in median RFS in the UFS compared to NAC group at 17 (0.3–77) months and 15 (3–76) months, respectively, (hazard ratio (HR) 0.79 (0.51–1.2), p = 0.29; Figure 3.2). Similarly, no difference in median OS was observed between these two groups at 25 (6–83) months in the UFS group compared to 21 (4–76) months in the NAC cohort (hazard ratio (HR) 0.79, p = 0.357; Figure 3.2).

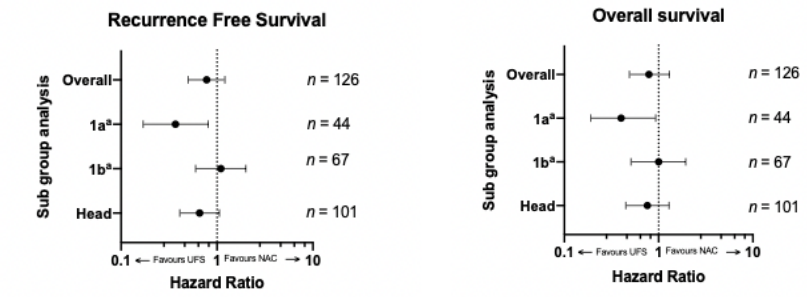


(a) (b)

Figure 3.2 (a) Recurrence free; (b) overall survival: NAC—neoadjuvant chemotherapy.

3.3.2.2 Upfront Radiological Stage

Sub-group analyses were pre-planned and included upfront stage and location of the tumour (Figure 3.3). Upfront stage 1a revealed a significant difference in median RFS in patients that received UFS at 18 months compared to 12 months for NAC (HR 0.30, p = 0.005; Figure 3.3). This survival advantage also translated to an improved OS at 24 months for UFS compared to 21 months for NAC (HR 0.42, p = 0.03, Figure 3.3). This difference was not reproducible for stage 1b for either RFS (p = 0.59) or OS (p = 0.97; Figure 3.3), and there were insufficient patients in the NAC group in stage 2a to allow for meaningful analyses (n = 2). There were 101 patients (38 NAC and 63 UFS) who had tumours arising from the head of the pancreas and 25 (2 NAC AND 23 UFS) had tumours arising from the tail. Median RFS for patients with head tumours was comparable (18 vs. 14 months, p = 0.09; Figure 3.3), as was OS (24 vs. 19 months, p = 0.20; Figure 3.3). There were only two patients in the body/tail subgroup that received NAC and analysis was not performed.



(a) (b)

Figure 3.3 (a) Recurrence-free and (b) overall survival comparing UFS to NAC by subgroup analysis. Radiological stage 2a and tail were not included due to inadequate Legend: UFS—upfront surgery; NAC—neoadjuvant chemotherapy; a-8th addition TNM staging system[26].

3.3.2.3 Prognostic Factors from literature

Factors identified as potentially prognostic after review of the literature are listed in Table 3.3. Both univariate and multivariate testing were performed. In multivariate analysis, no factors were identified that predicted for either recurrence-free or overall survival. Factors that were significant in univariate testing are discussed further.

3.3.2.4 CA 19-9 and Imaging

Both baseline CA 19-9 (for all patients) and post NAC (for patients treated with NAC) CA 19-9 were collected. A cut-off of 500 kU/L was chosen as per international consensus to delineate low versus high probability of advanced disease[154]. In the NAC cohort, 30% (12/40) had CA 19-9 ≥500 kU/L compared to 18% (15/82) in the UFS. There was no difference in the percentage of patients with a CA19-9 above 500 kU/L in either group (chi-squared, p = 0.14). Baseline CA 19-9 ≥500 kU/L did not predict for either RFS (p = 0.33; Table 3.3) or OS (0.06; Table 3.3). All patients had a baseline CT abdomen for radiological staging. Upfront stage did not predict for RFS (p = 0.11; Table 3) or OS (p = 0.36; Table 3). Baseline FDG-PET scans were performed in 38/40 (95%) of the NAC and 35/86 (41%) of UFS cohort. FDG-PET was a useful prognostic marker with tumours that had a SUVmax ≥ 5 having a significantly shorter RFS at 14 months compared to 37 months in patients with SUVmax < 5 (HR 2.4, p = 0.02; Table 3.3). This difference was also evident in OS at 23 months in patients with SUVmax ≥5 compared to 51 months in patients with tumours SUVmax < 5 (HR 3.4, p = 0.007; Table 3.3).

3.3.2.5 Histopathological Markers

At the time of pathological reporting an attempt was made to estimate the % of viable tumour cells remaining after surgery in patients that received NAC. The median viability was 80%. Higher tumour viability was associated with shorter RFS with an increase in hazard of 1.02 for every 1% rise in tumour viability (HR 1.02, p = 0.04; Table 3.4). This was also significant for OS (HR 1.03, p = 0.004; Table 3.4). For all patients, a higher tumour grade predicted for shorter RFS (HR 1.8, p = 0.005; Table 3.4) and OS (HR1.7, p = 0.017; Table 3.4). A higher T stage (size of the primary tumour) was associated with a decrease in OS (HR 1.6, p = 0.02; Table 3.4), however, had no impact on RFS (p = 0.12; Table 3.3). Patients with a higher nodal stage (increased number of nodes infiltrated with carcinoma) had a reduced RFS (HR 1.4, p = 0.01; Table 3.4), however, this did not translate to change in OS (p = 0.05; Table 3). A positive margin (R1) predicted for shorter RFS (HR 1.5, p = 0.049), however not for OS (p = 0.21; Table 3.4). Vascular invasion was associated with shorter OS (HR 2.4, p = 0.001; Table 3.4), however not for RFS (p = 0.14) and perineural invasion did not predict for either RFS or OS (p = 0.18, 0.51; Table 3.4).

3.3.2.6 Chemotherapy Regimen

Neoadjuvant and adjuvant chemotherapy regimens were matched for age. There were four neoadjuvant chemotherapy regimens utilized: gemcitabine; gemcitabine plus capecitabine; gemcitabine plus nab-paclitaxel; and FOLFIRINOX (Table 3.2). Thirty- seven patients received either gemcitabine-nab paclitaxel or FOLFIRINOX and there was no difference in RFS (p = 0.28) or OS between these two groups (p = 0.25; Table 3.2). The median duration of NAC was 8 (3–24) weeks. No difference in RFS (p = 0.29; Table 3.2) or OS (p = 0.26; Table 3.2) could be appreciated between patients who received ≥12 weeks of NAC compared to <12 weeks (Table 3.3).

Patients were more likely to commence adjuvant chemotherapy if they had an uncomplicated post-surgical recovery 65/78 (81%) compared to patients who had complications 32/45 (71%), however, this difference did not achieve significance (chi-squared, p= 0.11). In the NAC group, 25/38 (66%) of patients received further adjuvant chemotherapy compared to 69/85 (81%) in the UFS group. While adjuvant chemotherapy regimens were heterogeneous, the majority of patients received either gemcitabine (n = 36) or gemcitabine in combination with capecitabine (n = 24; Table 3.2). Patients who received gemcitabine plus capecitabine had a longer median OS compared to single agent gemcitabine therapy at 49 and 24 months, respectively (HR 0.6, p = 0.01; Table 3.3). There was no appreciable difference in RFS (p = 0.06; Table 3.4) between

these two groups. The median duration of adjuvant chemotherapy was 24 (4–52) weeks [155, 156]. Patients who received chemotherapy ≥24 weeks had a longer median RFS at 19 months (HR 0.5, p = 0.007; Table 3.4) compared to 12 months in those that had <24 weeks of chemotherapy. Similarly, there was an increase in OS for patients that received longer chemotherapy (≥24 weeks) at 49 compared to 20 months (HR 0.33, p = 0.0002; Table 3.3).

Table 3.4 Univariate and multivariate prognostic markers for recurrence-free and overall survival. HR-hazard ratio; RFS-recurrence-free survival; OS-overall survival; SUVmax-standardized maximum uptake value; NAC-neoadjuvant chemotherapy; GEM/ABR-gemcitabine and nab-paclitaxel; AC-adjuvant chemotherapy; GEM/CAPE-gemcitabine and capecitabine; R1-tumour within < 1 mm at closet margin. * Indicates statistical significance $p < 0.05$. a 8th addition TNM staging system[26].

RFS	**Univariate**		**Multivariate**		**OS**	**Univariate**		**Multivariate**
	p-Value	**Hazard Ratio (HR) (95% CI)**	**p-Value**	**HR (95% CI)**	**p-Value**	**HR (95% CI)**	**p-Value**	**HR (95% CI)**
Initial CA19-9 ≥500 kU/L	0.33	1.3(0.8–2.2)	0.95	5.5(0–5.6^21)	0.06	1.7(1–2.9)	0.63	3.6^6 (0–3.3^33)
Radiological stage	0.11	0.8(0.6–1.1)	0.95	0.2(1.8^20)	0.36	0.9(0.6–1.2)	0.44	3.0^5(0-3.16^19)
Viability (%)	* 0.04	1.02(1–1.03)	0.79	1.0(0.9–1.1)	* 0.004	1.03(1.01–1.05)	0.92	1.1(0.3–4.2)
Grade	* 0.005	1.8(1.2–2.7)	0.58	1.7^9(2.7^41)	* 0.017	1.7(1.1–2.7)	0.29	5.9^12 (0–2.6^36)
Baseline FDG PET ≥5	* 0.02	2.4(1.1–4.5)	0.88	0(0–3.0^77)	* 0.007	3.4(1.4–8.3)	0.73	694(0–1.8^19)
Surgical stage T a	0.12	1.3(0.9–1.8)	0.81	0(0–4.5^27)	* 0.02	1.6(1.1–2.5)	0.88	65.6(0–7.4^24)
Surgical stage N a	* 0.01	1.4(1.1–2.0)	0.86	9.6(0–3.6^11)	0.05	1.4(1.0–2.0)	0.57	0.1(0–9.2^4)
Perineural invasion	0.18	1.4(0.9–2.4)	0.79	1.8^4(0–8.8^35)	0.51	1.2(0.7–2.0)	0.38	1.2^16(0–2.0^52)
Small/large vessel invasion	0.14	1.4(0.9–2.2)	0.67	1.3^9(0–2.9^51)	* 0.001	2.4(1.4–4.1)	0.26	1.5^15(0–6.2^41)
R1	* 0.049	1.5(1.002–2.4)	0.73	75(0–4.3^12)	0.21	1.4(0.9–2.1)	0.39	1.4^6(0–9.9^19)
NAC: GEM/ABR	0.28	0.7(0.3–1.4)	0.88	2.6(0–5.6^5)	0.25	0.6(0.2–1.5)	0.62	44.2(0–1.5^8)
NAC ≥ 12 weeks	0.29	1.0 (0.9–1.0)	0.98	0.20(0–6.3^51)	0.26	0.6(0.3–1.5)	0.66	0(0–1.8^18)
AC: GEM/CAPE	0.06	0.8(0.6–1.0)	0.97	10.9(0–1.4^48)	* 0.01	0.6(0.4–0.9)	0.74	2.6^3 (0–4.5^23)
AC ≥ 24 weeks	* 0.007	0.5(0.3–0.8)	0.93	0.4(0–1.16^30)	* 0.0002	0.3(0.2–0.6)	0.96	0.4(0–6.6^10)

3.3.3 Surgical Outcomes

There was a statistically significant increase in complication rates at the time of surgery for patients who received NAC 19/38 (50%) compared to UFS 25/85 (29%) (chi-squared, p = 0.036). No difference between the two groups in the severity of complications could be appreciated (chi-squared, p = 0.256). Similar numbers of patients had R1 resections in both NAC 14/38 (37%) and UFS 36/85 (42%; p = 0.49). The rate of vein resection was significantly higher in NAC at 48% compared with UFS 18% (chi-squared, p = 0.001). Rates of perineural and vascular invasion between these two groups were also examined. In the NAC cohort 27/38 (71%) and 62/85 (73%) in the UFS cohort had perineural invasion and no difference between the two groups could be appreciated (chi-squared p = 0.97). There was, however, a significantly higher rate of vascular invasion (small or large vessel) in patients that underwent UFS at 68% (58/85) compared to 47% (18/38) of patients in the NAC cohort (chi-squared, p = 0.023).

3.3.4 Predictive biomarkers in patients treated with neoadjuvant chemotherapy

Baseline and pre-operative CA 19-9, CT and FDG-PET were analysed to explore their potential use as a marker to predict tumour viability, a surrogate for tumour response to chemotherapy. Serial CT scans (baseline and pre-operative) were performed on all forty patients undergoing NAC with a formal comparison report available for 33 of these patients. Using RECIST criteria four patients had progressive disease, 24 had stable disease, four had a partial response and one patient had a complete radiological response [148]. Similarly, baseline FDG-PET scans were performed on 38 of the 40 patients and again preoperatively in 22 of these patients. The standardized uptake value (SUVmax) was recorded for each patient and median on the baseline and pre-operative PET scan were 7.1 and 5.0 respectively. These radiological and biological markers were assessed for correlation to tumour viability on operative tumour specimen after NAC exposure (Table 3.5). Spearman rank analysis was used as all variables were considered non-parametric after Shapiro–Wilk testing.

Of these, only baseline SUVmax ≥ 5 (spearmans co-efficient, p = 0.023; Table 3.5) were useful in predicting higher tumour viability, and hence reduced chemotherapy response.

Table 3.5 Biological and radiological predictors for tumour regression (tumour viability). SUVmax—standardized maximum uptake value. * Indicates statistical significance $p < 0.05$. [a] RECIST 1.1[148].

	p-value	Correlation co-efficient (Spearmans)
CT response- RECIST[a]	0.72	0.317
Baseline CA 19-9 ≥500	0.28	−0.18
Pre-operative CA 19-9 ≥500	0.32	−0.17
Initial SUVmax: ≥5	* 0.023	0.369
Pre-operative SUVmax: ≥5	0.45	0.175

3.4 Discussion

In the context of recent randomized controlled trials PREP-02/JSAP-05 and PREOPANC-1 which reveal conflicting preliminary results, we conducted a retrospective cohort study and compared outcomes in patients diagnosed with resectable pancreatic adenocarcinoma who had been treated with either neoadjuvant chemotherapy (NAC) or upfront surgery (UFS) at one large pancreatic cancer centre.

Our study revealed that for all stages, survival was comparable between the two cohorts; however, there was a statistically and clinically significant increase in RFS and OS in patients who had small, node-negative tumours (stage 1a) that received UFS. Furthermore, the proposed benefit of improved margin negative rates in patients who received NAC was not evident. This improvement in survival in early-stage patients in our cohort treated with UFS makes biological sense. One of the purported benefits of NAC is that it allows exposure of potential micro-metastatic disease to cytotoxins early; however, the likelihood of disseminated disease in early tumours is lower than in advanced disease and the benefit in this context is as such diminished.

This study complements and expands on an earlier study conducted at our centre. A retrospective study by Itchins et al. compared survival in patients with upfront and borderline resectable disease and found no survival difference between NAC and UFS[147]. Expanding on this study we aimed to compare NAC and UFS in patients with upfront resectable disease only, as the survival benefit of NAC over UFS in borderline disease has since been established[16, 147, 157]. Another strength of our study is that it has a larger number of patients with resectable disease who received NAC (n = 40) and outcomes were assessed using prospectively collected

data. Although not performed on all patients, the widespread use of PET imaging and laparoscopy to rule out metastatic disease in particular prior to commencement of chemotherapy in our NAC cohort is also a strength. In the NEONAX trial 8% of patients in the NAC cohort were found to be metastatic at the time of surgery and an explanation given for this high number is that these patients may have had metastatic disease at the time of diagnosis that was not detected by routine imaging[158]. In our NAC cohort, 88% underwent a staging laparoscopy with negative peritoneal washings and 95% of patients underwent an FDG-PET making underdiagnosis of metastatic disease at diagnosis highly unlikely.
The importance of determining clinically useful biomarkers as surrogates for underlying tumour biology are becoming more prominent of late. At this stage, only CA19-9 is used clinically; however, more recent studies have identified additional potential biomarkers that if validated may prove an important aid in clinical decision making [158-162].

This is a hypothesis-generating analysis, which serves to highlight potential subgroups within the resectable population that may benefit from a particular upfront treatment modality and explore the role of markers identified in the literature in prognostication and prediction of chemotherapy response, within the confines of a retrospective review.

There are limitations to this study to note. This is a retrospective cohort study which introduces the potential of selection and recall bias. Although stages of the two groups were comparable it should be noted that the lack of randomization introduces the potential for selection bias toward NAC treatment for patients with more aggressive disease. The higher rate of vein resection in our neoadjuvant chemotherapy cohort indicate that this might have had more advanced disease at time of surgery. This may have been due to progression on neoadjuvant chemotherapy or a more advanced disease at diagnosis. The higher body/ tail lesions in the UFS group are a possible confounding factor and may resulted in a poorer survival in the UFS cohort[145, 146]. The higher rate of vein resection in our neoadjuvant chemotherapy cohort indicate that this might have had more advanced disease at time of surgery. This may have been due to progression on neoadjuvant chemotherapy or a more advanced disease at diagnosis. The higher body/ tail lesions in the UFS group are a possible confounding factor and may resulted in a poorer survival in the UFS cohort [145, 146]. In addition, the lack of recorded Eastern Co-operative Group (ECOG) and co-morbidity status for all patients introduces a potential treatment selection bias. The surgical data is from a high-volume pancreatic cancer centre by two pancreatic cancer specialist surgeons and surgical outcomes may not be representative of other

centres around the world. In addition the high percentage of upfront surgical patients that received adjuvant chemotherapy (81%) is significantly higher than the 60% often reported in the literature[163].

3.5 Conclusion

This real-world study demonstrates that replacement of UFS with NAC as a standard of care for all patients with resectable disease may be premature. Further research in the form of a prospective trial is urgently required to compare these two upfront modalities with the incorporation of biomarkers to help to personalize the treatment of this devastating disease.

CHAPTER 4:

Biomarker discovery on surgical specimens: proteomics and validation

4.1.1 Introduction

As discussed in Chapters 1 and 3, neoadjuvant chemotherapy is becoming increasingly used in pancreatic cancer in earlier disease. One of the benefits of this approach is that it provides clinicians to evaluate the tumours response to chemotherapy. During neoadjuvant chemotherapy regular assessments are made based on serial serum CA19.9, as well as computerise tomography (CT) and positron emission topography scanning (PET) scanning[16]. Whilst these biomarkers can assess biochemical and radiological response their ability to predict histopathological regression have not been validated in larger studies[164]. Radiological imaging often downstages disease compared to histopathological staging, particularly in patients with smaller tumours[165].

At present, histopathological response to neoadjuvant chemotherapy is evaluated using pathological staging (TNM 8th edition) in addition to tumour regression[166]. Tumour regression is assessed as the percentage of cancer cells viable after administration of chemotherapy, and is used as a measure of chemotherapy response. A tumour with minimal residual cells is indicative of a chemosensitive tumour whereas, higher residual tumour at the time of surgery is a marker of chemoresistance[167-169]. At present, there is no consensus as to the ideal tumour regression tool with the college of American pathologists (CAP), MD Anderson cancer centre (MDACC) and the Evans system being the most utilised[167, 168, 170, 171]. Whilst smaller studies have demonstrated that higher viable tumour is associated with poorer survival, large studies are needed to validate these findings[151].

For patients with upfront or borderline disease the identification of a chemoresistance biomarker at diagnosis may make treatment with early surgery a more attractive option rather than prolonged exposure to neoadjuvant chemotherapy. In addition, a better understanding of the mechanisms or biological pathways involved in chemoresistance may facilitate targeted biological therapy to overcome this resistance. This is particularly important in pancreatic cancer, a cancer notorious for poor outcomes and an absence of available targeted therapy.

In recent years, 'OMICS' studies have risen due to the reduced cost of these experiments. Whole genome sequencing led to a better understanding of the subtypes of pancreatic cancer, however, less is known about the proteins involved in pancreatic cancer and chemoresistance[172]. Mass spectrometry is an important initial first step in identification of potential 'proteomic' biomarkers. Improvements in sensitivity of mass spectrometers over

recent years has allowed identification of a higher number of proteins per sample. Furthermore, the volume of sample required is also reducing, making its use more appealing to researchers. This high throughput method identifies and quantifies proteins in individual samples. These results are then validated using more specific techniques including immunohistochemistry.

The aim of this chapter was to identify potential pathways involved in chemoresistance in patients treated with neoadjuvant chemotherapy, by comparing samples from chemoresistant and chemosensitive tumours (determined by residual viable tumour) after exposure to neoadjuvant chemotherapy.

Hypotheses

1. Patients with higher residual tumour at time of surgery after neoadjuvant chemotherapy will have different protein expression, compared to patients with lower residual tumour at time of surgery.
2. The input of differentially expressed proteins in chemoresistance tumours compared to chemosensitive tumours into ingenuity pathway analysis will identify key regulators that are associated with chemoresistance in pancreas cancer.
3. The identification of upstream regulators will form the basis for validation experiments including immunohistochemistry and cell culture.
4. Positive IHC staining of TGM2 will be associated with higher residual tumour (and hence chemoresistance), in surgical specimens following administration of neoadjuvant chemotherapy.
5. Using siRNA knockdown of TGM2 and OSMR in cells will result in a reduction in cell viability in PANC-1 and MIA PaCa-2 cell lines.
6. Treatment of cells with OSM cytokine, siRNA OSMR or siRNA TGM2 will result in a change in expression in proteins involved in the TGM2/OSMR pathway as detected on western blot.

Aims

1. To perform proteomic discovery using mass spectrometry on frozen surgical specimens in patients diagnosed with pancreatic cancer treated with neoadjuvant chemotherapy.
2. To identify proteins that are differentially expressed in specimens that are chemoresistant compared to chemosensitive tumours.

3. To ascertain which upstream or downstream regulators are involved in chemoresistance by inputting results from mass spectrometry into ingenuity pathway analysis (IPA)
4. To evaluate expression of TGM2 (as performed by immunohistochemistry) on surgical specimens after neoadjuvant chemotherapy.
5. Using matched clinical data to assess the role of TGM2 as a chemoresistance and prognostic biomarker.
6. To measure cell viability in PANC-1 and MIA PaCa-2 cell lines, after siRNA knockdown of TGM2 and OSMR.
7. To evaluate expression of proteins identified in TGM2/OSMR pathway (Figure 4.3) after treatment with OSM cytokine.
8. To assess expression of proteins, identified in TGM2/OSMR pathway (Figure 4.3) after treatment with siRNA TGM2 and OSMR.

4.1.2 Proteomics Methods

Inclusion criteria

Patients were identified through review of local pancreatic database. Only patients with either upfront resectable, borderline, or locally advanced tumours treated with neoadjuvant chemotherapy and had available frozen surgical tumour tissue were evaluated.

Ethics

Ethics approval was obtained from the Northern Sydney Local Health District (NSLHD) Human Research Ethics Committee (HREC; Ref# 2019/ETH08639).

Chemotherapy response

Clinical data including chemotherapy, recurrence and overall survival and residual tumour (tumour viability) were also obtained. As a single ideal scoring method for assessment of tumour viability in pancreatic cancer does not exist, tumours were divided into higher response to chemotherapy (≤30% residual tumour) or 'chemosensitive' tumours versus poorer response to chemotherapy (>30% residual tumour) or 'chemoresistant' tumours on surgical histopathology based on previous research by our team[169]. In this chapter, the terms 'residual tumour' and 'tumour viability' are used interchangeably.

Samples were run through mass spectrometry as detailed in Chapter 2. The quantified proteins identified in mass spectrometry were further analysed using multiple t tests ($p<0.05$), and area

under receiver operating characteristic (AUROC) in GraphPad Prism (GraphPad Software, San Diego, California). Multivariate analysis was performed in JMP® 17th edition using partial least squares. Results were also inputted into IPA (QIAGEN Inc., https://www.qiagenbio-informatics.com/products/ingenuity-pathway-analysis) to analyse associated pathways.

4.1.3 Results

4.1.3.1 Baseline characteristics

Baseline characteristics are listed in table 4.1. Twenty-five patients were included in analysis. Most patients received gemcitabine plus abraxane (52%). Seven patients had residual tumour ≤30% and 18 had residual tumour of >30% (Table 4.1). Median recurrence free survival (RFS) was 19 months and overall survival (OS) was 36months.

Table 4.1 Baseline characteristics

Baseline characteristics	N = 25
Age (median)	68
Gender	
Female	12
Male	13
Chemotherapy	
FOLFIRINOX	8
Gemcitabine + abraxane	13
Unknown	4
Residual tumour	
≤30%	7
>30%	18

4.1.3.2 Mass spectrometry

In total 6028 proteins were identified via mass spectrometry. A partial least squares (PLS) analysis was conducted and the top fifty proteins with the highest variable importance in the projection (VIP) score were chosen for further analysis (Figure 4.1). Using residual tumour (tumour viability) of 30% as a cut-off, multiple t-tests were conducted to identify the proteins that were upregulated in patients with higher residual tumour, as surrogate biomarkers for chemoresistance. The top five proteins were then further interrogated using area under the receiver operating characteristic (AUROC) to identify if any were useful biomarkers to predict residual tumour of either ≤30 or >30% (Table 4.2). Of these, two proteins; Protein S100-P and

NAD(P) H dehydrogenase 1 were identified as potential predictive biomarkers with AUROC values of >0.9 (Figure 4.2).

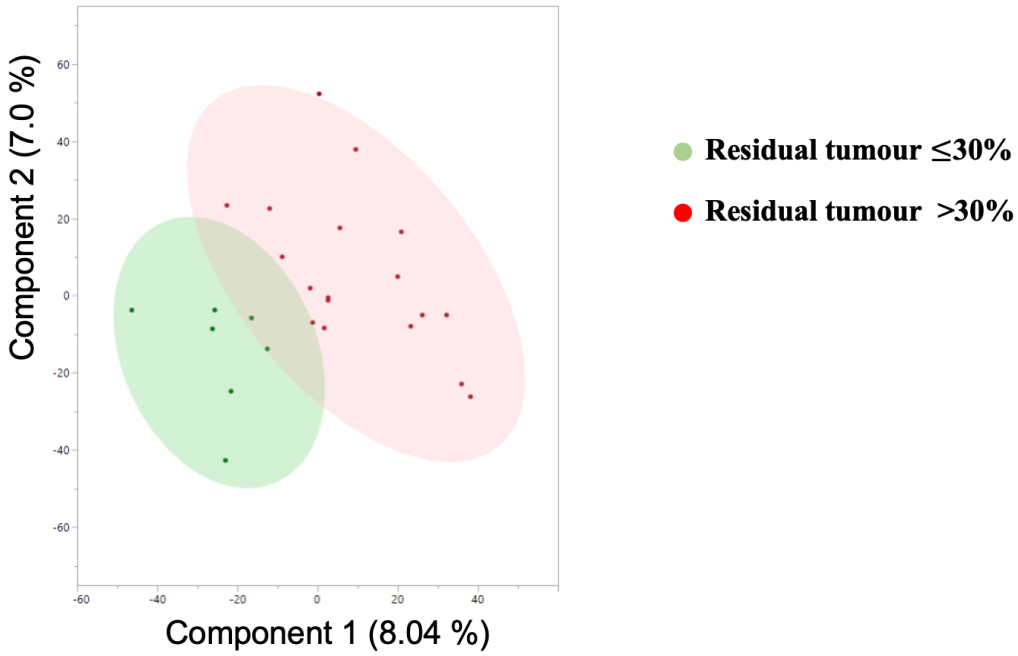


Figure 4.1 Partial least squares.

Table 4.2 Proteins upregulated in chemoresistant tumours. * Indicates AUROC >0.9.

Protein	**Multiple t test p-value**	**Multiple t test q-value**	**AUROC**	**AUROC p-value**
Protein S100-P	<0.000001	0.000004	*0.9444	0.0007
NAD(P)H dehydrogenase [quinone] 1	0.000002	0.000013	*0.9048	0.0020
Inhibitor of nuclear factor kappa-B kinase subunit beta	0.000007	0.000036	0.8571	0.0065
Xanthine dehydrogenase	0.000007	0.000036	0.8175	0.0155
ADP-ribosylation factor-binding protein GGA2	0.000016	0.000068	0.8413	0.0093

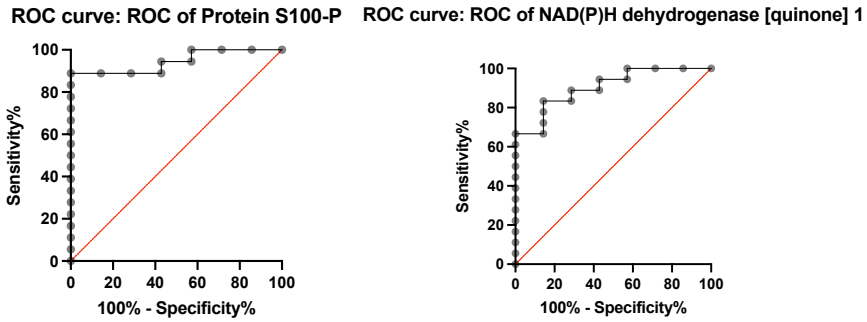


Figure 4.2 AUROC curve for S100-P and NAD(P)H dehydrogenase [quinone] 1.

4.1.3.3 Ingenuity Pathway Analysis (IPA)

Tumour specimens were normalised by comparison to matched normal pancreas specimens. After normalisation and multiple t-test analysis 104 proteins were identified that had significantly differential expression in tumours with higher viability (chemoresistant) compared to low tumour viability (A1). Using IPA upstream regulator analysis, we identified 612 upstream and downstream regulators of these 104 proteins. These results were then divided into highest activation z-score (a statistical measure of match between expected relationship from known literature and observed gene expression in our samples) and p value of overlap (Table 4.3). The p value of overlap defines the overlap between dataset and regulator molecules and values <0.01 are considered significant.

Table 4.3 Top regulators identified from the 104 proteins upregulated in patients with residual tumour>30%. Activation z score >1.5 and p value <0.01 have been included. [+]will be discussed further in this chapter, * to be discussed in the following chapter.

Upstream Regulator	Activation z-score	p-value of overlap
SCAP	2.586	0.0000218
WT1	2.425	0.000126
SREBF1	2.353	0.000864
IL2	2.201	0.0045
ANGPT2	2.158	0.00186
CNTF	2.121	0.00593
PPARGC1A	2.106	0.00145
NFE2L2	2.083	1.22E-07
KLF11	2	0.00634
RPTOR	1.939	0.00234
miR-30c-5p (and other miRNAs) *	1.937	0.00494
FGF1	1.915	0.000246
NCOA1	1.909	0.00316
CSF1	1.908	0.00216
CEBPA	1.78	0.000285
FAS	1.746	0.00307
CCN5	1.732	0.0043
TGM2[+]	1.706	0.00281
NR1I2	1.698	0.00376
BCR (complex)	1.633	0.00583
MYC	1.585	0.000556
OSM[+]	1.571	0.000206
TNF-α*	1.561	5.97E-07
ERBB2	1.555	0.0000104
BRD4	1.521	0.00184
FOXA1	1.509	0.00177

Once these upstream or downstream regulators were identified, consultation with the literature identified key molecules that have been identified in pancreatic cancer cell development and chemoresistance. The molecules with the highest level of evidence are discussed below.

<u>IL-2/ IL-6</u>

Interleukins 2 and 6 are cytokines associated with the immune system. The role of cytokines as biomarkers are discussed in detail in Chapter 6. Other immune cells are discussed in Chapter 7.

miRNA

MicroRNAs (miRNA) as biomarkers will also be discussed in more detail in Chapter 6.

Transglutaminase 2 (TGM2)

TGM2 is an enzyme that catalyses protein crosslinking and is involved in cellular apoptosis, extracellular matrix formation and cell adhesion, and migration[173, 174]. It has also found to be associated with increased distant metastases and chemoresistance[173]. As early as 2006 Verma et al demonstrated with that TGM2 expression in pancreatic tumours and cell lines were associated with a more invasive phenotype via activation of focal adhesion kinase (FAK)[174]. Both Verma and Zhang et al demonstrated that higher expression of TGM2 was associated with poor prognosis and gemcitabine resistance in patients with pancreatic cancer[173]. TGM2 has been found to enhance angiogenesis and is associated with increased expression of angiopoietins[175].

Oncostatin M (OSM)/ Oncostatin M receptor (OSMR)

OSM is a member of the IL-6 cytokine family. It forms part of the OSM receptor complex (OSMR) which activates JAK/STAT3 pathway via glycoprotein 130 (gp130, Figure 4.3) [176]. It has also been discovered to induce key epithelial-mesenchymal transition (EMT) transcription factors and increases tumour metastases *in vivo*[176]. Macrophage secreted OSM stimulates increased inflammatory gene expression in cancer associated fibroblasts[177]. OSMR is expressed in fibroblasts and perivascular cells[177]. Similar to OSM, OSMR expression has been demonstrated to correlate with inflammation, immune activation, and poor prognosis in pancreatic cancer[177].

TGM2 has been shown to be an important mediator in OSMR overexpression in squamous cell cervical cancer, making it an important and potentially targetable pathway[178].

The OSM/OSMR/TGM2 pathway was chosen for further validation studies (IHC and cell culture).

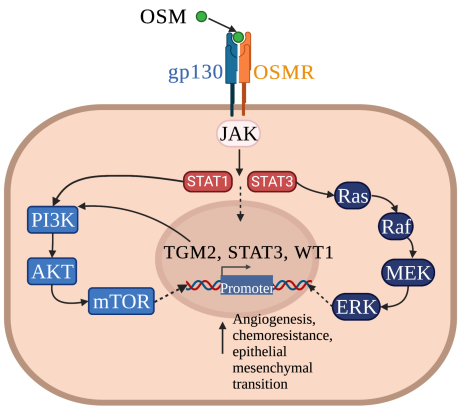


Figure 4.3 Pathways involved in chemoresistance. OSM forms part of the OSM receptor (OSMR) complex which activates the JAK/STAT pathway. TGM2 in the nucleus is activated by via STAT3 and the RAS pathway[179]. TGM2 is an activator of the PI3K/AKT pathway[178, 180, 181] RAS/RAF/MEK/ERK forms the mitogen activated protein kinase (MAPK) pathway.

4.2.1 Immunohistochemistry (IHC)

4.2.2 Methods

See 4.1.2 for inclusion criteria, ethics, and chemotherapy response.

IHC

Immunohistochemistry was performed as detailed in Ch 2: Materials and methods.

OSMR

Optimisation experiment with OSMR was performed, however there was minimal staining in positive controls. Further immunohistochemistry on OSMR was not performed.

TGM2

Immunohistochemistry was performed on surgical specimens of patients treated with neoadjuvant chemotherapy and was scored by intensity into weak and strong staining (Figure 4.4).

The log-rank test was used to evaluate survival. Spearman correlation was used to evaluate correlations. Data analysis was conducted in IBM (SPSS Version 27).

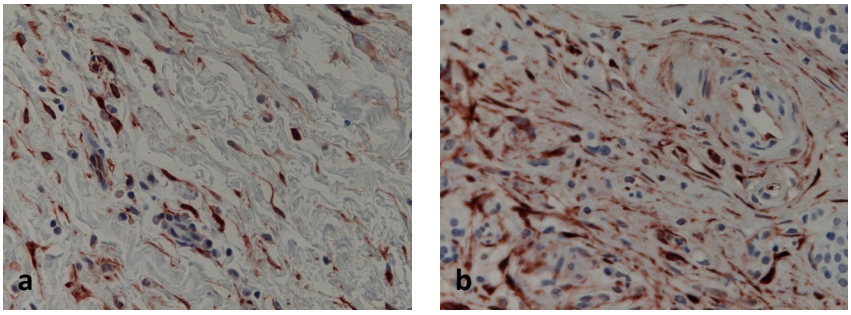


Figure 4.4 Staining TGM2; (a) weak staining, (b) strong staining on pancreatic surgical tissue after exposure to chemotherapy.

4.2.3 Results

4.2.3.1 Baseline characteristics

Immunohistochemistry using a TGM2 antibody was performed on the surgical specimens of 38 patients treated with neoadjuvant chemotherapy, this was a different cohort of patients to the 25 patients in the proteomics experiment (Table 4.4). Of these fifteen patients were treated with FOLFIRINOX, eighteen with gemcitabine plus abraxane, three with gemcitabine alone and one with chemoradiotherapy (using capecitabine), one patient's neoadjuvant chemotherapy regimen was unknown.

Table 4.4 Baseline characteristics

Baseline characteristics	N = 38
Age (median)	68
Gender	
Female	19
Male	19
Chemotherapy	
FOLFIRINOX	15
Gemcitabine + abraxane	18
Gemcitabine	3
Capecitabine + Radiotherapy	1
Unknown	1
Residual tumour	
≤30%	10
>30%	26
Unknown	2
TGM expression	
Weak	17
Strong	21

4.2.3.2 TGM2 as a prognostic biomarker

There was no difference in median recurrence free survival (p=0.21) or overall survival (p= 0.32) based on neoadjuvant chemotherapy regimen (Figure 4.5).

Thirty six out of 38 patients had a residual tumour score available to review. Of these ten patients had residual tumour of ≤30% and twenty-six >30%. Patients with a residual tumour of ≤30% had a significantly longer median recurrence free survival of 33 months compared to 18months in patients >30% residual tumour (p=0.008). Patients with residual tumour of ≤30% had a median overall survival of 42 months compared to 30 months, however this was not significant (p=0.14, Figure 4.6).

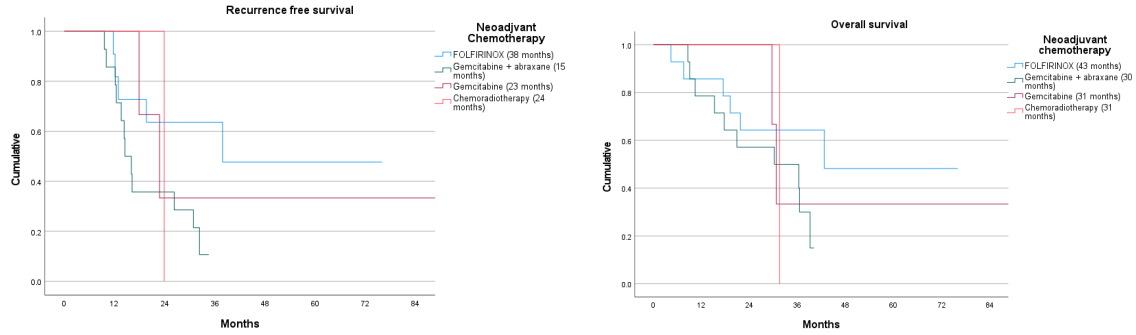


Figure 4.5 Recurrence free and overall survival based on chemotherapy regimen.

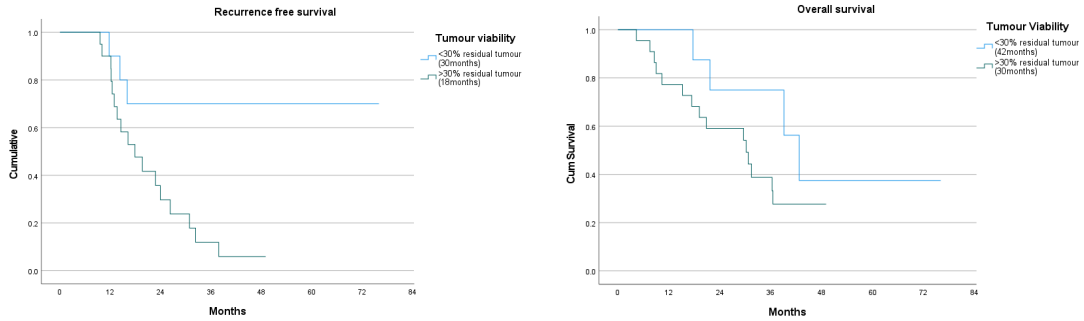


Figure 4.6 Recurrence free and overall survival based on tumour viability.

TGM2 expression was scored by intensity of staining in both the stromal and ductal cells and were divided into weak, moderate, and intense staining as per published literature[182] . Due to the distribution of scoring weak and moderate were combined and defined as ‘weak' staining. Seventeen patients had weak staining, and twenty-one had intense staining. There was no difference in recurrence free (p=0.70) or overall survival (p=0.74) based on TGM2 staining (Figure 4.7).

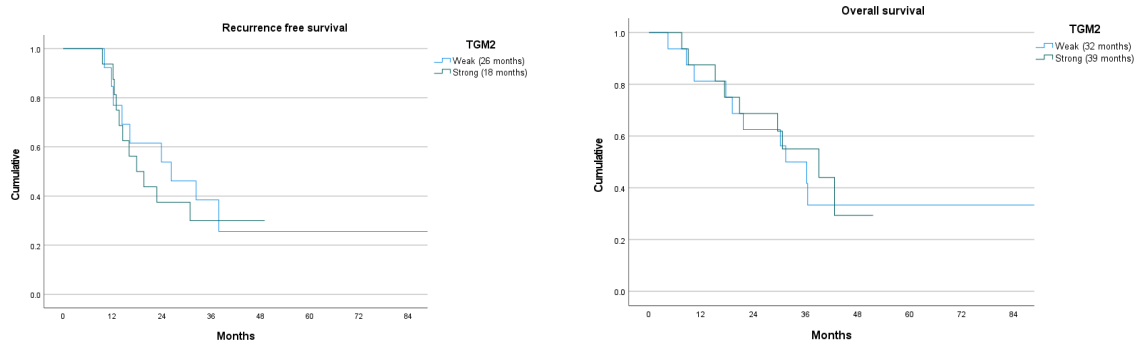


Figure 4.7 Recurrence free and overall survival based on TGM2 staining.

4.2.3.3 TGM2 as a predictive biomarker

TGM2 staining did not predict for tumour viability after chemotherapy (chi-squared 0.91).

4.3.1 Cell culture

4.3.2 Methods

Cell culture and Western blot methods are detailed in Chapter 2: materials and methods.

Reverse transfection was performed using PANC-1 and MIA PaCa-2 wildtype and gemcitabine resistant cells treated with TGM2-siRNA and OSMR-siRNA and negative control siRNA. Cell viability at 96 hours was assessed using an MTT assay. Western blot was conducted using cells from PANC-1 and MIA PaCa-2 cell lines.

Results were analysed by ANOVA in GraphPad Prism (GraphPad Software version 9, San Diego, California).

4.3.3 Results

4.3.3.1 Cell culture

Western blot confirmed the knockdown of TGM2 and OSMR genes with siRNA of TGM2 and OSMR respectively (Figure 4.8).

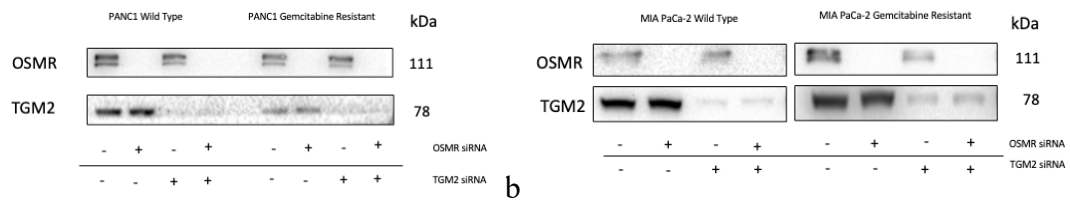


Figure 4.8 Western blot siRNA knockdown (a)PANC-1 (b)MIA PaCa-2

Cell viability of PANC-1 wildtype and PANC-1 Gemcitabine resistant cells

Cell plates were read at 96 hours after incubation. There was no difference in cell viability in PANC-1 wildtype in siRNA of TGM2 (p=0.13) or OSMR (p=0.26) knockdown compared to control siRNA. Similarly, cells treated with either TGM2 (p=0.59) or OSMR (p=0.69) siRNA had no difference in viability compared to control siRNA in PANC-1 gemcitabine resistant cells (Figure 4.9).

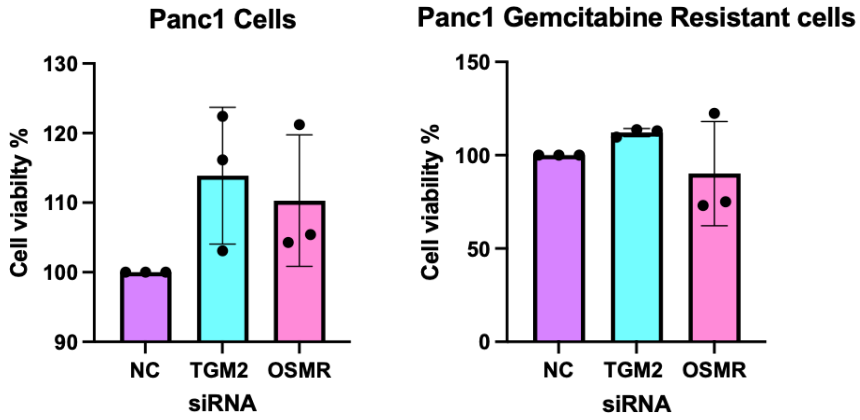


Figure 4.9 Viability of PANC-1 wildtype and PANC-1 gemcitabine resistant cells treated with control (NC) siRNA, TGM2 siRNA and OSMR siRNA.

Cell viability of MIA PaCa-2 and MIA PaCa-2 Gemcitabine resistant cells

Knockdown of TGM2 was associated with a significant reduction in cell viability to 65% (p=0.01) compared to control siRNA in MIA PaCa-2 wildtype cells. This was also true for cells treated with OSMR siRNA (p=0.02, Figure 4.10). Similar results were seen in the MIA PaCa-2 gemcitabine resistant cells treated with both TGM2 siRNA (59%, p=0.0001 and OSMR siRNA (79%, p=0.0001).

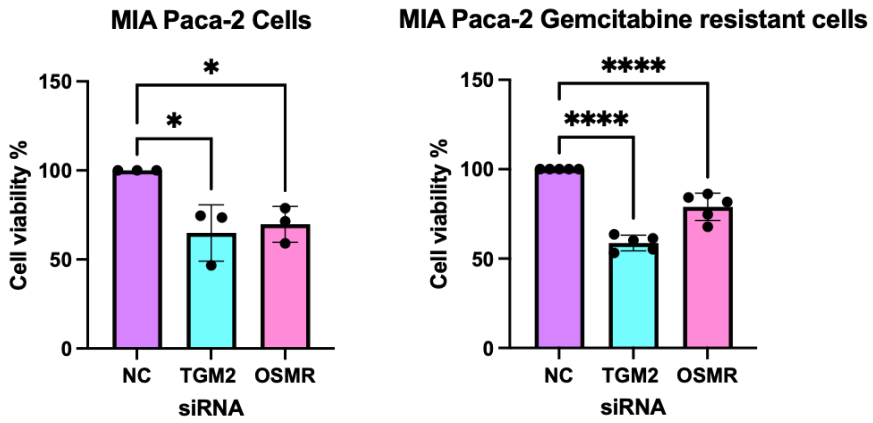


Figure 4.10 Viability of MIA-PaCa-2 wildtype and MIA-PaCa-2 gemcitabine resistant cells treated with control (NC) siRNA, TGM2 siRNA and OSMR siRNA.

4.3.3.2 Western blot treated with OSM cytokine.

In this experiment we aimed to investigate the role of OSM on expression of proteins in both PANC-1 and MIA PaCa-2 cells through western blotting by comparing cells treated with OSM to those that were not (Figure 4.11).

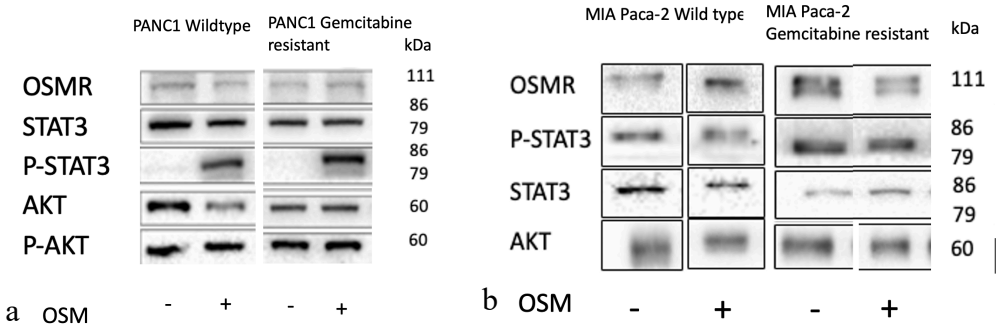


Figure 4.11 (a) PANC-1 and (b) MIA-PaCa 2 cell lines in presence and absence of OSM.

PANC-1 wildtype and gemcitabine resistant cells

The addition of OSM did not increase expression of OSMR, compared to the control group in PANC-1 cells (Figure 4.11). STAT 3 had similar expression in both groups, however phosphorylated STAT3 (p-STAT3) had higher expression in cells treated with OSM, compared to the control group. AKT and p-AKT expression was similar in OSM treated and control groups (Figure 4.11).

MIA PaCa-2 wildtype and gemcitabine resistant cells

OSMR expression was higher in OSM treated MIA PaCa-2 wildtype cells compared to control, however, were similar in MIA PaCa-2 gemcitabine resistant cells. STAT 3, p-STAT3 and AKT were comparable in the control and OSM treated groups. (Figure 4.11).

4.3.3.3 Western Blot treated with OSMR siRNA and TGM2 siRNA

PANC-1 and MIA PaCa-2 cells were treated with OSMR siRNA, control siRNA or TGM2 siRNA or both TGM2 and OSMR siRNA (Figure 4.12).

PANC-1 wildtype and gemcitabine resistant cells

Treatment with TGM2 and OSMR siRNA did not change expression of STAT3, p-STAT3, MAPK or p-MAPK in PANC 1 Cells (Figure 4.12). There was increased expression of p-AKT in siRNA TGM2 treated cells in PANC-1 wildtype cells.

MIA PaCa-2 wildtype and gemcitabine resistant cells

Treatment with TGM2 and OSMR siRNA did not change expression of STAT3, AKT or MAP-K in MIA PaCa-2 cells however the presence of TGM2 siRNA reduced p-STAT3 expression in both wildtype and gemcitabine resistant MIA PaCa-2 cells (Figure 4.12).

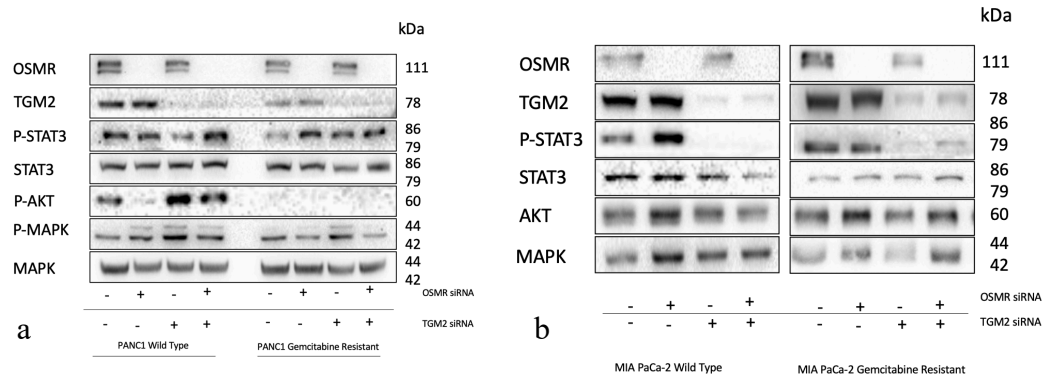


Figure 4.12 (a) PANC-1 and (b) MIA-PaCa 2 cell lines in presence and absence of OSMR and TGM2 siRNA.

4.4 Discussion

For patients with early pancreatic cancer the ideal upfront treatment between neoadjuvant chemotherapy and upfront surgery is unknown, posing a significant clinical challenge for the clinicians treating this terrible disease. Due to this equipoise the need for personalised biomarkers to assist with decision making is critical to improve the stagnating five-year

survival. A biomarker that could predict for chemoresistance would help identify if upfront surgery maybe more beneficial and may offer a potential therapeutic target.

In this chapter we discovered key proteins that were upregulated in tumours that were chemoresistant compared to chemosensitive tumours. Two proteins; Protein S100-P and NADPH dehydrogenase [quinone] 1 had high predictive ability for chemoresistance with AUROC >0.9. This is consistent with the literature demonstrating that both proteins are associated with increased angiogenesis and chemoresistance[183, 184].

Using IPA software, we were able to identify TGM2-OSMR as a key pathway that was upregulated in chemoresistant tumours. To further appreciate the capability of these key proteins as predictive and prognostic biomarkers, immunohistochemistry was performed. This identified that TGM2 expression did not predict for residual tumour or survival. This may be due to the small sample size, or it may be because TGM2 is just one protein involved in the many chemoresistance pathways of the pancreatic cancer cell. Our results from the western blot analysis demonstrated that addition of OSM to PANC-1 cells (both wildtype and gemcitabine resistant) increased expression of the activated form of STAT 3 (p-STAT3) whereas the siRNA knockdown of OSMR in these cells did not result in any change of expression in p-STAT3. Addition of OSM to MIA PaCa-2 did not change expression of any of the proteins tested which further highlights the complexity of these chemoresistant pathways (Figure 4.3). Finally, cell viability in MIA Paca-2 wildtype and gemcitabine resistant cells revealed that siRNA knockdown of either TGM2 or OSMR resulted in lower cell viability in both wildtype and gemcitabine resistant cells, offering a potential future therapeutic target.

There are weaknesses in these studies to note. In this chapter, we utilised tumour obtained from surgical specimens after exposure to chemotherapy. As such it cannot be assumed that the same number of proteins were present in the chemotherapy naive tumour, however, it still provides a much-needed insight into protein differences between chemoresistant versus chemosensitive tumours. Furthermore, our sample size was small with only twenty-five samples in the proteomic experiment and thirty-eight in the immunohistochemical experiment.

4.5 **Conclusion**

This exploratory study identified potential key proteins and pathways that may be involved in the chemoresistance mechanisms in pancreatic tumours. Whilst the immunohistochemical

results failed to support these findings, further analysis with larger cohorts is necessary. In addition, the results of reduction in tumour viability with siRNA knockdown of TGM2 and OSMR warrant further investigation as a potential future therapeutic target.

CHAPTER 5:

Biomarker discovery on biopsy and surgical specimens: immunohistochemistry

5. 1 Introduction

Whilst the survival rates of pancreatic cancer stagnate at 12% at five years, our understanding of the pathogenesis of pancreatic has dramatically changed in recent years[1]. Mouse models have demonstrated that cancer cell migration occurs early on this disease, often prior to tumour formation at the primary site[185]. In addition, circulating tumour cells (CTCs) have been found in patients with early-stage disease adding to the theory that pancreatic cancer is a systemic disease from the onset[186]. It is this concept, combined with the success of neoadjuvant chemotherapy (NAC) in downstaging locally advanced tumours, that has seen the introduction of early intervention with chemotherapy in patients with early (upfront or borderline) resectable disease. As discussed in the previous chapters, whether this approach affords superior outcomes to upfront resection, is yet to be demonstrated.

Previous research conducted by our research team, Nahm et al, identified a biomarker panel that can assist with prognosis and predict optimal treatment strategy in patients with upfront or borderline resectable disease[161, 187, 188]. Three proteins; S100A4, Ca-125 and mesothelin were identified as prognostic biomarkers, with expression of these biomarkers associated with a shorter overall survival[161]. In addition, patients with triple positive expression (signifying a more aggressive phenotype) treated with neoadjuvant chemotherapy, had significantly longer overall survival then those who had upfront surgery. In contrast, patients with triple negative expression performed better if treated with upfront surgery[187]. In both these studies, the biomarker expression was assessed using immunohistochemistry (IHC) on surgical specimens in both the chemotherapy naive population (upfront surgery) and in patients that had been exposed to chemotherapy (neoadjuvant chemotherapy cohort). Our research aimed to build on this knowledge, by evaluating the feasibility of assessing this biomarker panel in patients who received NAC using tissue obtained via endoscopic ultrasound fine needle aspiration prior to commencement of any systemic therapy.

CA 125

CA 125 is a membrane bound glycoprotein, present in normal mesothelial cells. Increased expression is seen in certain tumour types, most notably ovarian cancer and pancreatic cancer[189]. Up to 68-92% of pancreatic tumours have positive CA125 staining on IHC[189, 190]. As early as 2011, Einama et al identified CA125 as a potential prognostic biomarker in pancreatic cancer with high expression associated with a significantly shorter median overall survival at

21 months compared to 40 months in patients with no expression (p=0.03)[189]. This study also found a positive correlation with CA125 and mesothelin expression (p=0.006).

Mesothelin

Mesothelin is another membrane bound glycoprotein and is expressed in normal mesothelial cells, and in pancreatic cancer cells[189]. It is encoded by the *MSLN* gene[191]. Whilst the pathophysiology is not well understood it is thought that mesothelin expression increases cell proliferation and migration[189]. Positive staining is seen in 86-100% of pancreatic cancer tumours[192]. Einama demonstrated increased expression was associated with higher tumour grade and vessel invasion as well as shorter recurrence free and overall survival. Patients with a positive expression had a median overall survival of only 20 months compared to patients with no expression at 35 months (p=0.012)[189].

S100A4

S100A4 is encoded by the *S100A4* gene and is located in the cell membrane, nucleus and cytoplasm of pancreatic cancer cells[193]. It is thought to be involved in metastatic spread and it is found in 67- 93% of pancreatic cancer lesions, with no expression in normal pancreatic tissue[194, 195]. Dreyer et al demonstrated that positive expression for S100A4 was associated with poor median overall survival of only 16 months compared to 29 months in patients with no expression (p<0.001)[195]. In this study, 17 endoscopic ultrasound biopsies were analysed and correlated with the surgical specimen in 82% of cases.

Endoscopic ultrasound

The diagnosis of pancreatic cancer requires tissue confirmation. In patients with localised disease this is performed either through a fine needle aspirate during an endoscopic ultrasound (EUS) or via brushings from bile duct endoscopic retrograde cholangiopancreatography (ERCP). The tissue obtained via these methods are often sufficient for a diagnosis, however inadequate for further molecular testing. Constantinescu et al demonstrated the feasibility of immunohistochemistry on fine needle aspiration from pancreatic tumours in 57 patients[196]. In this study up to 3 passages through the tumour were taken. The main complications associated with endoscopic ultrasound guided biopsy are bleeding and risk of pancreatitis[197]. Whilst evidence is conflicting regarding the association with more passages and increased risk of pancreatitis, clinicians are still reluctant to obtain additional tissue to allow for additional molecular testing outside the clinical trial setting[197, 198].

Hypotheses

1. To validate the findings of CA 125, mesothelin and S100A4, from previous research conducted by our team on pancreatic cancer surgical specimens and endoscopic aspirates.
2. Patients with triple positive staining will have improved overall survival if treated with neoadjuvant chemotherapy versus upfront surgery and patients with triple negative staining will have longer survival with upfront surgery.
3. Positive staining of either CA 125, mesothelin or S100A4 on fine needle aspirates prior to treatment will correspond with higher residual tumour (tumour viability) on surgical specimen (and hence reduced chemotherapy response) in patients that received neoadjuvant chemotherapy.
4. Presence of staining of CA125, mesothelin and S100A4 in fine needle aspirates taken at diagnosis will strongly correlate with expression in matched surgical expression both in patients that have undergone neoadjuvant chemotherapy and those who have proceeded directly to upfront resection.

Aims

1. To assess the feasibility of performing immunohistochemical analysis of three biomarkers (CA125, mesothelin and S100A4) on diagnostic biopsy obtained from archived fine needle aspirate in patients diagnosed with pancreatic cancer and to investigate the relationships of these biomarkers with residual tumour and survival.
2. To correlate expression of biomarkers in biopsy tissue to their matched surgically resected specimen.

5.2 Methods

Methods in detail are presented in Chapter 2: Materials and methods.

Inclusion criteria

Patients were identified through review of local pancreatic cancer database. Only patients who had undergone endoscopic ultrasound guided diagnostic biopsy and had unused cell blocks available for analysis were included. Tumours of all stages including upfront resectable,

borderline, locally advanced were included regardless of whether they underwent surgical resection. When available results of these biopsies were compared to the matched surgical specimen.

Ethics

As per the Human Tissue Act 1983, a waiver of consent can be granted for a human research ethics committee if tissue was removed for purpose other than research (such as diagnostic biopsy) and is now to be used for research and is currently held in a tissue block or slide. Ethics approval was obtained from the Northern Sydney Local Health District (NSLHD) Human Research Ethics Committee (HREC; Ref# 2019/ETH08639).

Immunohistochemistry

Immunohistochemistry was performed on fine needle aspirates and surgical specimens as specified in chapter 2. The two assessors were blinded to patient survival.

Clinical data such including chemotherapy, radiology, or histopathology as well as progression free and overall survival was obtained for these patients.

Tumour viability was determined by the assessment of residual tumour in the surgical specimen in patients treated with neoadjuvant chemotherapy. Using cut-off from our previous research, tumours with ≤30% were considered 'chemosensitive' and tumours >30% were considered chemoresistant[169]. In this chapter, the terms 'residual tumour' and 'tumour viability' were used interchangeably.

Data analysis

The log rank and cox hazards method were used to evaluate survival. Chi-squared was used to compare dichotomous groups. Cohens kappa was used to evaluate the relationship of binary data. All analyses were performed in IBM (SPSS Version 27).

5.3 Results

5.3.1 Baseline characteristics

One-hundred and eighteen patients were diagnosed with either upfront, borderline resectable or locally advanced pancreatic cancer at Royal North Shore Hospital between the years 2016-2020. Of these only 64 patients had sufficient remaining tissue from biopsy in formalin fixed

paraffin embedded tissue blocks and 73 patients had sufficient tumour surgical tissue to be included. In total 80 different patients had either biopsy or surgical tumour available for testing (Figure 5.1).

The median age at diagnosis was 70 years. Forty-five patients were female (57%) and 35 (43%) were male. Forty-nine patients were classified as upfront resectable, and 46 (57%) underwent neoadjuvant chemotherapy. The most frequently used neoadjuvant chemotherapy regimen was either gemcitabine or gemcitabine plus nab- paclitaxel (abraxane, 52%). The most common adjuvant chemotherapy was gemcitabine plus capecitabine (22%, Table 5.1).

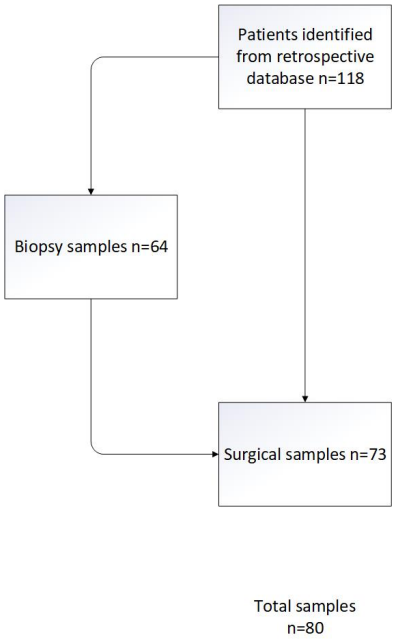


Figure 5.1 Biopsy and surgical samples.

Table 5.1 Baseline characteristics. [+]FOLFIRINOX (5- fluorouracil, irinotecan, oxaliplatin).

Baseline characteristics	
Age (Median)	70
Gender	
Female	45 (57%)
Male	35 (43%)
Upfront resectable status	
Upfront resectable	49 (61%)
Borderline	11 (14%)
Locally advanced	16 (20%)
Unknown	4 (5%)
Upfront treatment	
Upfront resection	34 (43%)
Neoadjuvant chemotherapy	46 (57%)
Neoadjuvant regimen	
Gemcitabine plus abraxane	17 (37%)
Gemcitabine	7 (15%)
FOLFIRINOX [+]	13 (28%)
Chemoradiation	1 (2%)
Unknown	8 (17%)
Adjuvant regimen	
Gemcitabine plus capecitabine	18 (25%)
Gemcitabine	14 (19%)
Nil	10 (15%)
Unknown	31 (41%)

Prognostic biomarkers

5.3.2 Survival

Forty-eight (62%) of patients had recurred and 27 (36%) of patients were alive. The median recurrence free survival was 19 months and median overall survival was 30 months.

Neither Eastern co-operative oncology group (ECOG) performance status (p=0.46, p=0.41), nor Charleson co-morbidity status (p=0.49, p=0.85) were predictive for recurrence free or overall survival.

5.3.2.1 By upfront resectability

There was no difference in recurrence free survival based on upfront resectability status (p=0.67, Figure 5.2). Upfront resectability did however, predict for overall survival, with median overall survival in patients of 36 months in those with upfront resectable and 18 months in those with locally advanced disease (p=0.03, Figure 5.2).

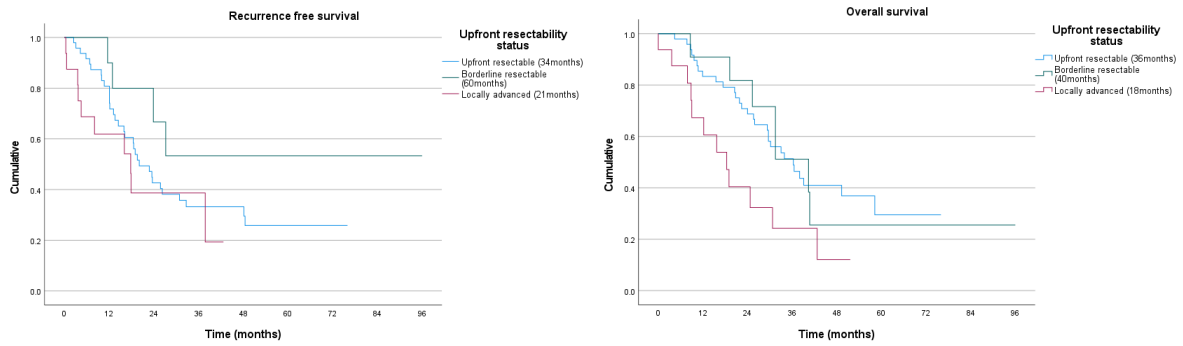


Figure 5.2 Recurrence free and overall survival by upfront resectability status. Medians are shown in the text for each group.

5.3.2.2 Upfront versus neoadjuvant chemotherapy

Thirty-five patients were treated with neoadjuvant chemotherapy. There was no difference in either recurrence free (p=0.89) or overall survival (p= 0.22) in patients treated with neoadjuvant chemotherapy versus patients undergoing upfront resection. For patients with upfront resectable disease, median survival of both upfront resectable patients and those receiving neoadjuvant chemotherapy was comparable (median 34 v 36 months respectively, p=0.68). All patients with borderline or locally advanced disease were treated with neoadjuvant chemotherapy.

5.3.2.3 Tumour viability and surgical stage

Patients with residual tumour>30% had similar recurrence free survival to patients with residual tumour ≤30%, p=0.91), however, residual tumour >30% did predict for a worse overall survival (p=0.04, HR 4.86). Surgical stage was determined using TNM 8th edition and was not associated with either recurrence free (p=0.15) or overall survival (p=0.25)[199].

5.3.2.4 CA125, Mesothelin and S100A4

The scoring used for each biomarker is listed in Table 5.2 and Chapter 2: Materials and methods. The percentage of patients with each score are listed in Table 5.3. Scores were initially marked from 0-3 based on % of cells stained. After all slides were scored, dichotomous cut-offs were then chosen based on frequency of expression and consultation with the literature (Table 5.2, Figures 5.3, 5.4).

Table 5.2 Scoring for antibodies.

Antibody	**Scoring**
CA 125 Biopsy and surgical tissue	0 Negative or <10% of cells staining. 1 ≥10% of cells staining
Mesothelin Biopsy and surgical tissue	0 Negative 1 Any positive staining
S100A4 Biopsy and surgical tissue	0 Negative or cytoplasm staining only. 1 Positive cytoplasm and nuclear staining

Table 5.3 Staining in biopsy and surgical specimens.

			Surgical staining		
			Negative	Positive	Total
Biopsy staining	**CA125**	Negative	25	9	34
		Positive	4	12	16
		Total	29	21	50
	Mesothelin	Negative	23	9	32
		Positive	4	14	18
		Total	27	23	50
	S100A4	Negative	9	9	18
		Positive	10	16	26
		Total	19	25	44

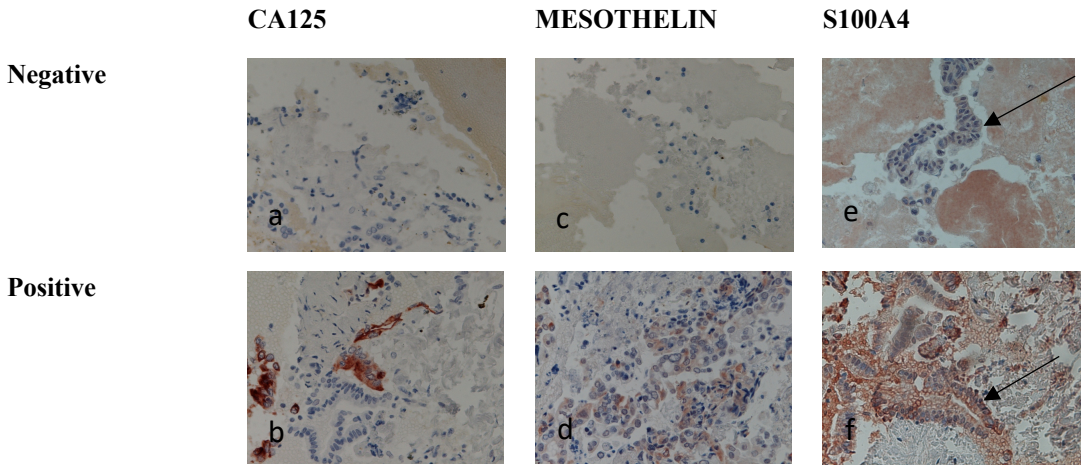


Figure 5.3 Representative images for immunohistochemical staining on biopsy samples at 40x magnification for CA125 a) negative, b) positive staining, Mesothelin c) negative, d) positive staining and S100A4 e) negative, and f) positive staining.

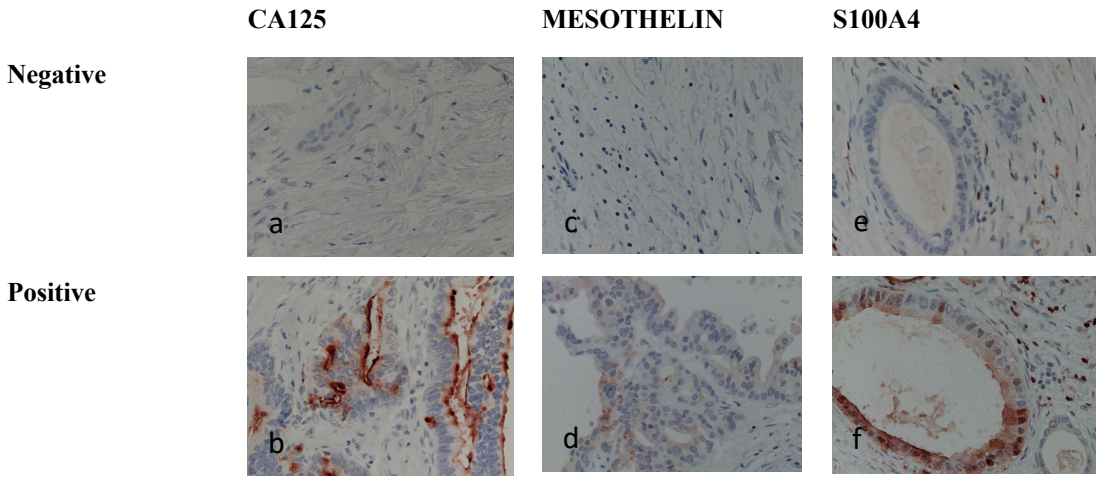


Figure 5.4 Representative images for immunohistochemical staining on surgical samples at 40x magnification for CA125 a) negative, b) positive staining, Mesothelin c) negative, d) positive staining and S100A4 e) negative, and f) positive staining.

5.3.2.5 CA 125

Negative staining of CA125 in biopsy samples was associated with a longer survival (median 40 months) compared with positive staining (median 30 months, p = 0.048). Median recurrence free survival was also higher in patients with negative staining (31 months versus 19 months); however, this was not significant (p=0.29). Similarly, negative staining in surgical samples was associated with a longer survival (median 43 months) compared to positive staining (median 25 months, p=0.023). There was no difference in recurrence free survival based on CA125 expression in surgical specimens (p=0.29, Figure 5.5).

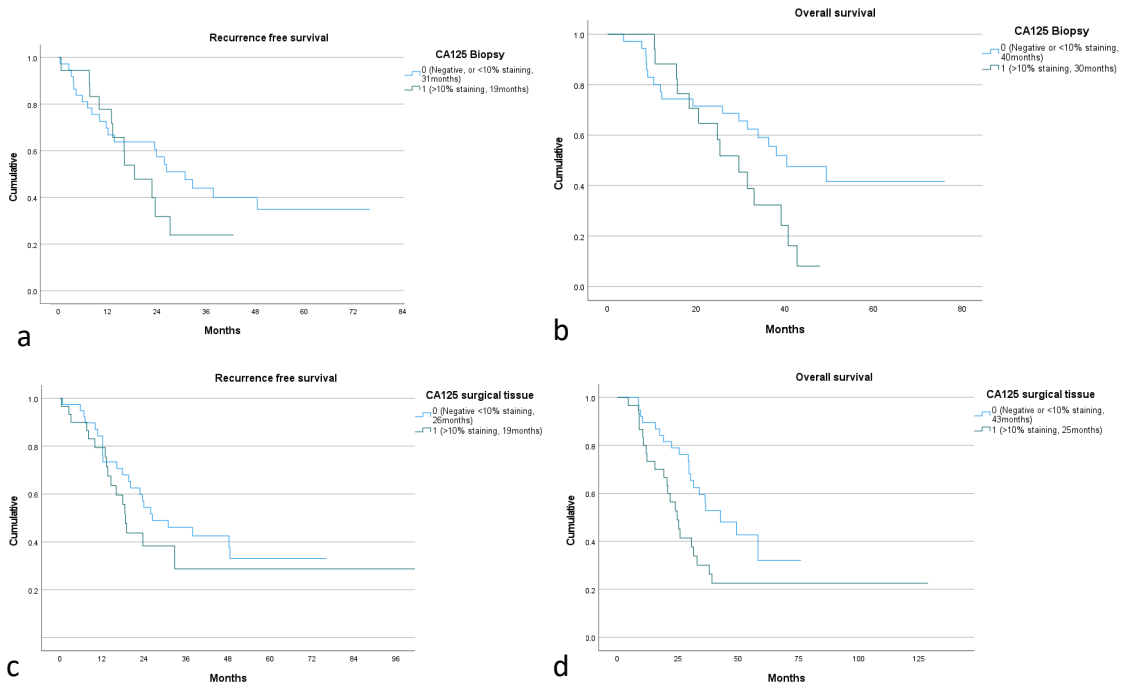


Figure 5.5 Recurrence free (a) and overall survival (b) based on CA125 expression on biopsy samples. Recurrence free (c) and overall survival (d) based on CA125 expression on surgical samples.

5.3.2.6 Mesothelin

Patients with positive mesothelin expression on biopsy did have a shorter median recurrence free and overall survival, however, the difference was non-significant (p=0.53, p=0.86, respectively, Figure 5.5). Similar results were also seen with mesothelin expression on surgical samples (p= 0.21, p=0.41 respectively, Figure 5.6).

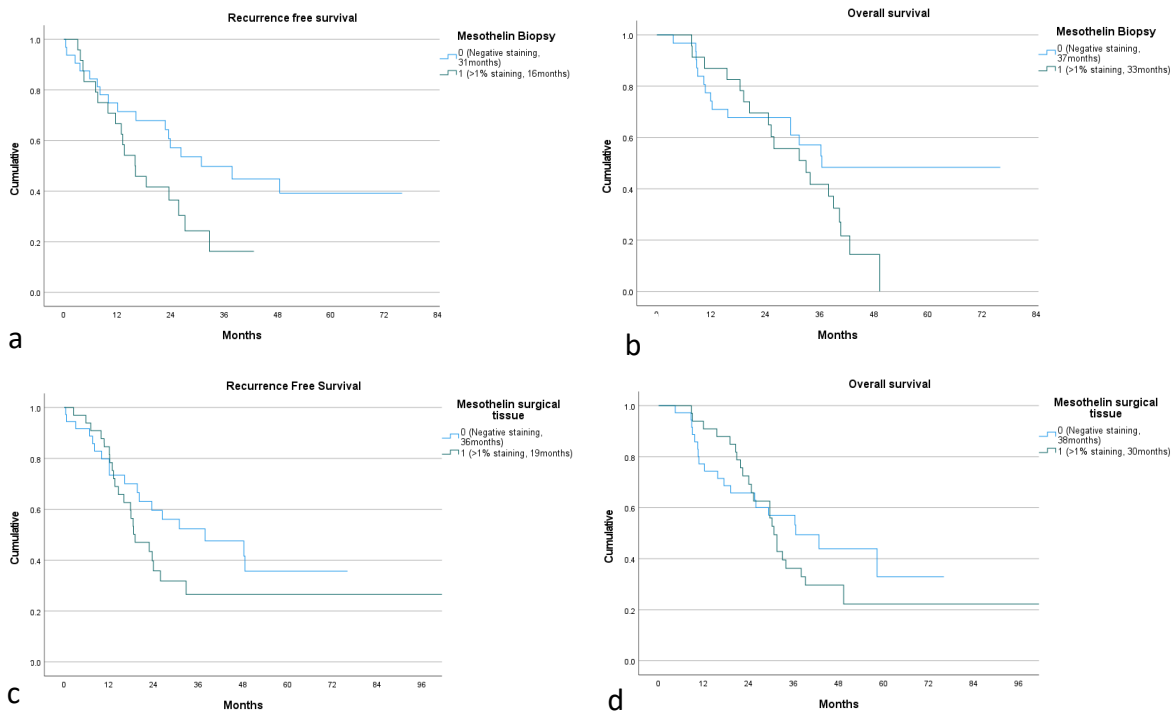


Figure 5.6 Recurrence free (a) and overall survival (b) based on mesothelin expression on biopsy and surgical samples. Recurrence free (c) and overall survival (d) based on mesothelin expression on surgical samples.

5.3.2.7 S100A4

S100A4 expression on biopsy specimen did not predict for either recurrence free (p=0.47) or overall survival (p=0.27). Similarly, S100A4 staining on surgical specimen was not a useful prognostic biomarker (Figure 5.7).

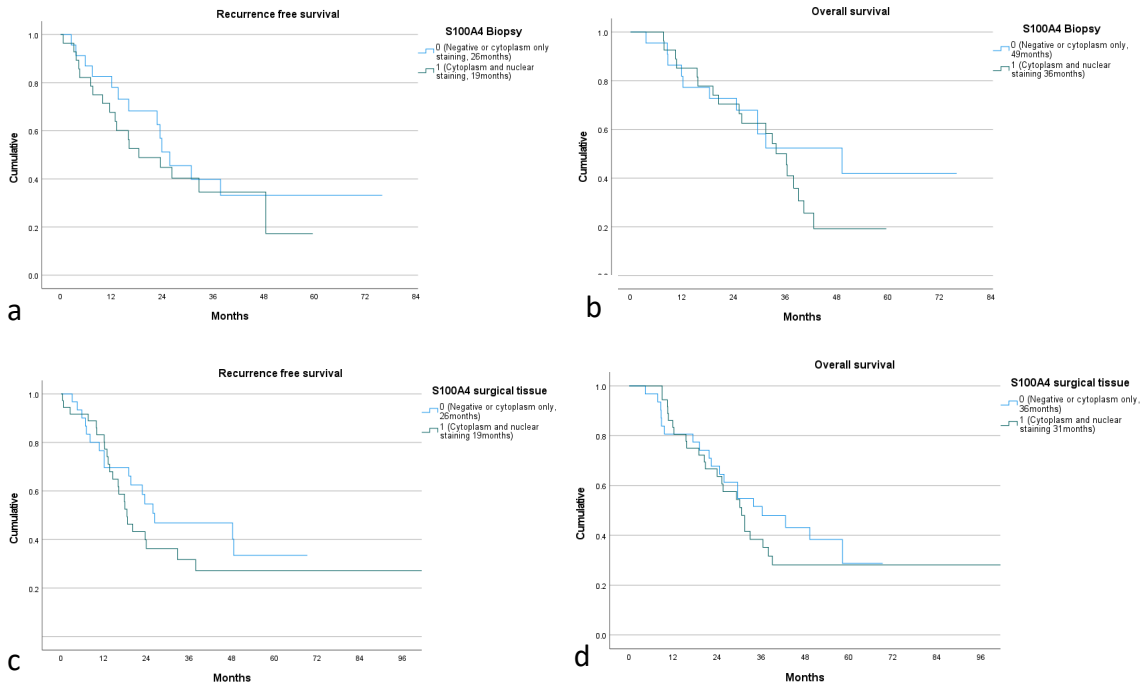


Figure 5.7 Recurrence free (a) and overall survival (b) based on S100A4 expression on biopsy. Recurrence free (c) and overall survival (d) based on S100A4 expression on surgical specimen.

Using *a-priori* factors known to impact on survival including ECOG, Charleson co-morbidity status and histopathological stage, staining of three antibodies on both biopsy and surgical specimens were assessed using multivariate analysis. Only CA125 (RFS: p=0.002, HR 82; OS: p=0.008, HR 85) and S100A4 in the surgical specimen were useful prognostic factors on multivariate testing, with shorter survival in patients that had negative S100A4 (RFS: p=0.009, HR 0.71; OS: p=0.014, HR 0.037) expression.

Table 5.5 Univariate and Multivariate analysis, * indicates significance.

	Univariate				Multivariate			
	Recurrence free survival		Overall survival		Recurrence free survival		Overall survival	
	p value	Hazard ratio	p value	Hazard Ratio	p value	Hazard ratio	p value	Hazard Ratio
ECOG	0.45	1.28	0.41	1.28	0.90	0.94	0.32	1.65
Charleson comorbidity status	0.49	1.06	0.85	0.98	0.39	1.16	0.58	0.90
Histopathological stage	0.15	1.27	0.25	1.21	0.15	1.72	0.14	11.86
CA125 Biopsy	0.30	1.48	*0.048	2.03	0.98	1.02	0.74	1.23
CA125 Surgical specimen	0.29	1.41	*0.026	2.0	*0.002	82.00	*0.008	85.07
Mesothelin Biopsy	0.57	1.95	0.09	1.81	0.11	0.185	0.05	0.16
Mesothelin Surgical specimen	0.21	1.50	0.42	1.29	0.77	5.01	0.37	2.21
S100A4 Biopsy	0.47	1.30	0.28	1.51	0.13	0.31	0.97	0.97
S100A4 Surgical specimen	0.35	1.35	0.44	1.27	*0.009	0.71	*0.014	0.037

5.3.3 Biomarkers to predict ideal upfront treatment

Only sixteen patients had triple negative expression of all three proteins in biopsy samples, with nine patients having triple positive expression. For patients with either triple negative expression (p=0.60) or triple positive (p=0.75) expression there was no difference in median overall survival between upfront resection and neoadjuvant chemotherapy. There was no difference in recurrence free (p=0.2) or overall survival (p=0.18) in patients with triple positive or triple negative expression.

5.3.4 Predictive biomarkers (of chemoresistance)

There was no correlation between presence of staining of either CA125 in biopsy (chi-squared p=0.30), mesothelin (chi-squared, p=0.88) or S100A4 (chi squared, p=0.07) with chemotherapy response (residual tumour) in the surgical specimen, in patients treated with neoadjuvant chemotherapy.

5.3.5 Biopsy and matched surgical specimen

Forty-four patients had matched biopsy and surgical specimens. The number of samples that stained positive and negative are listed in Table 5.3. The relationship between positive and negative expression in the biopsy and matched surgical specimen were assessed using Cohen's kappa. For all patients (upfront surgery and neoadjuvant chemotherapy), there was a moderate and statistically significant relationship between both mesothelin biopsy and surgical specimen ($k=0.47$, $p<0.001$) and CA125 biopsy and surgical specimen ($k=0.45$, $p=0.001$). There was no association between S100A4 biopsy and surgical resection ($k=0.11$, $p=0.45$). For the patients that underwent NAC, there was a moderate and significant correlation between mesothelin biopsy and surgical specimen ($k=0.42$, $p=0.03$), however CA125 biopsy and surgical specimens were not significantly concordant ($k=0.35$, $p=0.67$). Similar to the whole cohort there was no association with S100A4 between biopsy and surgical specimen ($k=0.21$, $p=57$).

5.4 Discussion

Earlier research conducted by our team highlighted the prognostic role of a biomarker panel consisting of three proteins: mesothelin, CA 125 and S100A4 on surgical specimens of patients diagnosed with pancreatic cancer. These studies found that positive staining for one or all the proteins were predictive for a more aggressive phenotype[161, 187]. In addition, patients with positive staining had longer survival if they were treated with chemotherapy first, highlighting that more aggressive biology may indicate more micro-metastatic disease and hence treatment with early systemic therapy may be advantageous[161, 187]. These studies were conducted using specimens from surgery in both the chemotherapy naïve population (for patients that underwent upfront resection) and in patients that received neoadjuvant chemotherapy. Whilst the results were promising, there are limitations to their use. As the panel was tested on some tumours after neoadjuvant chemotherapy exposure, it is difficult to appreciate if the same expression was present in the tumour prior to chemotherapy. The aims of this chapter were to assess the feasibility of testing these three biomarkers on biopsy material taken via endoscopic biopsy at time of diagnosis and to evaluate their use as prognostic and predictive biomarkers in patients with localised pancreatic cancer.

Our study found that immunohistochemical testing on fine needle aspirates was challenging. Of the 118 biopsy samples requested for analysis only sixty-four patients (54%) had sufficient residual tumour in the cell block to allow for biomarker assessment. Of these, only 53 patients

had sufficient cells to allow testing of all three antibodies with their matched surgical specimens, with slides of less than twenty cells excluded from analysis. Despite the small sample size, we were able to identify that the presence of staining of CA125 in both biopsy and surgical samples predicted for a significantly shorter overall survival, compared to negative staining. Patients with negative Mesothelin and S100A4 expression in both biopsy and surgical tissue did have longer overall survival then patients with positive staining, however, these failed to meet significance. The lack of statistical significance of these results, may, in part, be due to the small sample size, or the due to differences in fixation methods between surgical and biopsy specimens. Multivariate analysis revealed CA125 and S100A4 were useful as prognostic biomarkers, however, in contrast to the rest of the results, it was positive staining of S100A4 that was associated with a favourable prognosis. These results highlight the need for testing in a larger cohort of patients. For this to be achieved, improvements in methods to obtain tissue from endoscopic ultrasound are needed and whilst there is a reluctance from physicians to obtain more tissue, there is no evidence to suggest that more passes lead to an increase risk of pancreatitis[197, 198, 200].

The final aim of this experiment was to assess the relationship between expression of these proteins in the biopsy and the matching surgical specimen in all patients regardless of upfront treatment. This study found that a moderate but significant correlation in levels of expression existed between biopsy and surgical specimens of both mesothelin and CA125. This is important as it informs us about changes (or lack thereof) in tumoral biology over time and after exposure to chemotherapy.

There are several limitations in this study. Firstly, despite the presence of two independent assessors, scoring of immunohistochemistry remains subjective. Secondly, the number of samples were small, due to the difficulty in obtaining samples with sufficient cells, and as such we were unable to appreciate any survival advantage in patients with triple positive or triple negative expression based on upfront treatment. In addition, the number of cells obtained via biopsy were few and thus expression may not be representative of the rest of the tumour. With the surgical specimens, a higher proportion of tumour cells are obtained thus the proportion of positive or negative staining can be better assessed. Despite this, our findings demonstrate a positive relationship between biopsy and surgical specimen reflecting that biopsy specimens may be a more accurate representative of the rest of tumour than originally anticipated.

5.5 Conclusion

This study highlights that immunohistochemical testing on fine needle aspirates from endoscopic biopsies in the diagnosis of pancreatic cancer is challenging. Whilst only half the number of patients had sufficient tumour to test this biomarker panel, it did provide us with some preliminary results. Our research highlighted the potential for CA125 expression in both biopsy and surgical samples to predict prognosis in patients with localised pancreatic cancer. In addition, we were able to identify that CA125 and mesothelin staining on biopsy material correlated with matched surgical tissue, highlighting that testing of this tissue at time of diagnosis may provide prognostic information for the patient. Further studies in the prospective setting are needed to validate these findings.

*Protocol for prospective study found in Appendix 5.

CHAPTER 6:

Circulating biomarker discovery: microRNA and cytokine panel

6.1 Introduction

Despite the identification of novel biomarkers in recent years highlighted in Chapter 1, there continues to be a lack of validated prognostic and predictive biomarkers to assist clinicians with management of pancreatic cancer. In part this is secondary to the difficulty in obtaining biological material, and often the only source of tissue available is in surgical specimens in patients that have undergone resection, and often in patients that have been exposed to chemotherapy. The ideal tissue to analyse would be at time of diagnosis to allow for early identification of biomarkers with the view for them to direct clinical management. Due to the anatomical location of the pancreas, diagnosis is commonly made via endoscopic ultrasound plus biopsy usually involving a fine needle aspirate of the lesion. This type of biopsy often contains sufficient tissue to obtain a diagnosis, with limited residual tissue to allow further molecular testing as discussed in Chapter 5.

Circulating blood based biomarkers have become more prominent of late. As highlighted in Chapter 1, serum CA 19.9 is currently the only validated biomarker used in the clinical management of pancreatic cancer, however several others have demonstrated early promise[33, 34].

Recent years has seen the identification of the important roles of microRNAs and cytokines in the modulation of the tumour-immune response and as potential biomarkers.

6.1.1 miRNA

MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression by binding to messenger RNA (mRNA)[201, 202]. Since the discovery of their involvement in human malignancy in 2002, several studies have demonstrated that miRNA expression is often dysregulated in cancers, including pancreatic cancer[132, 203].

MAPK/RAS signalling

More than ten miRNAs are thought to play a role in expression of KRAS[204]. KRAS mutations occur in up to 95% of pancreatic tumours and result in the activation of downstream pathways including MAPK/ RAS (Figure 4.3) [205]. *In vitro* overexpression of miRNA 217, miRNA 96 and let-7, are associated with a reduction of KRAS expression, and thus reduction in KRAS mediated cell signalling and proliferation [204, 205].

Apoptosis

Similar to KRAS, many miRNAs influence cell apoptosis[205]. Low levels of miRNA-34a and miRNA 21 which are common in pancreatic tumours lead to higher expression of Bcl-2 which has an anti-apoptotic effect[206]. Tumour suppressor genes *PDCD4* and *PAF1* are also influenced by multiple miRNAs and are integral to tumour cell apoptosis[205, 206].

Chemoresistance

A number of miRNAs have been found to be associated with chemoresistance[207]. In particular, higher levels of miRNA 21 has been shown to be a poor prognostic biomarker in early pancreatic cancer patients receiving adjuvant gemcitabine chemotherapy[132]. In vitro studies have identified that downregulation of miRNA21 (through indole-3-carbinol) resulted in increased cytotoxicity to gemcitabine[208].

6.1.2 Cytokines

Disruption of the immune response is a hallmark of cancer pathogenesis, and cytokines play an important role in linking the innate and adaptative immune system[172]. Of these, emphasis has been on the role of cytokines both to assist in our understanding of the role of the immune system in cancer development, as well as potential therapeutic targets[172]. Higher levels of IL-6 and IL-10 are found in pancreatic cancer patients and are associated with JAK2- STAT3 activation resulting in increased proliferation and epithelial mesenchymal transition (EMT)[209, 210]. IL-8 has been shown to increase ERK and MAPK activation and both IL-6 and IL-8 are involved in the regulation of VEGF and thus angiogenesis in pancreatic cancer cells[211].

Cachexia

Cancer cachexia is common in pancreatic cancer patients and is associated with systemic inflammation and weight loss. Multiple cytokines have been implicated including TNF-α, IL-6 and IL-8[122]. The presence of cachexia is a poor prognostic factor and limits the ability of clinicians to give intensive treatments[212].

Chemoresistance

Whilst the mechanisms involved in chemoresistance are complex, it is thought that presence of cytokines may predict for chemoresistance. Delitto et al, demonstrated that patients with a poor response to neoadjuvant chemotherapy (NAC) as demonstrated by high residual tumour at time of surgery, had higher levels of circulating TNF-α and IL-β1 then patients with a

moderate to good response to NAC[213]. Similarly Van der Sijde et al discovered that IL-1RA levels following one cycle of FOLFIRINOX were predictive of chemotherapy response, with lower levels indicative of chemoresistance[214].

Further discussion of the immune system in pancreatic cancer is covered in Chapter 7.

Hypotheses

1. Patients with residual tumour ≤30% will have different expression of plasma miRNA and serum cytokines to patients with residual tumour >30%.
2. Using tumour viability on surgical samples as a marker of chemotherapy response, we will be able to identify key miRNA and cytokine in matched blood that can predict for higher versus lower residual tumour, and hence chemoresistance.
3. These miRNA and cytokines biomarkers will also be useful prognostic biomarkers.

Aims

1. To identify plasma miRNA or serum cytokines that are significantly upregulated in patients that have higher versus lower residual tumour after exposure to chemotherapy.
2. To assess the prognostic potential of identified miRNA and cytokine biomarkers in Aim 1.

6.2 Methods

Inclusion criteria

Patients were identified through review of local pancreatic database. Only patients with either upfront resectable, borderline, or locally advanced tumours treated with neoadjuvant chemotherapy and underwent surgical resection were obtained. Both serum and plasma (including CA 19.9) were taken from patients at time of surgery (after completion of neoadjuvant chemotherapy).

Ethics

Ethics approval was obtained from the Northern Sydney Local Health District (NSLHD) Human Research Ethics Committee (HREC; Master Ethics Ref# 2019/ETH08639 and associated LNRs Ref# 2023/ETH01082 and 2022/ETH01811).

Chemotherapy response

Clinical data including chemotherapy, recurrence and overall survival and histopathological regression scores were also obtained. As an ideal scoring method for assessment of tumour viability in pancreatic cancer does not exist, tumours were divided into good response to chemotherapy (≤30% residual tumour) or 'chemosensitive' tumours versus poor response to chemotherapy (>30% residual tumour) or 'chemoresistant' tumours on histopathology[169]. In this chapter, the terms 'residual tumour' and 'tumour viability' were used interchangeably.

6.2.1 miRNA analysis

miRNA extraction was performed using the QIAGEN® minElute® virus spin and QIAmp® circulating nucleic acid protocol on plasma samples collected by our research team. Further details on plasma processing and miRNA extraction are detailed in Chapter 2. RNA was then frozen at -80℃ prior to miRNA analysis. miRNA was analysed using nCounter® miRNA expression assay protocol as listed in Chapter 2.

Analysis of raw data was performed using Nanostring nSolver analysis software version 4.0. Lower threshold values were set as 1. Housekeeping probes ACTB, B2M, GAPDH, RPLP0 as well as positive and negative controls were used for normalisation. Partial least squares analysis was performed to identify discriminative miRNA in patients with ≤30% and >30% residual tumour. The top five miRNA identified were then interrogated using multiple t-tests and area under the receiver operative characteristic curves (AUROC). miRNA results were then compared to CA 19.9 that were taken at the same timepoint. Survival data was performed using log rank and cox hazards. All data analysis was performed in Nanostring nSolver, JMP® 17th edition, GraphPad (GraphPad Software, San Diego, California, 9th edition). and IBM SPSS Statistics (Version 28).

6.2.2 Cytokine assay

Serum samples used for this experiment were obtained from Kolling institute tumour bank. These were stored at -80℃ . Twenty-seven cytokines were analysed in seventy-three patients using Bio-plex pro Human Cytokine 27- plex assay with protocol as described in Chapter 2. All serum samples were done in duplicate.

Bio-plex manager software was used to analyse results with unknown cytokine concentrations calculated using a standard curve derived from recombinant cytokine standards. Multiple t-tests were performed to identify cytokines that had significant differences in cytokine concentrations between patients with ≤30% and >30% residual tumour on histopathological specimen. Significant cytokines were then evaluated using AUROC curves. These were compared to CA 19.9 that was taken at the same timepoint. Survival analysis was performed using log rank and cox hazards modelling. All data analysis was performed using GraphPad Prism (GraphPad Software, San Diego, California, 9th edition). and IBM SPSS Statistics (Version 28).

6.3 Results

6.3.1 miRNA

6.3.1.1 Baseline characteristics

Twenty- one patients had plasma taken at time of surgery, after completion of chemotherapy for upfront, borderline resectable and locally advanced pancreatic cancer between 2016-2020. This consisted of ten women and eleven men, and the median age of diagnosis was 69 (Table 6.1). Of these, nine were treated with neoadjuvant 5- fluorouracil, oxaliplatin and irinotecan (FOLFIRINOX), and twelve were treated with gemcitabine plus abraxane. The median duration of neoadjuvant chemotherapy was twelve weeks.

Table 6.1 Baseline characteristics of miRNA cohort. +FOLFIRINOX (5-flourouracil, irinotecan, oxaliplatin).

Baseline characteristics	
Age (Median)	69
Gender	
Female	10 (48%)
Male	11 (52%)
Neoadjuvant regimen	
Gemcitabine plus abraxane	12 (57%)
FOLFIRINOX +	9(43%)
Neoadjuvant duration (median)	12 weeks
Tumour viability	
≤30%	7
>30%	14
Adjuvant chemotherapy	
Gemcitabine plus capecitabine	9
Nil	5
Unknown	7

6.3.1.2 Prognostic biomarkers (clinical characteristics)

There was no difference in recurrence free or overall survival based on type of neoadjuvant chemotherapy (p=0.14, p=0.39 respectively, Figure 6.1).

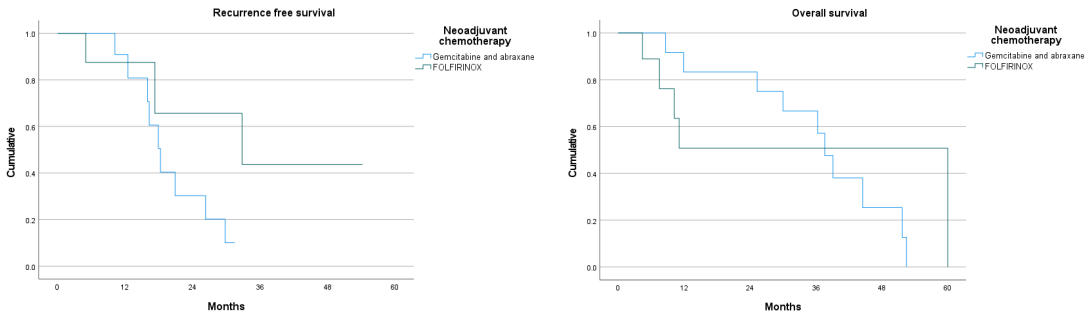


Figure 6.1 Recurrence free and overall survival based on type of neoadjuvant chemotherapy.

Patients that received neoadjuvant chemotherapy <12 weeks had a longer median overall survival than patients that received ≥12 weeks at 53 versus 36 months (p-0.047, figure 6.2). There was no difference in recurrence free survival based on duration of neoadjuvant chemotherapy (p=0.37).

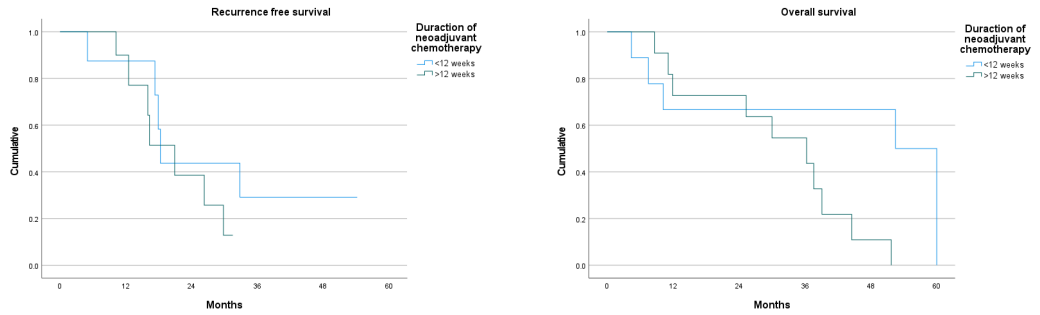


Figure 6.2 Recurrence free and overall survival based on duration of neoadjuvant chemotherapy.

Tumour viability predicted for overall survival with a median overall survival of 52 months in patients with tumour viability ≤30% and 25 months with residual tumour >30% (p=0.03, Figure 6.3.) There was no difference in recurrence free survival based on tumour viability at 17 versus 18 months in tumours ≤30% and >30% respectively (p=0.45).

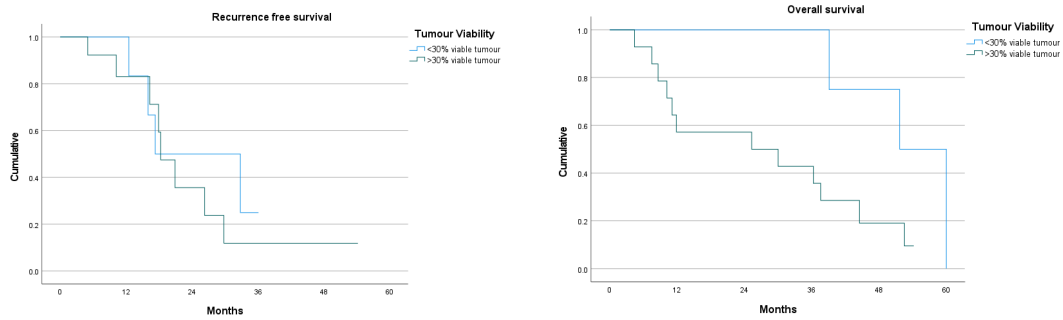


Figure 6.3 Recurrence free and overall survival based on tumour viability (residual tumour).

Patients that received adjuvant chemotherapy had similar recurrence free survival to patients who did not receive adjuvant chemotherapy (p=0.97), however, they did have a significantly longer median overall survival at 53 months compared to 25 months (p=0.001, Figure 6.4).

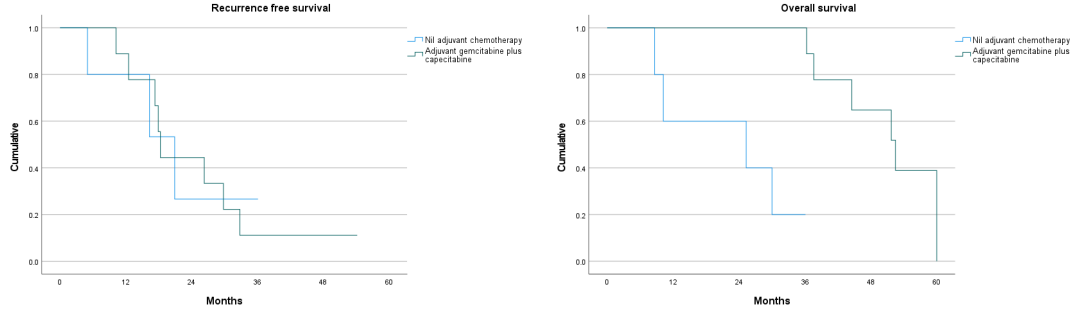


Figure 6.4 Recurrence free and overall survival based on administration of adjuvant chemotherapy.

6.3.1.3 miRNA as a predictive biomarker

Seven hundred and ninety-eight miRNA were analysed using Nanostring nCounter® miRNA Expression Assay. To help determine potential miRNA that could discriminate between patients with 'chemoresistant' (residual tumour >30%) and 'chemosensitive' (residual ≤ 30%) disease, a partial least squares analysis was performed (Figure 6.5). The miRNA that contributed most to the model were determined using a variable importance in projection (VIP) score and miRNA with a VIP score >1.5 were interrogated using AUROC (Table 6.2).

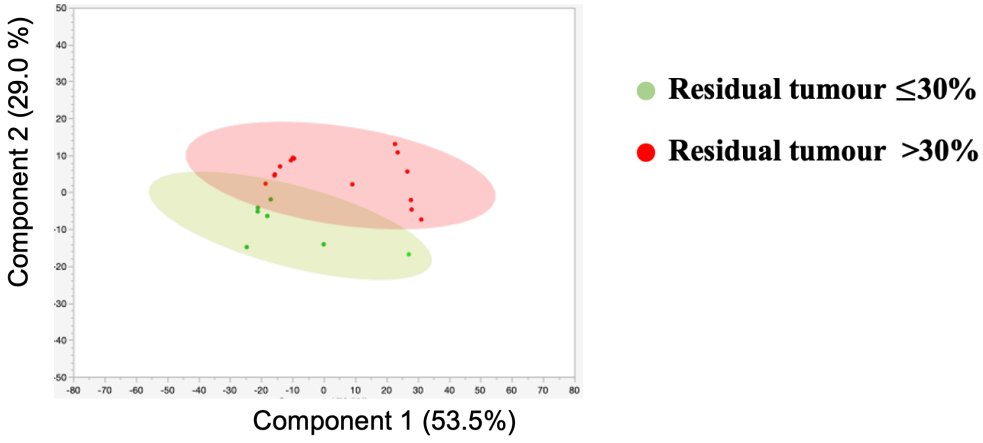


Figure 6.5 Partial least squares.

Multiple t-testing was also conducted on these miRNAs and there were no differences in expression in the two cohorts (Table 6.2, Figure 6.6). CA 19.9 levels were also not significantly different in patients ≤30% and those >30% residual tumour (Table 6.2).

Performing AUROC analysis, only one miRNA, (miR-631) was able to predict for tumour viability (p=0.04, AUROC 0.78) Table 6.2, Figures 6.6, 6.7).

Table 6.2 miRNA with VIP score >1.5 contributing to PLS model. Log fold change; chemoresistant compared to chemosensitive, **significance p<0.05.

miRNA or CA 19.9	Log_2 Fold change*	Multiple t test p-value	Multiple t test q-value	AUROC	AUROC p-value	VIP score
CA 19.9	1.27	0.25	0.71	0.62	0.38	
miR-1305	-0.91	0.14	0.73	0.63	0.35	2.46
miR-2110	-0.61	0.31	0.73	0.72	0.11	2.28
miR-1322	-0.70	0.40	0.73	0.59	0.50	2.09
miR-4425	-0.78	0.32	0.73	0.63	0.33	2.02
miR-374a-5p	-0.45	0.59	0.81	0.57	0.60	1.90
miR-483-3p	-0.52	0.54	0.76	0.62	0.40	1.90
miR-1976	-0.57	0.49	0.73	0.59	0.53	1.86
miR-3202	-0.62	0.39	0.73	0.66	0.25	1.84
miR-491-5p	-0.55	0.39	0.73	0.68	0.19	1.82
miR-323a-3p	-0.51	0.46	0.73	0.67	0.22	1.81
miR-548ai	-0.51	0.46	0.73	0.67	0.22	1.81
miR-631	-0.95	0.09	0.73	0.78	0.04**	1.80
miR-181a-3p	-0.72	0.28	0.73	0.68	0.13	1.80
miR-516a-5p	-0.57	0.40	0.73	0.67	0.22	1.78
miR-892a	-0.55	0.43	0.73	0.66	0.25	1.78
miR-6503-5p	-0.33	0.76	0.90	0.51	0.97	1.77
miR-548i	-0.34	0.64	0.85	0.60	0.48	1.74
miR-513c-5p	-0.43	0.53	0.76	0.63	0.35	1.74
miR-1197	-0.71	0.22	0.73	0.69	0.16	1.73
miR-568	-0.27	0.77	0.91	0.52	0.88	1.71
miR-214-3p	-0.50	0.48	0.73	0.64	0.31	1.71
miR-3127-5p	-0.42	0.53	0.76	0.67	0.20	1.70
miR-3614-5p	-0.19	0.81	0.93	0.55	0.74	1.66
miR-892b	1.40	0.05	0.73	0.73	0.09	1.64
miR-1205	-0.07	0.93	0.99	0.53	0.82	1.61
miR-4458	-0.59	0.44	0.73	0.62	0.39	1.60
miR-342-5p	-0.28	0.73	0.90	0.57	0.60	1.60
miR-1972	-0.55	0.12	0.73	0.67	0.21	1.55
miR-377-3p	-0.24	0.72	0.89	0.63	0.35	1.53
miR-3144-5p	-0.55	0.40	0.73	0.63	0.35	1.52
miR-4755-5p	-0.75	0.21	0.73	0.75	0.07	1.52
miR-224-5p	-0.26	0.74	0.90	0.58	0.55	1.51

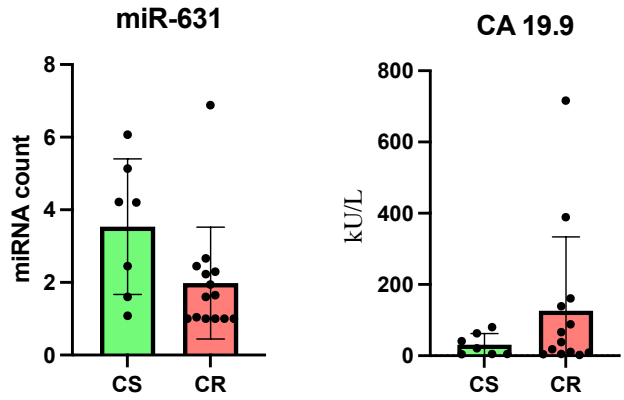


Figure 6.6 miRNA and CA 19.9 expression. CS-chemosensitive (≤30% residual tumour/ viability) and CR-chemoresistant tumours (>30% residual tumour/ viability).

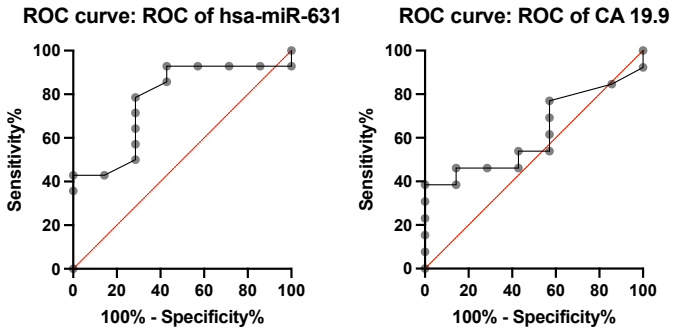


Figure 6.7. Area under receiver operating characteristic curves (AUROC) of miRNA (miR) and CA19.9.

6.3.1.4 miRNA as a prognostic biomarker

MicroRNAs (miRNA) 631, CA 19.9, tumour viability >30% and administration of adjuvant chemotherapy was also tested as prognostic biomarkers in recurrence free and overall survival using univariate and multivariate analyses (Table 6.3). Only miRNA- 631 on multivariate testing, predicted for recurrence free survival with higher values associated with longer survival (HR=0.46, p=0.048).

Table 6.3 Univariate and Multivariate analysis of miRNA, CA19.9 and clinical factors, * indicates significance.

	Recurrence free survival				**Overall survival**			
	Univariate		**Multivariate**		**Univariate**		**Multivariate**	
	p-value	HR	p-value	HR	p-value	HR	p-value	HR
Tumour viability >30%	0.45	1.60	0.23	3.50	0.048	4.57	0.27	4.13
Adjuvant chemotherapy	0.97	1.03	0.55	1.70	0.29	0.00	0.94	0.00
CA 19.9	0.87	1.00	0.11	1.00	0.72	1.00	0.85	1.00
miR-631	0.05	0.66	0.048*	0.46	0.10	0.73	0.21	0.37

6.3.2 Cytokine

6.3.2.1 Baseline Characteristics

A cytokine panel consisting of twenty- seven cytokines were performed using Bio-plex pro Human Cytokine 27- plex assay on serum of 73 patients diagnosed with upfront, borderline resectable or locally advanced pancreatic cancer, treated with NAC between 2016-2020. There were 38 women and 35 men, and the median age of diagnosis was 64 (Table 6.4). Of these twenty-two were treated with neoadjuvant 5- fluorouracil, oxaliplatin and irinotecan (FOLFIRINOX) and twenty-six were treated with gemcitabine plus nab- paclitaxel (abraxane), five with gemcitabine alone and two received chemoradiotherapy (Table 6.4).

Table 6.4 Baseline characteristics. + FOLFIRINOX (5- fluorouracil, irinotecan and oxaliplatin).

Baseline Characteristics	
Age(median)	64
Gender	
Female	38
Male	35
Neoadjuvant regimen	
FOLFIRINOX +	22
Gemcitabine plus abraxane	26
Gemcitabine	5
Chemoradiotherapy	2
Unknown	18
Tumour viability	
≤30%	18
>30%	54
Unknown	1
Stage	
1a	1
1b	8
2a	6
2b	30
3	9
Unknown	19
Tumour viability	
≤30%	18
>30%	54
Unknown	1

6.3.2.2 Prognostic biomarker (clinical characteristics)

The type of neoadjuvant chemotherapy regimen did not predict for either recurrence free (p=0.18) or overall survival (p=0.23, figure 6.8).

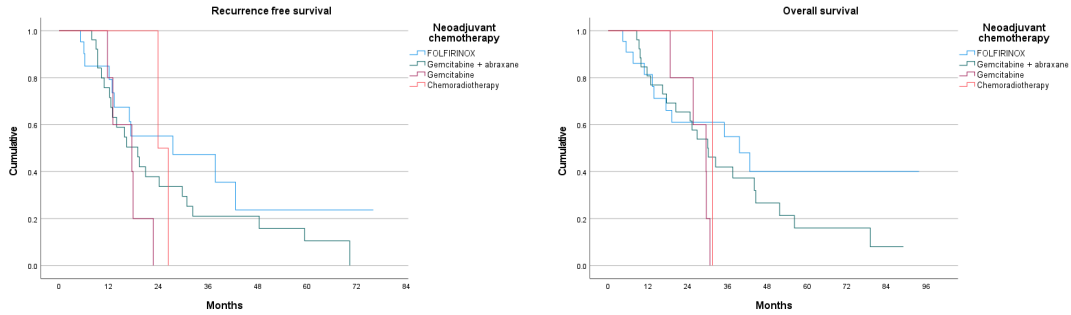


Figure 6.8 Recurrence free and overall survival based on neoadjuvant chemotherapy regimen.

Patients with tumour viability ≤30% had a median recurrence free survival, of 19 months compared to those with tumour viability >30%, median 18 months (p=0.18, figure 6.9). There was no difference is median overall survival in either group (43 v 30 months, p=0.29, figure 6.9).

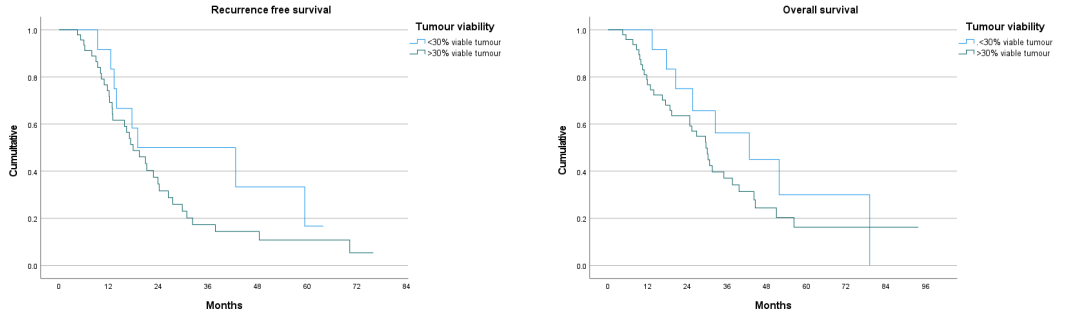


Figure 6.9 Recurrence free survival and overall survival based on tumour viability.

6.3.2.3 Cytokines panel as predictive biomarkers (chemoresistance)

Tumour viability was available for 72 out of the 73 patients and were divided into chemosensitive (≤30% tumour viability) or chemoresistant (>30% tumour viability)[169]. A 27-cytokine panel was analysed for 73 patients. Of these seven cytokines did not have results within the reference range (VEGF, IL-2, IL-5, IL-6, IL-10, IL 12, IL 15) and were excluded. IL 13, GM-CSF was similarly excluded due to only 25% of results falling within the reference range. IL-1Ra had only two values in patients with tumour ≤30% and was removed from analysis.

Multiple t-testing of the remaining 17 cytokines revealed six cytokines that had significant difference with ($p<0.05$, $q<0.1$) between chemosensitive and chemoresistant tumours (see

Table 6.5, A3). TNF- α , RANTES, IL-4, G-CSF, Eotoxin, IP-10 all had higher concentrations in patients with chemosensitive disease (≤30%) compared to chemoresistant disease (>30%, Table 6.5). There was no difference in CA 19.9, levels in patients with chemosensitive vs chemoresistant tumours (p=0.4). AUROC was performed on these cytokines to assess their ability to predict for a chemosensitive versus chemoresistant tumour (Table 6.5, Figure 6.10). TNF-α had an AUROC of 0.82 (p<0.0001)

Table 6.5 Top six cytokines and CA 19.9. Log2 fold change chemoresistant to chemosensitive tumours. *Indicates significance p<0.05).

	p-value	**q-value**	**Log2 fold change**	**AUROC**	**AUROC p-value**
CA 19.9	0.40	0.40	0.97	0.62	0.16
TNF-α	*0.000006	0.000077	-0.72	0.82	<0.0001
RANTES	*0.004781	0.029	-0.23	0.74	0.003
IL-4	*0.013351	0.040	-0.43	0.68	0.02
G-CSF	*0.010928	0.040	-0.39	0.66	0.04
Eotaxin	*0.022519	0.054	-0.41	0.68	0.03
IP-10	*0.027089	0.0546	-0.51	0.68	0.03

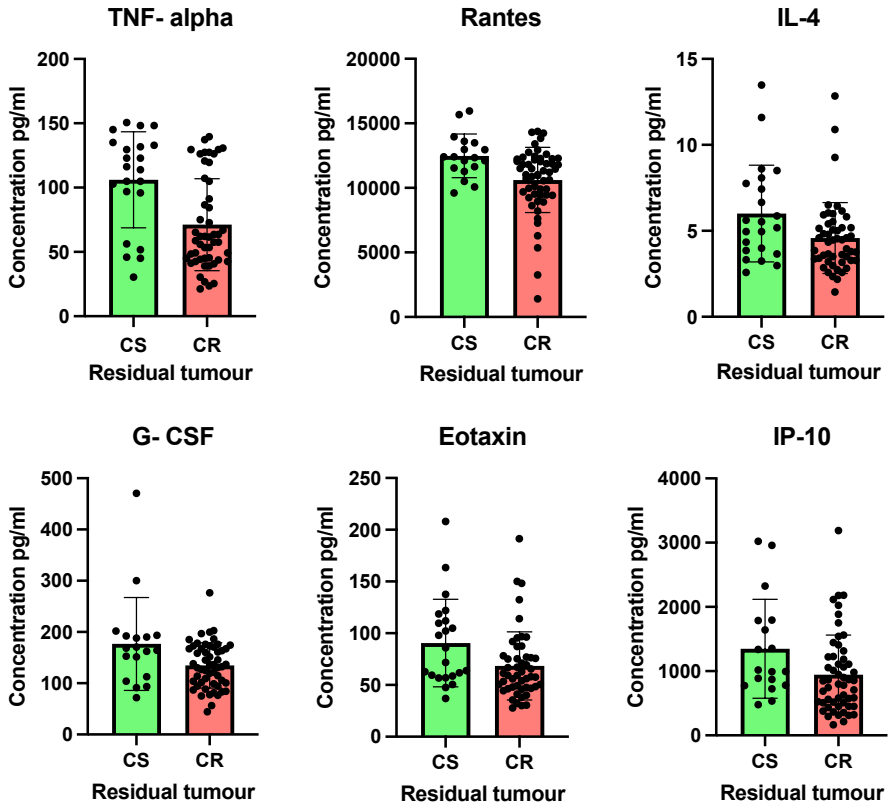


Figure 6.10 Mean cytokine concentration and range for chemosensitive (≤30% residual tumour) and chemoresistant tumours (>30% residual).

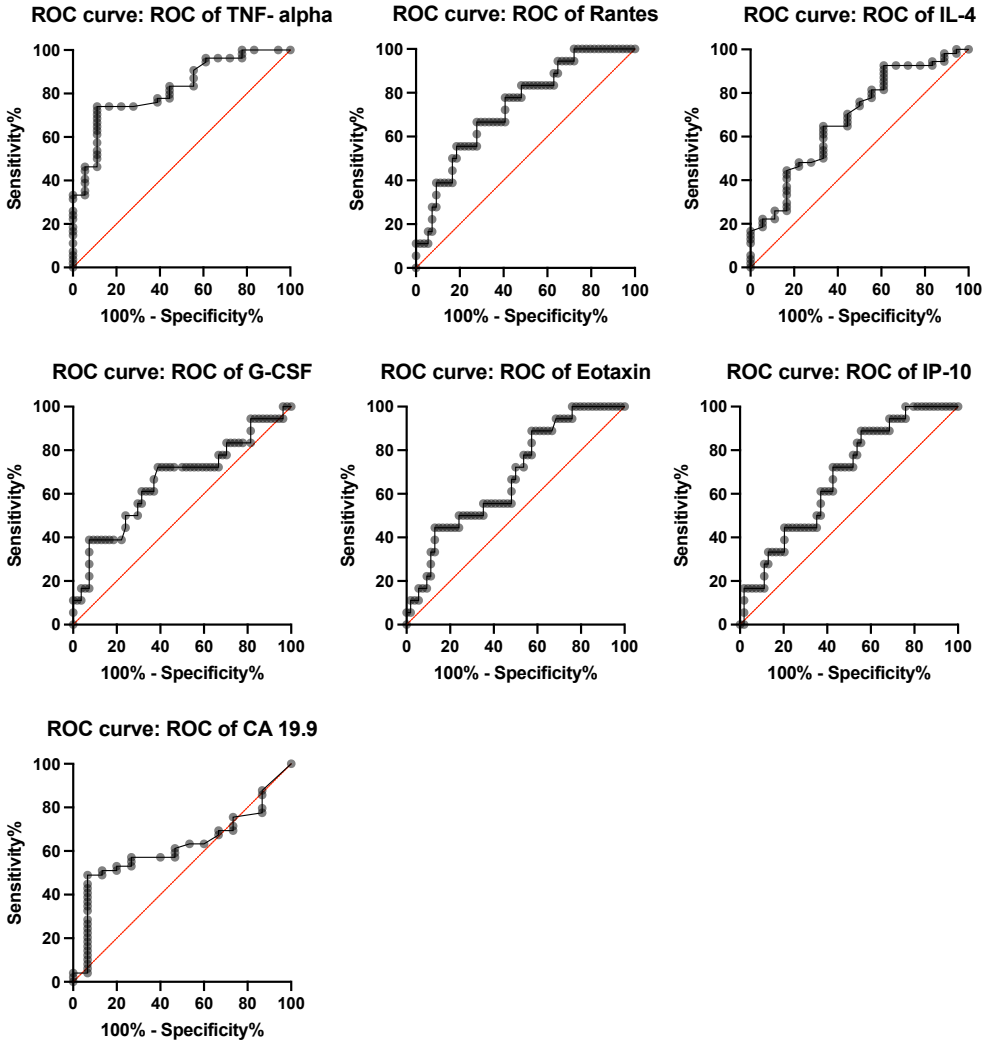


Figure 6.11 AUROC of top six cytokines and CA 19.9.

6.3.2.4 Cytokines as prognostic biomarkers

These six cytokines were then analysed to see if they had any prognostic significance and were compared to the prognostic ability of CA19.9. On univariate testing, CA19.9 did not predict for overall survival (p=0.07); however, it was strongly prognostic for recurrence free survival (p<0.001). All six cytokines did not predict for recurrence free or overall survival in univariate testing (see Table 6.6). On multivariate testing, CA 19.9 was the only marker to predict for recurrence free survival (p<0.001), whereas higher levels of CA 19.9 (p=0.017) and Eotaxin (p=0.01) both predicted for shorter overall survival (Table 6.6).

Table 6.6 Univariate and Multivariate analysis of cytokines and CA 19.9, *indicates significance.

	Recurrence free survival				**Overall survival**			
	Univariate		**Multivariate**		**Univariate**		**Multivariate**	
	p-value	HR	p-value	HR	p-value	HR	p-value	HR
Neoadjuvant chemotherapy	0.18	1.29	0.45	1.24	0.23	1.29	0.77	1.09
Histopathological stage	0.09	1.35	0.12	1.43	0.29	1.20	0.42	1.19
Tumour viability >30%	0.19	1.68	0.76	1.33	0.29	1.51	0.038	4.71
CA 19.9	<0.001**	1.002	0.003**	1.002	0.07	1.00	0.017**	1.002
TNF- α	0.17	0.99	0.37	1.00	0.26	1.00	0.93	1.00
Rantes	0.17	1.00	0.61	1.00	0.1	1.00	0.37	1.00
IL-4	0.86	0.99	0.96	1.02	0.36	1.06	0.23	0.76
G-CSF	0.64	1.00	0.74	1.00	0.89	1.00	0.58	1.00
Eotaxin	0.90	1.00	0.35	1.02	0.129	1.01	0.01**	1.00
IP-10	0.28	1.00	0.38	1.00	0.66	1.00	0.78	1.04

6.4 Discussion

This study explored the predictive and prognostic roles of a blood-based miRNA and 27-cytokine panel in patients diagnosed with pancreatic cancer after completion of neoadjuvant chemotherapy. In the miRNA cohort we identified that duration of chemotherapy was associated with prognosis, with patients receiving ≥12 weeks of chemotherapy having a shorter overall survival compared to patients receiving <12 weeks of chemotherapy. This result is likely due to selection bias with more advanced disease at diagnosis in the ≥12-week cohort. In these patients' tumour viability was a strong predictor of overall survival with a 27-month survival advantage in patients with ≤30% compared to their >30% counterparts. There was also a significant benefit in overall survival in patients receiving adjuvant chemotherapy which is consistent with the literature[215]. We identified that lower levels of miRNA-631 predicted for chemoresistance, (AUROC=0.78, p=0.04) and shorter recurrence free survival on multivariate testing (p=0.048, HR= 0.46), however not for overall survival (p=0.21, HR=0.37). Whilst there is little literature looking at miRNA-631 in pancreatic cancer it is consistent with results in by Chen et al which demonstrated lower levels of miRNA-631 were associated with hepatocellular

carcinoma tissue, compared to normal tissue [207]. Chen et al study discovered that lower expression of miRNA-631 was associated with higher PTPRE gene expression. PTPRE was found to promote migration of hepatocellular carcinoma cells (HCC), postulating that miRNA-631 may act as a tumour suppressor by inhibiting PTPRE enhanced HCC migration and metastatic spread[216]. It should be noted that Chen et al research was based on miRNA in tumour specimens, this is the first study to our knowledge that has demonstrated these results in serum of patients with pancreatic cancer.

In the cytokine cohort we were able to identify six cytokines that had significantly different concentrations in patients with chemosensitive disease (residual tumour≤30%) compared to those with chemoresistant (>30% viable tumour) disease. TNF-α, RANTES, IL-4, G-CSF, Eotoxin, IP-10 had significantly higher concentrations in patients that were chemosensitive (lower viable tumour), compared to chemoresistant patients[209, 217]. We also identified that TNF-α had a significant AUROC of 0.82 and which was higher than CA19.9 (AUROC 0.62) in this cohort, although in sample sizes of <200, AUROC curves should be interpreted with caution[218].

Whilst Zhao et al demonstrated the role of TNF-α as a poor prognostic biomarker in patients with pancreatic cancer, its role in tumorigenesis is far more complicated[219]. Our identification of TNF-α as a potential upstream regulator in chemoresistance in Chapter 4 is testament to this complexity. TNF-α is a pleotropic cytokine with pro and anti-tumoral activity, with the latter thought secondary to its role in inhibiting vascular supply to tumour [220]. As a pro inflammatory cytokine, the presence of higher concentration of TNF-α in patients with a good response to chemotherapy indicates the role that immune system has in anti-cancer as well as pro-cancer effects, particularly in the context of cell death secondary to chemotherapy[221]. Like TNF-α; RANTES, IL-4, G-CSF, Eotoxin and IP-10, similarly, have both pro and anti-tumoral emphasising the complexity between the immune response and pancreatic cancer[222-224]. This is also demonstrated by the seemingly contradictory result that although higher concentrations of Eotaxin were associated with of a good response to chemotherapy (lower tumour viability), it was found to be a poor prognostic factor for overall survival on multivariate testing, along with CA 19.9.

There are limitations of this study to note, both studies had a small sample size with less than 100 patients each. The population is heterogenous and although a pre-requisite that all patients

had to receive surgery for their pancreatic cancer, the upfront stage could be either upfront or borderline resectable or locally advanced, highlighting a potential confounding factor. Bloods were also obtained from patients after treatment with chemotherapy, thus making extrapolation to patients at time of diagnosis, inappropriate. In addition, the bloods were taken from one time point, and cytokine levels are known to vary day to day or at different time points in the one day[225]. Another limitation is the storage of cytokines, some of which have been stored more than 4 years. Whilst cytokines remain stable, if stored at -80 ºC for two years, the stability after this is less predictable[226].

There are also strengths to this study. Whilst Van der Sijde et al assessed the role of serum cytokines in pancreatic cancer patients following FOLFIRINOX in predicting radiological response, this is the first research to our knowledge that has compared miRNA and cytokine levels to histopathological response (i.e., tumour viability in surgical specimen)[214].

6.5 Conclusion

Due to the difficulty in obtaining upfront tissue in pancreatic cancer, exploration of the potential role of blood-based biomarkers in pancreatic cancer is critical in the management of patients in the clinic. We found one miRNA (631) and six cytokines to be present in higher concentration in patients that were chemosensitive compared to patients that were resistant to chemotherapy. Of these, TNF-α had an AUROC >0.8 which was higher than CA 19.9, thus suggesting that serum measurement of TNF- α may provide pertinent biomarker capabilities that could be used in patients to predict response to chemotherapy. Analysis of this cytokine panel in the blood of patients, taken at diagnosis (prior to administration of chemotherapy) is needed to validate these findings.

CHAPTER 7:

Circulating biomarker discovery: immune cells

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7.1 Introduction

Inflammation plays an important role in tumorigenesis and propagation in pancreatic cancer[227-229]. Initial cell damage leads to activation of the immune system with the recruitment of inflammatory cells in both the innate (macrophage, neutrophils, and natural killer cells) and adaptive (T and B-lymphocytes) immune system[227]. These immune cells release pro-inflammatory cytokines, creating a positive feedback loop leading to further upregulation of the immune system. The role of cytokines as predictive and prognostic biomarkers was discussed previously, in Chapter 6. Once the tumour has developed, the body's immune system continues to be activated and this dynamic interaction between tumour and immune mediators form the basis of the inflammatory tumour microenvironment (TME)[230]. These immune cells then circulate throughout the bloodstream, where they can be detected in a peripheral blood test.

The role of circulating inflammatory cells including neutrophils as prognostic markers is well established in a range of different tumour types[112, 231, 232]. Several markers emerged in recent years in patients with advanced disease, including neutrophil lymphocyte ratio (NLR); platelet lymphocyte ratio (PLR); and lymphocyte monocyte ratio (LMR); with higher numbers associated with worse prognosis and reduction in overall survival[112]. Neutrophils in particular are known to produce reactive oxygen species; a key trigger in DNA damage and a hallmark in the development and progression of cancer[233].

In pancreatic cancer there have been many other different mechanisms posited associating higher neutrophils with more aggressive disease. One of these involves tumour secretion of chemokines, including CXCR2. It has been demonstrated that higher levels of CXCR2 expression are associated with an increase in tumour size in pancreatic adenocarcinoma and a worse prognosis in a range of cancer types[108, 234]. Other *in vitro* studies (using transwell assays) have demonstrated that neutrophils promote the migration and invasion potential of pancreatic cancer cells[108].

The role of these inflammatory markers in early disease are less established, with smaller tumours not generating as significant an immune response as more advanced cancers that have undergone haematogenous spread[235, 236].

There have been minimal clinical studies assessing these inflammatory tests as markers to predict response to chemotherapy in patients with early pancreatic cancer. In the modern-day treatment of pancreatic cancer, upfront surgery has become the exception rather than the rule, with the majority patients undergoing neoadjuvant chemotherapy to downstage their disease. In addition, chemotherapy and the pancreatic TME have a complex relationship due to the pervasive nature of stromal cells and the extracellular matrix (ECM), which form a physical barrier between tumour and chemotherapy. Neutrophils are often involved in the formation of the ECM and can thus interfere with the TME, which has a direct impact on the success of chemotherapy. Conversely, chemotherapy can contribute to TME remodelling[237].

C-reactive protein (CRP) is also an acute phase reactant that is produced by pro-inflammatory cytokines (including IL-6)[238]. It is a key marker of inflammation and easily tested on routine bloods. Albumin is another important protein that has several important functions including regulation of oncotic pressures in the blood vessels. Low levels can be associated with increased losses (gastrointestinal or renal), as a marker of malnutrition or as a negative acute phase reactant in inflammation[239]. The latter two both commonly occur in patients diagnosed with cancer. The modified Glasgow performance scale (mGPS) which utilises both albumin and CRP levels is becoming increasingly used as a prognostic marker in gastrointestinal cancers. Patients with lower levels of albumin and higher levels of CRP have worse outcomes[240].

That there is a clear need for validated prognostic and biomarkers is indisputable. Biomarkers allow for personalised treatment plans and more reliable prognostic data. The utilisation of inflammatory blood tests such as NLR, PLR and LMR as biomarkers have widespread appeal. They are routinely performed on all patients at diagnosis, minimally invasive and are cost-effective.

We hypothesise that due to the active role the immune system has in the development of pancreatic cancer that these inflammatory markers can be used as prognostic and as predictive biomarkers, in response to chemotherapy, in early pancreatic cancer.

Aims

The aims of this study were 1) to determine if inflammatory markers including NLR, PLR, LMR, as well as other clinical markers could predict tumour viability at the time of surgery in patients treated with neoadjuvant chemotherapy at our high-volume pancreatic cancer centre and 2) to assess if these markers were useful in prognostication for recurrence-free and overall survival in pancreatic cancer patients that underwent surgical resection.

7.2 Materials and Methods

Patient Population and Clinical Characteristics

Patients that were treated at one large tertiary centre from 2013-to 2020 were identified from prospective clinical databases and medical records. Patients were included if they underwent surgery for localised pancreatic cancer and were identified as either upfront resectable, borderline resectable or locally advanced (Table 7.1)[16]. Patients that were diagnosed with metastatic pancreatic cancer or did not proceed to surgical resection were excluded.

All surgeries were performed by two specialised pancreatic cancer surgeons. Patient characteristics including age, sex, eastern co-operative oncology group (ECOG) performance status and Charleson comorbidity index were collected (Table 7.1) [241, 242]. Clinical symptoms including weight loss or back pain were also recorded. Baseline blood tests included inflammatory markers such as neutrophil-lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), lymphocyte monocyte ratio (LMR) as well as c-reactive protein (CRP), albumin and bilirubin. These values were analysed as dichotomous variables and cut-offs were chosen from previous published values and were NLR>5, PLR >150, LMR >3, CA 19-9>1000 [232, 235, 243, 244]. C-reactive protein (10mg/L) and albumin (<35g/L) were combined to generate a modified Glasgow performance score (mGPS) with a higher CRP>10mg/L and lower albumin (<35g/L) indicating a worse prognosis. Bilirubin (>20µmol/L) was also measured as a dichotomous variable and cut-off was based on the laboratory range.

Blood tests were taken at the time of staging laparoscopy, prior to chemotherapy in patients that received neoadjuvant chemotherapy (NAC). Baseline resectability and stage was determined on computerised tomography (CT) scan. Upfront treatment with either NAC versus upfront surgery was recorded (Figure 7.1, Table 7.1).

Tumour viability was assessed only on patients that received NAC and was based on residual tumour at the time of surgery and was measured as a continuous variable. No scoring system was used due to the lack of consensus on tumour regression grading system in pancreatic

cancer. The histopathological stage was based on surgical histology using TNM 8th edition[26]. Clinical factors including recurrence-free (RFS) and overall survival (OS) were analysed.

Patients were discussed at a multidisciplinary team meeting and the decision for NAC versus upfront surgery was determined based on time of diagnosis and resectability (due to the success of NAC in the locally advanced setting, in our centre it became standard of care for patients with upfront and borderline resectable disease from 2016 onwards).

Statistical analysis

Predictive markers were analysed using spearman's rank correlation with tumour viability. Prognostic markers were assessed using Kaplan-meier and cox proportional-hazards using both univariate and multivariate analyses. Statistical analysis was performed using SPSS (IBM Corp, Armonk, NY, USA). P values <0.05 were considered statistically significant.

Institutional Ethics

Institutional ethics approval for this study was obtained (HREC/16/HAWKE/105).

7.3 Results

7.3.1 Baseline characteristics

One hundred and ninety-six patients were included in the analysis and baseline characteristics are demonstrated in Table 7.1. Of these 110 underwent neoadjuvant chemotherapy (NAC) and 86 patients received upfront surgery (UFS). Most patients received either gemcitabine plus abraxane or FOLFIRINOX (5- fluorouracil, irinotecan, oxaliplatin) as per physician discretion. All patients received at least one cycle of NAC.

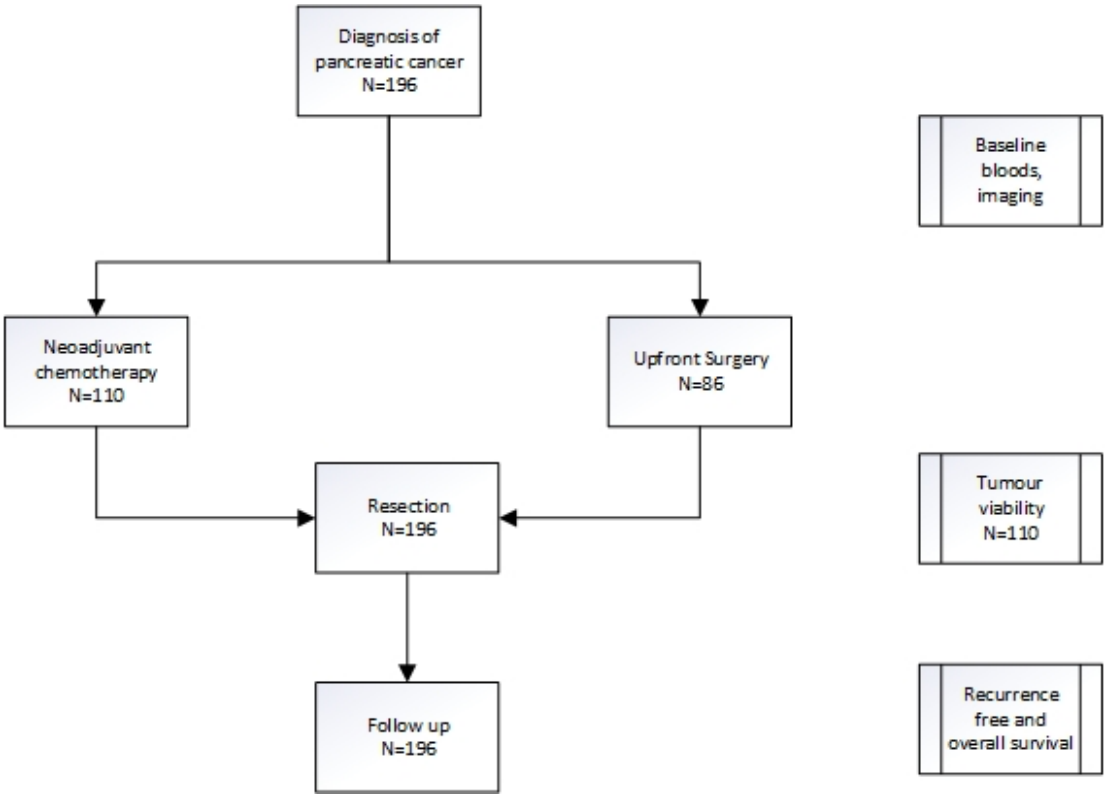


Figure 7.1 Treatment pathway for pancreatic cancer patients.

Table 7.1 Baseline characteristics. NAC-neoadjuvant chemotherapy, FOLFIRINOX- (5-fluorouracil, irinotecan and oxaliplatin), NLR- neutrophil monocyte ratio, PLR- platelet monocyte ratio, LMR- lymphocyte monocyte ratio

Stage at diagnosis	**Number of patients**
1	145
2	47
3	4
Resectability	
Upfront resectable	133
Borderline	26
Locally advanced	37
Upfront treatment	
Upfront resection	86
NAC	110
NAC regimen	
Gemcitabine plus nab-paclitaxel	41
FOLFIRINOX	60
Other	9
Charleson co-morbidity	
<2	36
2-5	117
>5	41
Unknown	2
Sex	
M	104
F	92
Inflammatory markers	**Median (range)**
NLR	2.9 (0.2-17.6)
PLR	181 (12.6-662)
LMR	2.4 (0.4-18)

7.3.2 Predictive biomarkers

To identify the presence of biomarkers that may predict chemotherapy response, baseline clinical characteristics and blood tests were compared to residual tumour (tumour viability) at the time of surgery for the 110 patients in the NAC cohort (Figure 7.1). The patients who had upfront surgery were excluded.

Of these, the platelet lymphocyte ratio (PLR) correlated to tumour viability with a platelet lymphocyte ratio >150 predicting higher tumour viability and hence poorer chemotherapy response (p= 0.03, co-efficient 0.21) at the time of surgery (Table 7.2). When adjusting for multiple testing (Bonferroni) p=0.27, and as such is not significant. Neither neutrophil lymphocyte ratio (NLR), lymphocyte monocyte ratio (LMR) or modified Glasgow performance scale (mGPS) had any correlation with tumour viability.

Table 7.2 Clinical and inflammatory predictive markers correlation with tumour viability. ECOG- Eastern co-operative oncology group, CCI- Charleson co-morbidity index, NLR- Neutrophil monocyte ratio, PLR- platelet monocyte ratio, LMR- lymphocyte monocyte ratio, mGPS- modified Glasgow performance scale. *Indicates statistical significance <0.05.

	p value	Correlation Co-efficient (spearmen's)
ECOG	0.102	0.158
CCI	0.165	0.134
Weight Loss	0.997	0
Back pain	0.276	-0.106
NLR>5	0.875	-0.015
PLR> 150	0.03*	0.21
LMR > 3	0.075	-0.172
mGPS	0.436	-0.93
CA 19.9 >1000kU/L	0.818	0.023

7.3.3 Prognostic biomarkers

To assess if baseline clinical inflammatory markers were useful as prognostic biomarkers all 196 patients were included in the analysis, regardless of whether they received NAC or upfront surgery (Figure 7.1). Both univariate and multivariate analyses were performed to assess the

role of these biomarkers in prognosis both in recurrence-free (RFS, Table 7.3) and overall survival (OS, Table 7.4).

7.3.3.1 Recurrence-free survival

Of these factors, the surgical stage was a significant prognostic marker both in both univariate (p=0.001, HR 1.36) and multivariate analyses (p=0.003, HR 1.66, Table 7.3). Patients diagnosed with stage 1 disease had a median recurrence-free survival of 30.9 months compared to only 17 months in those with stage 3 disease. LMR> 3 proved to be prognostic in multivariate analysis (p=0.019, HR 2.32), as did the presence of back pain (p=0.014, HR 4.55). These were not significant on univariate analysis. NLR was not significant in either univariate (p=0.28, HR 1.34) or multivariate (p=0.109 HR 2.41) testing (Figure 7.2).

Table 7.3 Clinical and inflammatory prognostic markers in recurrence-free survival. ECOG- Eastern co-operative oncology group, CCI- Charleston co-morbidity index, LMR- lymphocyte monocyte ratio, PLR- platelet monocyte ratio, NLR- Neutrophil monocyte ratio, mGPS-modified Glasglow performance scale. *Indicates statistical significance <0.05).

Recurrence-free survival				
	Univariate		**Multivariate**	
	p-value	**Hazard Ratio (95%CI)**	**p-value**	**Hazard ratio (95% CI)**
ECOG	0.228	1.27 (0.86-1.89)	0.825	1.1 (0.52-2.25)
CCI	0.981	1.00 (0.91- 1.10)	0.566	0.95 (0.79- 1.14)
Weight Loss	0.346	1.20 (0.82- 1.75)	0.935	1.03 (0.54-1.95)
Back Pain	0.581	1.22 (0.60- 2.51)	0.014*	4.45 (1.34-14.26)
Surgical stage	0.001*	1.36 (1.15-1.62)	0.003*	1.66 (1.18-2.34)
NLR >5	0.283	1.34 (0.79-2.27)	0.109	2.41 (0.82-7.04)
PLR >150	0.671	1.08 (0.76-1.54)	0.975	1.00 (0.47-2.07)
LMR >3	0.275	1.21 (0.86-1.71)	0.019*	2.32 (1.15-4.68)
Bilirubin >20µmol/L	0.079	1.40 (0.96-2.04)	0.138	1.66 (0.85-3.26)
mGPS	0.238	0.82 (0.59-1.14))	0.459	0.84 (0.52-1.34)
CA 19-9>1000kU/L	0.114	1.47 (0.91-2.38)	0.396	1.38 (0.65-2.92)

7.3.3.2 Overall survival

Neutrophil lymphocyte ratio was a strong prognostic biomarker for overall survival. Patients with NLR ratios >5 had a median overall survival of only 12.5 months compared to 32.4 months in those ≤5 (Table 7.4, Figure 7.2). NLR was statistically significant in both univariate (p=0.001, HR 2.43) and multivariate analysis (p=0.019, HR 3.04). Bilirubin levels were also a

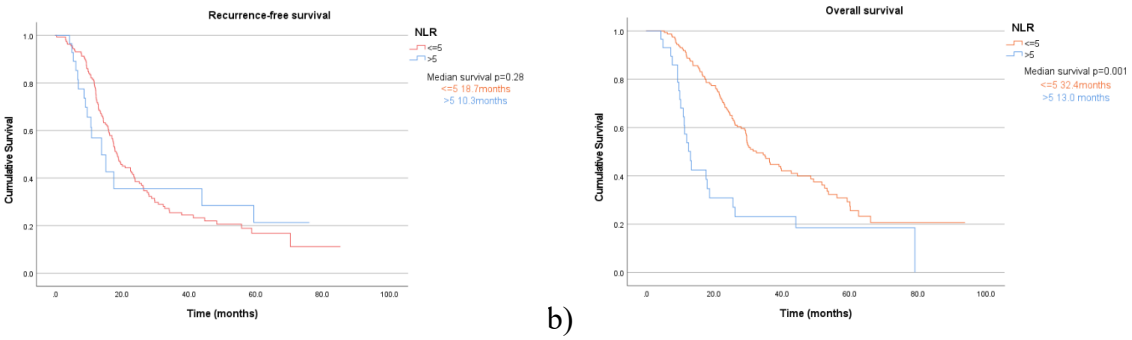
useful prognostic marker with levels above 20µmol/l demonstrating significantly shorter overall survival at 26 months compared to those with <20µmol/l at 42.7 months (p=0.037, HR 1.52). Similar to recurrence-free survival, the surgical stage was also statistically significant with the higher stages resulting in worse overall survival (p=0.021, HR 1.23). Neither bilirubin nor surgical stage were significant on multivariate testing. Other inflammatory blood tests in LMR, PLR were not prognostic for overall survival.

For patients diagnosed with recurrent disease, higher NLR at diagnosis was a strong prognostic factor for shorter overall survival from time of disease recurrence. Patients with a higher NLR (>5) at baseline had median time from recurrence to overall survival of only 5 months versus 12.7 months in those with NLR ≤5 (HR 2.37, 1.356-4.13, p=0.002).

Clinical factors including eastern co-operative group (ECOG) function status, baseline comorbidities and weight loss were not useful as prognostic markers either in recurrence-free or overall survival.

Table 7.4 Clinical and inflammatory prognostic markers in overall survival. ECOG- Eastern co-operative oncology group, CCI- Charleston co-morbidity index, LMR- lymphocyte monocyte ratio, PLR- platelet monocyte ratio, NLR- Neutrophil monocyte ratio, mGPS- modified Glasgow performance scale. *Indicates statistical significance <0.05

Overall survival				
	Univariate		**Multivariate**	
	p-value	**Hazard ratio (95%CI)**	**p-value**	**Hazard ratio (95% CI)**
ECOG	0.144	1.30 (0.92-1.84)	0.785	1.10 (0.55-2.2)
CCI	0.383	1.04 (0.95-1.15)	0.583	1.05 (0.88-1.27)
Weight Loss	0.147	1.33 (0.91-1.94)	0.964	0.99 (0.52-1.88)
Back Pain	0.484	1.30 (0.63-2.66)	0.010*	4.21 (1.4-12.63)
Surgical stage	0.021*	1.23 (1.03-1.46)	0.092	1.33 (0.95- 1.87)
NLR >5	0.001*	2.43 (1.54-3.85)	0.019*	3.04 (1.20-7.71)
PLR >150	0.163	1.30 (0.90-1.90)	0.504	1.33 (0.58-3.08)
LMR >3	0.966	1.01 (0.70-1.45)	0.427	1.346 (0.65-2.80)
Bilirubin >20µmol/L	0.037*	1.52 (1.03- 2.26)	0.051	2.07 (1.00- 4.27)
mGPS	0.234	0.81 (0.57- 1.15)	0.028*	0.58 (0.36-0.94)
CA 19-9>1000kU/L	0.270	1.30 (0.81-2.16)	0.953	1.02 (0.46-2.30)



a) b)

Figure 7.2(a) Recurrence-free and (b) overall survival by neutrophil-lymphocyte ratio (NLR).

7.4 Discussion

It is well recognised that the immune system and advanced cancer are closely linked with many studies demonstrating the relationship between inflammatory blood tests and metastatic pancreatic cancer[112, 245]. What is less established are the role of these inflammatory markers in prognosis in early pancreatic cancer patients that undergo curative surgery. In addition, there have been no studies assessing the role of these potential biomarkers to predict chemotherapy response after neoadjuvant chemotherapy by comparing to tumour viability in the surgical specimen. The purpose of our study was to establish the roles of these inflammatory markers as prognostic and predictive biomarkers in patients diagnosed with earlier disease that went on to have surgery.

We identified that platelet lymphocyte ratio (PLR) is a potential predictor for tumour viability with a ratio >150 associated with higher residual tumour at the time of surgery. Our study also demonstrated that there is a role of neutrophil-lymphocyte ratio (NLR) for prognostication with levels >5 resulting in a significantly shorter median overall survival of 13 months compared to 32 months in those with a ratio of ≤5. This significant difference was not observed in recurrence free survival, however, for patients that did recur, higher NLR at baseline predicted for a shorter time from recurrence to overall survival (5 versus 12.7 months). This poorer survival on relapse may be due to a more biologically aggressive or chemoresistant tumour or may reflect patient factors including a poorer performance status.

This confirms our hypothesis that NLR can be used as a surrogate prognostic marker for overall survival, even in patients that have an earlier stage of disease that undergo surgery. It also highlights the important role the systemic immune system has in response to pancreatic cancer

which may prove fundamental to exploit in future therapies, particularly with the more widespread use of immunotherapy, a treatment that has so far proved disappointing in the treatment of this terrible disease.

The ability to identify prognostic and predictive markers is crucial to personalising treatment for patients in the clinic. Due to the toxicity of modern-day chemotherapy and the poor response rates of second- and third-line regimens, the ability to predict patients that will derive less benefit, particularly at time of recurrence, may be useful in sparing unnecessary side effects and preserving quality of life.

There are limitations of these studies to note. Firstly, this is a retrospective review and there may be recall bias. In addition, white blood cells and c-reactive protein can be elevated for several reasons and pancreatic cancer can be linked to pancreatitis. We opted to use blood tests on admission to the hospital for staging laparoscopy to try to standardise the time of collection however this is one-time point. Bloods are dynamic and may change over time. In addition, the correlation between higher baseline PLR and residual tumour at the time of surgery whilst statistically significant has a coefficient of only 0.2 and hence should be interpreted with caution.

There are also strengths to our study. Although there have been publications involving NLR, PLR in advanced and even resectable pancreatic cancer, this is the first paper to our knowledge, that assess the relationship between baseline inflammatory blood tests and tumour viability after neoadjuvant chemotherapy[235]. The ability to predict which patients may respond better to chemotherapy may be invaluable in the identification of ideal upfront modality, a contentious issue in upfront resectable disease. A large number of patients (196) were included in the analysis and the blood tests were processed at one of only two laboratories at the same time in the patient's clinical journey to minimise bias.

7.5 Conclusions

This real-world study demonstrates that there is an association between the immune system and earlier pancreatic cancer with higher neutrophil-lymphocyte ratio predicting shorter survival and higher platelet lymphocyte ratio correlating with higher viability at the time of surgery in patients receiving neoadjuvant chemotherapy. Further prospective trials to validate

these findings are crucial to provide insight into the role of inflammatory blood tests as potential biomarkers in early pancreatic cancer.

CHAPTER 8:

Conclusions and future directions

8.1 Discussion

Pancreatic cancer survival has changed little in recent years[1]. This lack of progress can be attributed to two primary factors. The first is the anatomical location of the pancreas deep within the abdomen, such that, at the emergence of symptoms, disease has often spread beyond the pancreas and local nodes. The second is the underlying biology of pancreatic cancer and its adjacent pervasive stromal tissue, which, is innately chemoresistant. For those who do get an early diagnosis the treatment is a combination of six months of chemotherapy in addition to surgery (pancreaticoduodenectomy, total pancreatectomy or a distal pancreatectomy). Traditionally, chemotherapy was introduced after surgery for patients with resected tumours, however recurrence was common and often occurred soon after completion of this systemic therapy. Due to these outcomes and the success of neoadjuvant chemotherapy in downstaging inoperable tumours to allow surgery, neoadjuvant chemotherapy in patients with early (upfront or borderline resectable) disease was introduced[246]. The aims of this thesis were to address the following questions.

8.1.1 Does neoadjuvant chemotherapy provide improved survival outcomes compared to upfront surgery in patients with upfront or borderline resectable pancreatic cancer?

As chapter one highlights, there have been several randomised controlled trials designed to answer this question. The methodology and chemotherapy regimens used in these trials are heterogenous and different from our Australian practice of care. The number of patients recruited was often small, reducing statistical certainty and leaving concerns about a lack of external validity of these results. In addition, the results of these studies, which include the PREOPANC, PREP 02/JSAP 05, ESPAC and most recently the NORPACT 1 have been conflicting, leaving clinicians with unanswered questions as the appropriate method of management for these patients in the clinic[28, 30, 247, 248].

In the absence of large randomised controlled trials demonstrating a definitive superior approach of either neoadjuvant chemotherapy or upfront surgery, there becomes a greater need for identification of biomarkers, to help clinicians improve outcomes, and at present although each patient's tumour is biologically unique, their treatment is not.

The retrospective analysis conducted in Chapter three was embarked on, prior to the publication of the afore mentioned randomised controlled trials. Its aim was to address this first

question directly in the upfront resectable cohort of patients that we treated in our local health district. This review was focussed on upfront resectable only, as preliminary results in the borderline setting were available at the time of review[28]. In our centre, neoadjuvant chemotherapy replaced upfront surgery for most patients with upfront resectable cancer from 2016 onwards. This approach adopted by our multidisciplinary team at the time was novel, and allowed an opportunity to compare patients with upfront resectable disease that were treated with upfront surgery to those treated with neoadjuvant chemotherapy. The results of this analysis demonstrated that for all patients there was no difference in recurrence free or overall survival between the two groups, however there was a survival advantage of upfront surgery in patients diagnosed with earlier disease (radiological stage 1a at diagnosis).

These results are consistent with recently released preliminary results from the NORPACT-1 randomised controlled trial, which also demonstrated no statistically difference in survival between patients in the two groups[247]. It was this management dilemma that led to the second question addressed in this thesis.

8.1.2 Are there biomarkers that can assist with prognosis and predict response to treatment in patients with early pancreatic cancer?

Chapter one investigated the literature for prognostic and predictive biomarkers that could be used in the management of early pancreatic cancer. It highlighted several promising biomarkers however, at present serum CA 19.9 remains the only validated biomarker, and its use is limited in monitoring response on therapy and in early detection of disease recurrence [4, 34, 38, 39, 116, 249]. In addition, 10% of patients have a Lewis antigen negative phenotype, and as such CA19.9 lacks utility in these patients[33].

In addition to the comparison between upfront surgery and neoadjuvant chemotherapy in resectable patients, Chapter three also investigated the role of clinical factors as potential prognostic biomarkers and biomarkers to predict response to chemotherapy in our cohort. It identified that a SUV of ≥5 on baseline fluorodeoxyglucose positron emission tomography (FDG-PET) resulted in a reduction in recurrence free and overall survival and a weak but significant correlation between reduced response to chemotherapy or ‘chemoresistant’ disease. Whilst needing validation in a larger cohort this lends insight into the potential role of radiomics in pancreatic cancer management and from November 2022 Medicare benefit

scheme (MBS) listed rare cancers (including pancreatic cancer) as an item for FDG-PET. This introduction will see an increase in use of FDG-PET and will allow further investigation into its role as biomarker in pancreatic cancer. Our team seeks to explore FDG-PET in combination with machine learning as a biomarker in this cohort, the pilot retrospective study was commenced during this candidature, and this will be further explored in a post-doctoral research project (see appendix A4).

Chapters 4-7 of this thesis aim to address this second question with the view to identify biomarkers that may change clinical management or indeed may assist with answering the first question. Whilst this question pertains to early (upfront and borderline resectable) disease, the experiments also include patients with locally advanced disease that underwent surgical resection, to allow for a larger recruitment of patients and samples.

8.1.2.1 Role of tissue-based biomarkers

Waddel et al first explored the role of genes as potential biomarkers by performing whole genome sequencing in 100 pancreatic adenocarcinoma tissues[80]. This research confirmed the presence of known oncogenes (discussed in Chapter one) but also highlighted new genes previously unidentified in pancreatic cancer[80]. The individualized molecular pancreatic cancer therapy (IMPaCT) trial utilized next generation sequencing (NGS) on biopsy and surgical samples in patients with pancreatic cancer to perform real time genomic discovery, thus, returning results that could impact directly on patients' management[20]. There were several barriers to this trial including the length of time it took to return results, in some cases too late to intervene with appropriate therapy due to the aggressive nature of this disease. Another limitation identified in this trial was that of insufficient biological material in biopsy samples. This issue is touched on in Chapter five and forms the basis of future work, which was unable to be completed during this thesis.

8.1.2.2 Surgical tissue

Resected tumour tissue is the most common utilised tissue for biomarker identification in pancreatic cancer and it was used in chapter four of this thesis. In this chapter, tumour tissue was obtained at surgery from patients treated with neoadjuvant chemotherapy for pancreatic cancer. Proteomic discovery was chosen as a method to detect biomarkers with further experiments used for validation. This was a novel approach with little literature available in this cohort[169]. We identified key proteins that predicted for chemoresistance including S100-

P and NAD(P)H dehydrogenase which was consistent with the available literature[184, 250]. S100-P is one of the proteins in the S100 family, another of which, S100A4, is explored further in Chapter five. Using ingenuity pathway analysis and in consultation with the literature, a potential pathway as predictor of chemoresistance was identified which included the enzyme TGM2 and the cytokine OSM and its receptor OSMR. Validation of these potential biomarkers through cell culture studies demonstrated that silencing of either TGM2 or OSMR resulted in a reduction in cell viability, highlighting that TGM2 and OSMR may be directly involved in cell proliferation. Further studies of these cell lines by combining siRNA of TGM2 and OSMR with gemcitabine, will help elucidate their role in chemoresistance and form the basis of future work.

This study provided useful insights into the proteomic changes and associated pathways involved in chemoresistance in pancreatic cancer, but extrapolation of the results must be made with caution. These experiments were conducted on tumour tissue after exposure to chemotherapy, rather than chemotherapy naive specimens. A pilot study of proteomic discovery on chemotherapy naïve specimens was planned for this thesis (A5), however due to time constraints will be conducted as post-doctoral research. The limitations of using surgical specimens, is that it is difficult to appreciate if these proteins were present at time of diagnosis. The ideal time of detection would be on biopsy material taken at the time of diagnosis.

8.1.2.3 Biopsy tissue

The best example of a biomarker driven decision making akin to the initial question of this thesis is seen in modern day management of breast cancer. Immunohistochemistry is performed on the biopsy of breast cancer to assess oestrogen receptor, progesterone receptor and her-2 receptor expression. Not only do these results impact on prognosis but they also, along with other radiological and clinical factors, determine whether patients proceed to neoadjuvant chemotherapy versus upfront surgery[251].

There are two main reasons that this approach has failed to achieve the success in pancreatic cancer that has been established in breast cancer. The first is that there are no known biomarkers that have been validated that guide selection of treatment and the second is the difficulty obtaining sufficient biological biopsy material in which to test, as discovered in the IMPaCT trial[20]. The anatomical location of the pancreas, in contrast to breast tissue, can only be accessed endoscopically and even with larger bore needles, tumour yield is small.

During this thesis, six biopsy samples were collected and stored as fresh frozen at -80℃ with the view to perform proteomic discovery (A5). The aim was to compare these samples to matched surgical samples in patients that had either upfront surgery or neoadjuvant chemotherapy. As a large part of this process was a question of feasibility, four non-malignant biopsy samples were processed in the mass spectrometer to assess if sufficient proteins were present in this scant biological tissue. A total of 2200 proteins were identified, a third of what was detected in the surgical specimens from Chapter four. In addition, the proteins with the highest expression were also blood cell-based proteins, consistent with the macroscopic blood seen in the fine needle aspirate (FNA) specimen prior to analysis. These results highlighted two main difficulties with this approach, the first was that amount of tissue obtained, although sufficient enough to get 2000 proteins, contained significantly less proteins than the surgical samples, raising concern that the proteins contained may not be a true representation of the tumour as a whole. The second was that difficulty with interpreting the blood-based proteins, as the biopsy samples often contained macroscopic blood. Whilst it may be that proteomic discovery on biopsy specimen is at present immature, the improved sensitivity of mass spectrometry that will no doubt occur as well as improvements in FNA techniques may see this being a very important source of biological material in future. Further procurement of these biopsy samples, and analysis will form the basis of future post-doctoral research studies. The potential for biopsy material to be used in more targeted screening, formed the basis of Chapter five.

In the fifth chapter, three proteins; mesothelin, CA125 and S100A4 on biopsy and matched surgical specimens were investigated as potential prognostic biomarkers in patients with pancreatic cancer. We identified the presence of CA125 staining on both biopsy and surgical specimen to be associated with a worse survival. Whilst there were insufficient patient numbers to get useful data on the predictive ability of CA125 to determine response to chemotherapy, its identification as a potential prognostic biomarker warrants further investigation in validation studies. The next step will be to repeat this experiment in a different cohort of patients, with the ultimate aim being a prospective study of this panel (A2 for prospective protocol).

This chapter also revealed that that CA125 and mesothelin expression was similar in biopsy and matched surgical samples thus highlighting that biopsy material may be a strong representation of the tumour as a whole and thus a useful biomarker to target in further studies.

The benefit of immunohistochemical staining on biopsy samples, as is the case with breast cancer, is that should we find clinically relevant targets, the turnaround time to deliver results is fast enough that it can be implemented to change patient management.

Despite these results, we still encountered an issue with lack of biological material with just over 50% of patients having sufficient tumour in their formalin fixed paraffin embedded samples to allow for immunohistochemistry. The improvement in techniques of endoscopic ultrasound and biopsy, enabling larger volumes of tumour tissue to be obtained are necessary to further this area of research[197, 198].

8.1.2.4 Role of blood-based biomarkers

Due to the limitations associated with tissue-based biomarkers previously mentioned, we pursued further investigation on blood (both serum and plasma) to identify any predictive or prognostic biomarkers. The benefit of blood-based biomarker discovery is that it is less invasive and can be performed at multiple time points in a patient's diagnosis and treatment journey. In chapter six we discovered that the cytokine TNF-α was found in increased concentrations in patients with chemosensitive disease, compared to patients with chemoresistant disease and was superior to CA19.9 at predicting response tumour viability. Whilst TNF-α has previously been identified in the literature as a poor prognostic factor in pancreatic cancer, its impact on tumorigenesis and propagation is in fact multifaceted with both pro and anti-tumoral activity. In this chapter RANTES, IL-4, G-CSF, Eotoxin and IP-10 were also present in increased concentration in patients with lower residual tumour. Similarly, to Chapter four, these samples were collected on patients after systemic chemotherapy so further evaluation on diagnostic blood samples (prior to chemotherapy) need to be performed. During this thesis bloods have been collected in newly diagnosed patients pre and post chemotherapy (A6) to allow validation of this cytokine experiment.

8.2 The role of the immune system and pancreatic cancer

Chapter seven of this thesis identified that neutrophil lymphocyte ratio (NLR) in blood samples taken at diagnosis of pancreatic cancer patients was a poor prognostic biomarker. Higher platelet lymphocyte ratio (PLR) predicted for higher viable tumour at time of surgery, however the association was weak. This was the first study to assess the role of these biomarkers in predicting chemotherapy response and one of only a few studies assessing these inflammatory bloods on patients with earlier disease (that underwent surgical resection). In addition to

identifying potential biomarkers, Chapters six and seven also developed our understanding of the role of the immune system in pancreatic cancer. With previous literature supporting the immune system involvement in advanced or metastatic disease, these results highlight the systemic nature of early pancreatic cancer, prior to radiologically evident spread. This lends credence to the use of neoadjuvant chemotherapy in patients with early disease. Further identification of the role of the immune system, will hopefully be addressed in the translational component of the currently recruiting NEO-IMPACT study, in which microbiome samples are collected in patients undergoing chemo-immunotherapy for early (upfront and borderline resectable) pancreatic cancer.

8.3 Chemoresistance to inform decision making

Chemoresistance in pancreatic cancer is complex and multifactorial, with more than 165 genes, implicated[252]. The relationship between stromal and tumour cells are pivotal to this innate chemoresistance as the hypo-vascularity of stromal cells provide a physical barrier shielding tumour cells from chemotherapeutic agents[253]. In this thesis chemoresistance was measured by tumour viability at time of surgery. This has limitations as there is no one validated regression score used in pancreatic cancer management. We used a cut-off of 30%, based on previous research by our group[169]. Due to patient numbers chemoresistance to specific chemotherapy agents were not analysed. Whilst these retrospective analysis provide an initial insight into potential biomarkers, the ideal study to test this hypothesis would be a biomarker driven randomised controlled trial, however recruitment in this cohort is challenging, as demonstrated in the ESPAC-5F trial[30]. The importance of identification and validation of chemoresistant biomarkers, are that it may lead a clinician and multidisciplinary team to opt for upfront surgery rather than prolonged exposure to chemotherapy.

8.4 Conclusion

The purpose of this thesis was to answer a clinically important question ‘is neoadjuvant chemotherapy superior to upfront surgery in patients with upfront and borderline resectable cancer?’ The ensuing literature review and our own cohort analysis showed conflicting results and highlighted that the answer is not simple. This absence of a superior upfront modality emphasized the importance of searching for biomarkers that may assist with this question and importantly personalise treatment for each patient. This is crucial in pancreatic cancer as little progress has been made to survival rates in many years. The experiments in chapters four to seven were hypothesis generating and highlighted several tissue- and blood-based biomarkers

that have the ability to be used either as prognostic or predictive biomarkers. Whilst these results were promising, further studies are needed to validate these findings, and will form the basis of post-doctoral research.

The work set in place during this thesis including the collection of biopsy samples and pre chemotherapy bloods were logistically challenging and echo the barriers seen in previous trials but now they are established, allow these results to be tested in a different cohort of patients (prior to systemic therapy) and will develop our understanding of these biomarkers.

It is access to these biological specimens that will allow further exploration of these biomarkers which will no doubt prove the key to unlocking our understanding and thus improve management of this terrible disease.

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