# Investigation and therapeutic targeting of the metabolic relationship between Pancreatic Stellate Cells and Pancreatic Ductal Adenocarcinoma

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# Abstract

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most aggressive solid organ malignancies with persistently poor survival despite advancements in chemotherapy and surgical techniques. Pancreatic stellate cells (PSCs) are key pro-tumourigenic players within the inflammatory microenvironment/stroma of PDAC, and thus represent an attractive therapeutic target. This project aimed to examine the under-investigated relationship of PSCs to cancer metabolism, and identify novel translational treatment approaches.

Methodology

Human PSC and PDAC (Panc1, Miapaca2 and Bxpc3) cell lines were utilised in numerous *in vitro* transwell co-culture experiments to investigate the effect on activity and more specifically metabolic phenotype, including the use of qPCR, western blot, metabolic assays, and seahorse technology. The impact of drugs targeting enzymes and transporters specific to lactate metabolism were then examined. In addition immunohistochemistry (IHC) was performed on resected tumours to examine the expression patterns and prognostic relevance of certain metabolic markers within the tumour microenvironment.

Results

Initial co-culture experimentation revealed PSCs and PDAC impact on lactate metabolism, with a significant pro-glycolytic effect of PSCs on the PDAC cell lines. In keeping with this, a notable upregulation in the monocarboxylate transporters

MCT1 and MCT4 was also observed, indicating an impact on lactate flux. PSC interactions with the PDAC lines were influenced by the underlying genetic/metabolic phenotype of each cell line. IHC staining revealed distinct MCT expression patterns, with a positive correlation between the tumoural and stromal compartments, whilst expression of MCT1 and MCT4 within the stroma and tumour respectively correlating with reduced overall survival. The MCT1 inhibitor AZD3965 exerted a KRAS-dependent de-activating effect on PSCs, with a reduction in αSMA and IL6 expression. AZD3965 also exerted a KRAS-dependent anti-proliferative effect on the PDAC lines, a cell line-specific PSC-dependent reduction in PDAC metabolic processes, as well as a reduction in PSC lactate transporter expression. Inhibition of all LDH isoforms with Galloflavin revealed a mixed effect on both PDAC proliferation and apoptosis, although an over-riding reduction in lactate acidification.

Conclusion

This project has proven a metabolic symbiosis exists between PDAC and PSCs, which appears to be inextricably related to lactate metabolism. Inhibition of lactate transporters or enzymes has demonstrated effects on both cancer cells and PSCs which, in combination with the correlation to poor prognosis in resected specimens, holds great promise in a potentially beneficial translational impact for patients. The heterogeneity of results observed between cell lines, postulated to be due to either KRAS status or underlying metabolic phenotype, suggests a patient/tumour-specific personalized approach to MCT or LDH inhibition should be adopted in any future clinical trials.

# List of abbreviations

| | |
|---|---|
| ASA | American Association of Anaesthesiologists |
| αSMA | α smooth muscle actin |
| ATP | Adenosine triphosphate |
| Brdu | Bromodeoxyuridine |
| Ca19-9 | Carbohydrate antigen 19-9 |
| CAF | Cancer associated fibroblast |
| CBD | Common bile duct |
| CP | Chronic pancreatitis |
| CSC | Cancer stem cells |
| CT | Computed tomography |
| CTGF | Connective tissue growth factor |
| DMEM | Gibco Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DR | Desmoplastic reaction |
| ECAR | Extra-cellular acidification rate |
| ECM | Extra-cellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| EMT | Epithelial-mesenchymal transition |
| ETC | Electron transport chain |
| FCS | Fetal calf serum |
| FGF | Fibroblast growth factor |

| | |
|---|---|
| FNA | Fine needle aspiration |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GF | Galloflavin |
| γH2AX | Phosphorylated histone variant H2AX protein |
| Glut | Glucose transporter |
| HBSS | Hanks' Balanced Salt Solution |
| HIF1-α | Hypoxia-inducible factor |
| hPSC | Human pancreatic stellate cell |
| IHC | Immunohistochemistry |
| IL-6 | Interleukin 6 |
| IL-8 | Interleukin 8 |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| LDH | Lactate dehydrogenase |
| MCT | Monocarboxylate transporter |
| MMP | Matrix metalloproteinase |
| MTT | Thiazoyl blue tetrazolium bromide |
| NAC | N-Acetylcysteine |
| OCR | Oxygen consumption rate |
| OS | Overall survival |
| OXPHOS | Oxidative phosphorylation |
| PALB2 | Partner and localizer of BRCA2 |
| PanIN | Pancreatic intra-epithelial neoplasia |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PDAC | Pancreatic ductal adenocarcinoma |

| | |
|---|---|
| PDGF | Platelet derived growth factor |
| PDH | Pyruvate dehydrogenase |
| PDK | Pyruvate dehydrogenase kinase |
| PET | Positive emission tomography |
| PPPD | Pylorus preserving pancreaticoduodenectomy |
| PSC | Pancreatic stellate cell |
| Ras | Rat sarcoma |
| ROS | Reactive oxygen species |
| rPSC | Rat pancreatic stellate cell |
| RWE | Reverse Warburg effect |
| SHH | Sonic hedgehog |
| TBS | Tris buffered saline |
| TGF | Tumour growth factor |
| VEGF | Vascular endothelial growth factor |
| WB | Western blot |

# Chapter 1

# Introduction

## 1.1 Pancreatic cancer

Pancreatic cancer is one of the most aggressive malignancies of the modern age, and despite advancements in chemotherapy and radiotherapy, with a host of well designed clinical trials [1, 2], survival rates remain relatively unchanged over the past 40 years, lagging behind the progress of other tumour types such as the "big four" (lung, breast, colorectal and prostate). The reasons for this persistently poor prognosis are multifactorial, and will be discussed in more detail, however the statistics speak for themselves. In the UK there are 8,000 new cases of pancreatic cancer per year, and despite being just the 12$^{th}$ most common cancer, it is the fifth most common cause of cancer death. [3] More concerning is the fact that incidence is on the rise, and in the US it is predicted pancreatic cancer will become the 2nd most lethal cancer according to annual death rates by 2020. Poor prognosis is highlighted by the dismal one and five year overall survival (OS) rates of 18% and 3.5%. [3] Therefore it is clear more research is essential to better understand this devastating disease and improve outcomes in line with other forms of malignancy. As such Cancer Research UK has made pancreatic cancer one of its key priorities in their most recent mission statement [4], and significant efforts are now being made within the international pancreatic cancer research community to come together to form productive collaborations to move forward our understanding of the complex pathogenesis underpinning the aggressive nature of this disease, and develop improved therapeutics. [5]

### 1.1.1 Pathogenesis

The most common malignant pancreatic tumour is a pancreatic ductal adenocarcinoma (PDAC), which is of exocrine origin and accounts for over 95% of pancreatic tumours. [6] Multiple combinations of genetic mutations underpin PDAC, making it inherently heterogeneous and therefore partly explaining the lack of success with conventional chemotherapeutics. The genes involved can be divided into three main categories; oncogenes, tumour-suppressor genes and genome maintenance genes (involved in DNA damage repair). [7] Mutational activation of the KRAS oncogene is present in 90% of tumours, resulting in a persistently activated and abnormal Ras protein that allows for sustained proliferation and cell survival. [8] Other commonly encountered mutations include the inactivation of tumour suppressors CDKN2a (with resultant loss of p16) in 85%, TP53 in 80% and SMAD in 50% of PDAC tumours. [9] Furthermore telomere shortening, leading to chromosomal instability, is heavily implicated.

PDAC has been shown to develop through a stepwise accumulation of genetic mutations, associated with progression from a premalignant to invasive phenotype. [10] Pancreatic intra-epithelial neoplasia (PanINs) is the histological precursor to PDAC, consisting of mucin producing epithelium with cytological and architectural atypia of the small ducts of the pancreas. These develop through minimally dysplastic PanIN1a (flat) and b (papillary without atypia), through to more significant dysplasia in PanIN2 (papillary with atypia) and 3 (carcinoma in situ), and then to an infiltrating adenocarcinoma, with PanIN3's often being found adjacent to resected PDAC specimens. [11] This progression is inextricably linked to the aforementioned accumulation of mutations; KRAS activation and telomere shortening are the earliest known abnormalities identified in low grade PanINs, whilst TP53 and SMAD4 inactivation occur in more advanced PanINs as they progress into

invasive PDAC. [12] The fact these precursors demonstrate a similar genetic landscape, and given KRAS-genes shed from PanINs have been identified in stool, duodenal and pancreatic juice samples [13], means much research is ongoing in an attempt to develop biomarkers of more advanced PanINs to improve screening and early diagnosis approaches.

An extensive analysis of 24 tumours has emphasized the complexity and heterogeneity of PDAC, demonstrating each tumour has on average 63 different genetic abnormalities, mostly point mutations, which can be incorporated into 12 core signaling pathways including RAS (e.g. KRAS), DNA damage repair (e.g. TP53), cell cycle control (e.g. CDK2NA) and TGF-β (e.g. SMAD4). [14] Importantly it was seen that key pathways were not consistently seen across all tumours, thus explaining why tumours simply do not consistently respond to modern day chemotherapeutic agents. The study did however highlight the need to personalize treatment of each individual tumour, further emphasized by the fact one patient with a PaLB2 mutation (suggesting the cancer would be sensitive to DNA damaging agents) responded remarkably well to alkylating agents, resulting in tumour regression and prolonged survival. [15] Therefore given this heterogeneity, tailored precision approaches are now being investigated, whereby resected tumours are extensively analysed and specific therapies administered to improve patient outcomes. [16]

The most recent analysis of PDAC subtypes has potentially revolutionized the way we classify these tumours. Bailey et al performed integrated genomic analysis of 456 PDAC tumours with expression analysis defining 4 distinct subtypes; squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX). [17] These subtypes correlated with histological characteristics, whilst inferring differences in

the molecular evolution of PDAC, and will assist in identifying which patients will benefit from certain therapies; for example squamous tumours have more activated stroma and therefore may benefit from stroma-targeted treatments, whilst immunogenic tumours may benefit from drugs modulating immune response.

With respect to pathophysiology, PDAC tumours are typically white/yellow, hard and poorly defined masses, which frequently invade and cause obstruction of the adjacent common bile duct (CBD) or main pancreatic duct. Microscopically the affected infiltrating ducts vary in size and shape according to the degree of tumour differentiation, whilst the nuclei usually demonstrate marked pleomorphism and hyperchromasia. [18] Tumours are surrounded by dense fibrous tissue, caused by the reactive tumour stroma, creating tissue much like that encountered in chronic pancreatitis (CP) due to excessive collagen deposition and calcification. This in turn makes intra-operative identification of the tumour challenging, and may in part explain the high degree of tumour positive resection margins. The pathophysiology of this stroma will be discussed later in the chapter. Infiltration into the adjacent perineural, lymphatic and vascular spaces is frequently seen, and tumours typically metastasise to the liver (80%), peritoneum (60%), lungs and pleurae (50-70%), and adrenal glands (25%). [12]

### 1.1.2 Pancreas anatomy and physiology

To better understand why pancreatic tumours are so challenging to diagnose and treat, one needs to first consider the anatomy and positioning of the organ. The organ itself is retroperitoneal, and can be divided into head, neck, body and tail portions. The head lies in the C-loop of duodenum, the neck and body lie posterior to the distal portion of the stomach, and the tail then extends towards the hilum of the spleen. Approximately 75% of

tumours occur in the head of pancreas (HOP), 15-20% occur in the pancreatic body and 5-10% in the pancreatic tail. [19] The neck is in close proximity to a number of major vessels, including the superior mesenteric artery and vein, splenic vein, portal vein junction, inferior vena cava and aorta. There is a complex ductal system within the pancreas, which chiefly comprises of the main pancreatic duct (duct of Wirsung), with the inter- and intra-lobular ducts linking the acinar tubules to the main duct; it is in this ductal epithelium that PDAC develops. Furthermore the CBD, which drains bile from the liver, passes through the pancreas and joins the main duct near its entry into the duodenum, a site also termed the Ampulla of Vater [20]; therefore any CBD involvement can disrupt biliary drainage.

The main physiological functions of the pancreas are in relation to digestion (exocrine) and glucose metabolism (endocrine), therefore nutritional sequelae are common in pancreatic cancer. The exocrine pancreas consists of acinar cells, which produce digestive enzymes, and ductal cells, which drain the acinae as well as secreting mucous and bicarbonate. This resultant pancreatic juice is then excreted into the duodenum and aids digestion, particularly fatty foods. The endocrine pancreas comprises 4 specialised cell types (α, β, δ and PP cells) that amass to form an Islet of Langerhan, which are embedded within the acinar tissue and secrete hormones into the blood stream. α and β cells regulate glucose metabolism through the secretion of glucagon and insulin respectively. PP and δ cells secrete pancreatic polypeptide and somatostatin respectively, which regulate secretory mechanisms within the pancreas. [21]

With respect to nerve supply, relevant when considering the development of abdominal pain, the pancreas receives parasympathetic fibres from the posterior vagal trunk via a

coeliac branch, whilst the sympathetic supply comes from T6-T10 via the thoracic splanchnic nerves and the celiac plexus. [22]

### 1.1.3 Clinical presentation and diagnosis

As a result of these anatomical and structural associations, pancreatic cancer has to grow to a relatively large size to cause symptoms.[6] As such patients often present at a late and advanced stage of disease, whereby treatment options are limited. The classic triad of abdominal pain, jaundice and weight loss is often experienced. If large enough, tumours invade adjacent nerves to cause abdominal pain, or infiltrate the distal CBD causing obstructive cholestasis, resulting in jaundice, dark urine and pale stools. They may also compress adjacent bowel to cause gastrointestinal upset or even vomiting by gastric outlet obstruction. Alternatively patients may experience systemic upset due exocrine affects, causing weight loss, diarrhoea or diabetes. With respect to the latter, it has been suggested any patient over 50 years old who develops new onset diabetes should be investigated for pancreatic cancer. [23] A study of 123 patients with HOP tumours found frequencies of symptoms to be abdominal pain (71%), weight loss (81%), jaundice (89%), lethargy (42%), change in bowel habit (42%) and sudden onset of diabetes mellitus (33%); however it was noted that jaundice and abdominal pain were late symptoms, at which stage it may be too late to be able to offer potentially curative surgery. [24]

Risk factors for PDAC should also be considered as part of the patient assessment. A number of lifestyle factors are linked with PDAC, including smoking [25], raised body mass index (BMI) [26], red meat and dairy consumption [27], , and occupational exposure to chemical such as benzidine. [28] Chronic pancreatitis has also been associated with an

increased risk of PDAC development [29]; this is of particular relevance when we consider the role of the tumour stroma and associated inflammation, and will be discussed in more detail later in the chapter. Lastly a genetic predisposition must be considered, which may be present in 5-10% of tumours, with an increasing number of affected family members also increasing risk. [30] Mutations of BRCA2 (breast cancer susceptibility gene 2)[31] and PALB2 [32] also have familial associations with increased risk and should be considered.

As part of the clinical assessment one must also considered any differential diagnoses, such as chronic pancreatitis (CP), which is a progressive fibro-inflammatory condition. The aetiology is complex and often due to multiple influences; lifestyle factors such as excessive alcohol consumption and smoking have strong associations, as do certain genetic factors such as mutations in the trypsin-controlling gene of the cystic fibrosis transmembrane conductance regulator. [33] Symptoms of CP are similar to the non-specific nature of pancreatic cancer, including abdominal pain, weight loss, GI disturbance and jaundice. In particular autoimmune pancreatitis, a rare heterogeneous disease, can be challenging to differentiate with cancer, with the possibility of raised tumour markers, ductal strictures or a pancreatic mass. [34] This emphasizes the need to ensure a thorough clinical assessment and investigation of potential pancreatic disease to ensure an accurate diagnosis and avoid unnecessary invasive procedures or surgery.

Diagnosis is in the form of clinical staging which is multimodal, using a combination of clinical features, blood tests and imaging techniques. The results are then collated to formulate a diagnosis, and determine suitable management options, most pertinently whether a patient is able to proceed with surgical resection. Whilst a histological diagnosis in the form of a tissue biopsy is not strictly required prior to surgery, it is necessary for

those who are planned to receive chemotherapy, and in the investigation of indeterminate lesions. Fine needle aspiration (FNA) biopsy may be performed; this can be done under image guidance, either using CT or endoscopic ultrasound (EUS); if the patient is potentially receiving neoadjuvant chemotherapy to downsize a tumour for resection then EUS is preferable as the percutaneous CT approach risks peritoneal seeding.[35] Laparoscopic (keyhole surgery) biopsy may also be used in cases of clinically suspected locally advanced disease when an FNA has been negative on two occasions. Endoscopic retrograde cholangiopancreatography (ERCP) may also be performed in cases of indeterminate ductal lesions to allow improved visualization, acquire brushings for cytological analysis, and permit stent insertion to restore biliary drainage if required.

A useful blood based biomarker for pancreatic cancer is lacking, and a field of intense research efforts. Ca19-9 (carbohydrate antigen) is a tumour marker that lacks the sensitivity and specificity as a screening tool, as it may be found in benign pancreatic disease (such as pancreatitis or biliary obstruction) or other tumour types (colorectal, hepatocellular). [6] Alternately some patients with PDAC may not have a raised Ca19-9. However it can prove useful in the monitoring of recurrent disease (e.g. post-resection), and has been shown to be prognostically relevant when assessing response to chemotherapy. [36] Simple liver function tests may be performed to assess for any degree of obstruction to the biliary system.

With respect to imaging, more complex modalities are required due to the pancreas's positioning and adjacent structures that require in depth evaluation. Assessment is best performed using a triphasic (i.e. arterial, venous, and portal phase) contrast enhanced helical CT scan, with 3D reconstruction, allowing for optimally enhanced, thin cut images to

analyse the tumour and it's involvement with adjacent vascular and ductal structures, thus determining suitability for surgery.[37] Magnetic resonance imaging (MRI) has become increasingly utilised due to technological advancements, and has been shown to be equally accurate in assessing tumours as compared to CT. [38] Simple abdominal ultrasound is limited in its ability to image the pancreas, however endoscopic ultrasound (EUS) has been shown to equal to, if not better than, CT in diagnosing pancreatic lesions, with the additional benefit that a FNA (fine needle aspiration) biopsy may be performed concurrently. However with respect to becoming a screening tool, this procedure requires a high level of expertise, is costly, invasive and not without complications, such as bleeding or perforated bowel.

Lastly, positron emission tomography (PET) CT has been developed to analyse pancreatic tumours by means of measuring functional changes caused by enhanced glucose utilization. With 2-[fluorine 18]-fluoro-2-deoxy-D-glucose (FDG), PET can be used to identify pancreatic cancer and differentiate it from chronic pancreatitis with a sensitivity of 85-98% and a specificity of 53-93%. [39, 40] PET examines the metabolic activity on a molecular level, therefore will also take into account other factors such as oxygenation, blood flow and inflammation. However it is limited in its ability to detect smaller lesions (<1cm) and distinguish between chronic pancreatitis. The PET-PANC trial (NIHR HTA 08/29/02) demonstrated the ability of PET to improve sensitivity and specificity in detecting local/distant disease, with this modality leading to an alteration in patient management in 45% of cases, and deemed particularly useful in patients with cystic tumours and concomitant chronic pancreatitis. In view of these findings PET is now recommended as part of the investigative work-up in all patients with suspected pancreatic cancer.

Some institutions also perform diagnostic staging laparoscopy on potentially resectable tumours as a means to rule out radiological occult peritoneal or liver metastases that would preclude surgery. This has been recommended to be more appropriate in those patients considered to be higher risk of disseminated disease, e.g. borderline resectable tumours, significantly raised CA19-9 or large tumour size.[41]

One can see that there are severe limitations to the screening nature of all the aforementioned imaging modalities, and in combination with the lack of a specific and sensitive biomarker, other forms of tests need to be developed to enable clinicians to diagnose the disease earlier and permit potentially curative surgery before the tumour is too advanced.

### 1.1.4 Clinical management

Surgical resection offers the only prospect of long term survival, however as previously mentioned this is still poor and significantly lagging behind outcomes post-resection of other tumour types. At presentation tumours are only suitable for surgery in 20% of cases. [6] Optimal management is curative resection, and this incurs an improved prognosis to a degree, with 5 year survival of up to 25%, however patients often relapse, with local recurrence or disease within the liver or lung observed in over 60% of patients within 2 years of resection [42]; as such many patients are offered adjuvant chemotherapy to reduce the risk of post-resection recurrence and improve survival. [43] Outcomes are very much dependent on achieving a disease free (R0) resection margin, with particularly favourable outcomes demonstrated in patients with small tumours (≤25mm) and ≤1 lymph node involved. [44] Indeed it has been shown that patients with a positive resection margin (R1) have a similar survival benefit to those who undergo palliative therapy without

surgery, emphasizing the importance of achieving an R0 resection. [45] Due to the high risk of post-resection recurrence many patients are offered adjuvant chemotherapy to improve survival.

Once a patient has undergone the necessary investigative tests, clinicians aim to classify patients into one of 4 categories that then guides subsequent management and increases the chance of an R0 resection; resectable, borderline resectable, locally advanced and metastatic (Table 1). These categories are defined by the level of involvement of major vessels, with resectable tumours having clear tissue planes between critical vessels, whereas borderline tumours may abut, encase or distort adjacent vessels, increasing the risk of an R1 (positive margin) resection which then significantly worsens prognosis. Therefore borderline patients are often offered chemotherapy prior to surgery (neoadjuvant) in an effort to downsize the tumour, following which more aggressive surgery with vascular reconstruction may be required to achieve an R0 resection. Patients with locally advanced tumours typically receive up front chemotherapy; a small minority demonstrating response to treatment may be taken to theatre for an exploratory laparotomy/trial dissection in an attempt to resect, which invariably involved vascular reconstruction. Patients with metastasis are not suitable for resection and have the option of chemotherapy with palliative management.

Table 1 – Key features and management strategies of the 4 categories of pancreatic cancer

| Classification | Defining features | Suggested management |
|---|---|---|
| Resectable | No vascular involvement | Standard surgery |
| Borderline resectable | <180 degree encasement of SMV/SMA | Aggressive surgery (vascular reconstruction)<br>Neoadjuvant chemotherapy to downstage tumour |
| Locally advanced | >180 degree encasement of SMV/SMA | Chemotherapy/palliation (potential for attempted resection if response observed) |
| Metastatic | Disseminated disease to distant sites (liver/lung/peritoneum) | Chemotherapy/palliation |

#### 1.1.4.1 Surgical management

The surgical procedure performed is dependent on tumour location. Patients with tumours in the pancreatic body/tail undergo a distal pancreatectomy. As these tumours are more accessible, have less risk of vascular association and do not require duct or bowel reconstruction, the procedure is performed laparoscopically/robotically in the majority of cases with a low morbidity rate (less bleeding and pancreatic leaks). Conversely HOP tumours require more complex reconstruction through a pancreaticoduodenectomy (PD - either pylorus preserving (PPPD) or classical Whipple's procedure). This operation involves removal of the pancreatic head, duodenum, proximal jejunum, gallbladder, parts of the extra-hepatic biliary tree, distal stomach and regional lymph nodes. Preservation of the pylorus in a PPPD offers a theoretical improvement in gastric emptying and nutritional status. [41] More aggressive approaches to vascular involvement have been pioneered with vein resection and reconstruction allowing complete resection with no significant increases

in morbidity or mortality. [46] Morbidity rates of pancreaticoduodenectomy are reported at 30-60%[47, 48], with mortality generally accepted at less than 5%[49]. The procedure involves 3 anastomoses to reconstruct the anatomy; a pancreaticojejunostomy, hepaticojejunostomy and gastrojejunostomy. The pancreatic anastomosis is at a high risk of leak, fistula formation and abscess formation.[48] Whilst the research literature demonstrates mixed results as to the prognostic value of a pancreatic leak (with a theoretical increased risk of recurrence), it has been purported that increased post-operative morbidity results in impaired immunity and thus increased risk of recurrence[50] ; whilst low morbidity in combination with an R0 resection significantly improves survival. [51]

Once a tumour is resected, histological staging is performed using the TNM classification. As seen in table 2, increasing T stage and N status impacts on median survival. [6]

Table 2 – Characteristics of the TNM classification of pancreatic cancer, with associated impact on prognosis

| Stage | Tumor Grade | Nodal Status | Distant Metastases | Median Survival† | Characteristics |
|---|---|---|---|---|---|
| | | | | *mo* | |
| IA | T1 | N0 | M0 | 24.1 | Tumor limited to the pancreas, ≤2 cm in longest dimension |
| IB | T2 | N0 | M0 | 20.6 | Tumor limited to the pancreas, >2 cm in longest dimension |
| IIA | T3 | N0 | M0 | 15.4 | Tumor extends beyond the pancreas but does not involve the celiac axis or superior mesenteric artery |
| IIB | T1, T2, or T3 | N1 | M0 | 12.7 | Regional lymph-node metastasis |
| III | T4 | N0 or N1 | M0 | 10.6 | Tumor involves the celiac axis or the superior mesenteric artery (unresectable disease) |
| IV | T1, T2, T3, or T4 | N0 or N1 | M1 | 4.5 | Distant metastasis |

#### 1.1.4.2 Chemotherapy

As previously mentioned, one of the main difficulties in treating PDAC is the poor response to chemoradiotherapy, due to both the heterogeneous nature of the genetic landscape and the tumour stroma acting as a physical barrier to chemodelivery. Nonetheless post-operative adjuvant chemotherapy +/- radiotherapy is offered to select patients to extend survival. The nucleoside analog gemcitabine tends to be the mainstay of chemotherapy treatment and has demonstrated significant however limited survival benefits. [52] The large phase 3 CONKO-001 randomised controlled trial (RCT) showed that gemcitabine prolonged disease free survival as compared to the observation group (13.4 vs 6.9 mths, $p<0.001$), with improved median survival albeit by just over 2 months.[53] The European Study Group for Pancreatic Cancer (ESPAC) have performed a number of RCTs investigating optimal adjuvant chemotherapeutic approaches with mixed results. ESPAC-1 demonstrated gemcitabine did prolong survival, however the addition of radiotherapy had no effect.[1] ESPAC-3 showed no difference in outcomes comparing gemcitabine to 5-fluorurocil and folinic acid, concluding that given gemcitabine should be used given its improved side effect profile.[54] Combination chemotherapy regimens was then investigated in the recently reported ESPAC-4 trial, which demonstrated that gemcitabine in combination with capecitibine significantly improved OS from 25.5 months in the gemcitabine monotherapy group to 28 months in the combination group ($p=0.032$). [55] Overall one can see from the marginal increases in survival that more research is required to identify more effective chemotherapeutics, and also consider how to reverse the high levels of chemoresistance that PDAC tumours harbor.

Neoadjuvant chemotherapy has also been introduced, whereby patients with borderline resectable tumours are given chemotherapy in an effort to downsize their tumours,

reducing any vascular involvement, and thus permitting surgery with a better chance of an R0 resection and improved OS. [56] The earliest study from MD Anderson demonstrated a non-significant trend towards improved >30 day survival with neoadjuvant therapy. [57] Whilst more recent studies (ESPAC-5, PREOPANC) have demonstrated a more convincing benefit in OS with neoadjuvant therapy (chemotherapy +/- radiotherapy), more well designed RCTs are required to determine which patients and which treatment regimens are most likely to benefit from this approach as opposed to up-front resection.

Patients with locally advanced PDAC have a median survival of 6-11 months [58], whilst those with metastatic disease typically have a median survival of 2-6months. [59] Therefore in an effort to improve this dismal prognosis palliative chemotherapy is considered for those with biopsy confirmed locally advanced or metastatic PDAC, and who are considered fit enough to endure the side effects of the treatment (defined as having a performance status ECOG score of 0-2). Gemcitabine has again been the mainstay of treatment in these cases [52], however other drugs have demonstrated improved benefit. A phase II/III clinical trial of metastatic PDAC (n-=342) has demonstrated that FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin) improved median OS by over 4 months as compared to the gemcitabine group (11.1 vs 6.8 months). [60] Drugs in combination with gemcitabine have also been investigated, with both capecitabine and cisplatin resulting in no significant improvement in outcomes with patients most likely to benefit having a good performance score. [61, 62] However Abraxane (nab-paclitaxel), a plant alkaloid causing apoptosis through microtubule inhibition, has shown promising results, with a phase 3 study of Abraxane plus gemcitabine significantly improving OS, PFS and response rate, with attenuation of the desmoplastic reaction and improved chemodelivery. [63] Part of palliation also includes managing symptoms of advanced

disease, including restoring biliary drainage for in cases of jaundice with endoscopic stent insertion, palliative gastrojejunostomy for gastric outlet obstruction, coeliac plexus block for relief of intractable abdominal pain, and exocrine enzyme supplementation for pancreatic insufficiency. [41]

#### 1.1.4.3 Novel therapies

A number of promising targeted therapies are either in development or clinical trials for PDAC, whereby the drugs attack specific cancer cell targets, resulting in fewer side effects than systemic chemotherapy. Given the prevalence of KRAS mutations in PDAC, much effort has been made towards targeting this GTPase protein in the Ras family, however this is extremely difficult to do. Alternatively attempts have been made to block KRAS downstream through protein kinase MEK inhibition, however both selumetinib and trametinib, which both inhibit MEK1/2, have not demonstrated any benefit. [64, 65] Rigosertib, a first-in-class Ras mimetic and small molecule inhibitor of multiple signaling pathways, including polo-like kinase 1 and phosphoinositide 3-kinase (PI3K), showed extremely promising results in preclinical animal models, however combination with gemcitabine did not improve OS. [66] Further work is required in what could be a game-breaking discovery if an effective approach is elucidated.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor of the ErbB family with a tyrosine kinase domain activated by a number of ligands; Erlotinib is a tyrosine kinase inhibitor that has previously been shown to give a modest survival benefit in combination with gemcitabine [67] and is now approved for use, however the benefit has been questioned in a recent large phase 3 RCT. [68] Furthermore cetuximab, a monoclonal antibody binding EGFR has not demonstrated any survival benefit. [69] Nonetheless, given

the theoretical benefit this approach promises, a number of drugs targeting EGFR are currently in trial.

Pancreatic cancer is profoundly hypoxic and relies on aggressive angiogenesis for their growth, as evidenced by overexpression of vascular endothelial growth factor (VEGF), therefore agents have been developed to target new vessel formation and hence reduce tumour invasion and dissemination. However clinical studies of VEGF inhibitors such as bevacizumab have not shown any benefit. [70] In fact it has been shown that tumours can be resistant to anti-angiogenic therapy, with treatment increasing metastasis; however proinflammatory factors and EMT markers have been identified as potential biomarkers to select patients who may respond to this form of therapy, emphasizing the potential need for more personalized and specific tumour-directed therapy for each patient. [71]

Cancer stem cells are known to promote tumour growth and increase chemoresistance; therefore monoclonal antibodies such as decluzimab are currently under trial, with promising early results. Another promising approach is towards Poly ADP-ribose polymerase (PARP), which is a nuclear enzyme involved in DMA damage repair, and the results of clinical trials testing PARP inhibition is awaited with much anticipation. [72]

As previously mentioned PDAC has a profoundly inflammatory and reactive tumour stroma, which includes a variety of cellular and non-cellular components, which allow the cancer cells to evade the host immune system. Therefore modulating this immune microenvironment through immunotherapy is another promising avenue to approaching PDAC. This can be performed with checkpoint inhibitors/immune modulators (e.g. nivolumab), therapeutic vaccines (e.g. GVAX), adoptive cell transfer (i.e. with

genetically engineered T cells), monoclonal antibodies that generate an immune response, oncolytic viruses (e.g. ParvOryx), adjuvant immunotherapies (e.g. motolimod), and targeting cytokines (e.g. recombinant human IL10 AM0010).[73]

There are alternative approaches to the tumour stroma that have also been investigated. With respect to chemoresistance, it has been observed in a mouse model experiment that the pancreatic vasculature rarely comes close to the adenomatous component of the tumour, resulting in poor vascular perfusion and hence reduced effective delivery of chemotherapeutics. [74] So essentially it may be that current chemotherapeutics such as gemcitabine are being inappropriately sequestered in the surrounding stroma and not reaching the tumour itself, placing further emphasis on the importance of targeted stromal therapies to allow more effect drug delivery. Furthermore multiple protumourigenic relationships exist between PDAC and different stromal cell types, and disrupting this interplay has been shown to induce tumour growth suppression, slow local invasion and reduced metastatic spread.[75] Targeting the relationship with immune cells has been discussed above, however a more attractive approach may be towards pancreatic stellate cells (PSCs), which have been shown to have a key role within this desmoplastic reaction. [76] The role of the stroma and potential therapeutic approaches will now be discussed with particular relevance to pancreatic stellate cells.

## 1.2 Tumour microenvironment

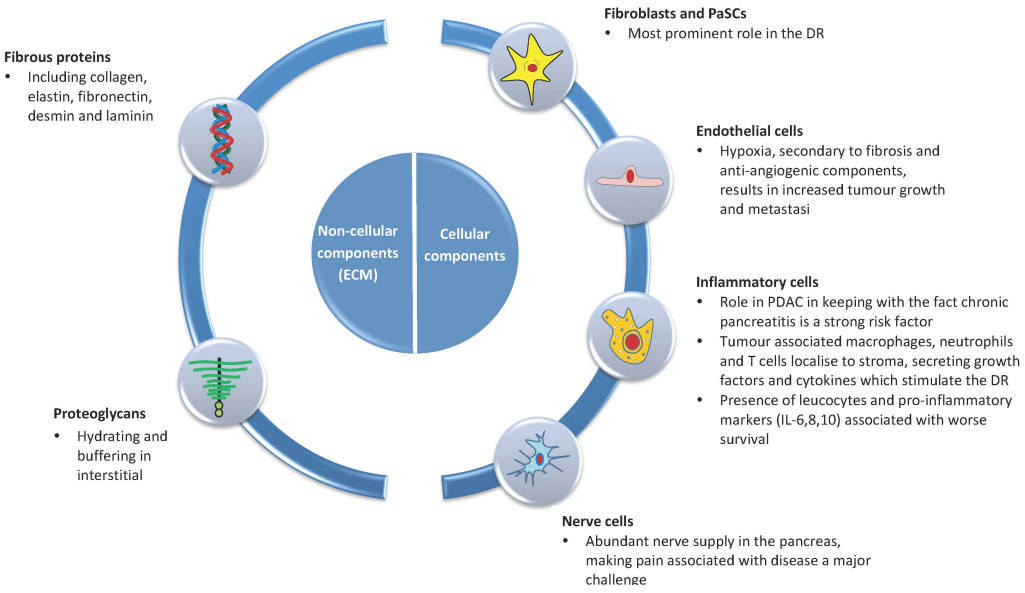
The pancreatic stroma consists of a variety of cellular and non-cellular constituents that become more abundant in both benign inflammatory conditions and malignancy. Tumour stroma is defined as the interstitial tissue surrounding a malignant lesion, consisting of a wide variety of inflammatory, vascular and neural components which interact with tumours resulting in a desmoplastic reaction [6] which encourages tumour invasion, metastasis, and chemoresistance. [77-79] Stromal activity is most linked with epithelial tumours (breast, prostate, ovarian, colorectal), and pancreatic cancer perhaps demonstrating the most prominent 'stromal' reaction.[79] Furthermore it has been observed that stromal reactions can precede cancer, confirming the importance of premalignant lesions such as pancreatic intraepithelial neoplastic lesions (PanINs).[80]

### 1.2.1 Stromal composition

The stromal components surround the central core of a malignant pancreatic ductal adenocarcinoma. The stroma is made up of a variety of distinctly different cellular substrates resulting in a dense desmoplastic reaction (DR) which accounts for up to 90% of the total tumour volume.[81] This stroma can typically be seen surrounding tumours on radiological imaging (computed tomography) in the form of ill-defined inflammatory tissue, and this suggests an interesting concept in tumour staging as large sections of "activated" stroma may be left behind following resection, which may encourage proliferation of extremely small numbers of tumour cells that may have been left in situ (R1 margin), as well as acting as a barrier to chemotherapy, hence reducing the likelihood of eliminating this residual disease with adjuvant treatment. It was previously thought that this DR had a protective effect.[82] However it has now been shown that once the stromal

elements are activated they take on pro-malignant attributes, and possibly even initiate carcinogenesis.[77] The interactions between stroma and tumour are bi-directional, as cancer cells stimulate stromal components to secrete more mitogenic constituents, which in turn promote tumour growth, invasion and resistance to chemo/radiotherapy. The DR is composed of a variety of cellular and non-cellular components, as depicted in figure 1. Essentially the cellular components interact with each other and the adjacent cancer cells, resulting in the deposition of an abundant extracellular matrix (ECM).

Figure 1 – The various cellular and non-cellular components that make up the desmoplastic reaction in pancreatic cancer




### 1.2.2 Fibrosis and cancer

In the context of a normal pancreas, the stroma can be deemed a protective environment, providing crucial signaling which maintains tissue architecture and suppresses the malignant phenotype. The ECM plays a key role in this process as the dynamic non-cellular scaffolding, which is present in all organs to support tissue homeostasis. It contains mostly

water, proteins and polysaccharides.[83] The two main macromolecules making up the ECM are fibrous proteins and proteoglycans, which act as a gel within the interstitial space and have a role in buffering, hydration and binding.[84] Collagen is the most abundant fibrous protein, accounting for 30% of protein mass, and has a key role in tensile strength and in the regulation of cell adhesion.[83] In normal epithelial tissue, non-activated fibroblasts are the main players in the secretion and organisation of fibrous proteins, in particular type 1 collagen that constitutes 90% of protein content.

The physical and biochemical characteristics dictate the properties of an organ, such as strength and elasticity, and these will vary according to its physiological state. For example, tumours have been compared to wounds that fail to heal, taking on the typical stiffness as compared to normal tissue.[85] When an injury is sustained to the ECM, the classic wound healing process is activated resulting in vascular damage and the formation of a fibrin clot. Activated monocytes then differentiate into macrophages, and subsequently release various growth factors, cytokines and proteases (e.g. metalloproteinases - MMPs). This results in both angiogenesis, and most importantly fibroblast proliferation, which leads to in increased deposition of ECM proteins (especially collagen) and resultant fibrosis. Normal tissue has a feedback mechanism to maintain homeostasis and regression in fibrosis. However, as supported by clinical observation such as in cases of chronic pancreatitis and liver cirrhosis, repeated injury results in continuous ECM synthesis, and ultimately irreversible damage. The impact of neural invasion resulting in the commonly presenting abdominal pain of these patients is reflected by stromal interactions; supernatants of fibroblasts from chronic pancreatitis patients have been shown to significantly stimulate neurite outgrowth.[86]

This continual damage to the ECM can be viewed as an aging process, whereby basement membranes become thin, and suboptimal cross-linking of collagen occurs. This results in a weak and fragile ECM with disorganisation promoting age-related diseases such as cancer.[83] The fact that chronic pancreatitis is a major risk factor for PDAC makes this all the more interesting, suggesting that stromal activity and the ECM damage that ensues is a key process of carcinogenesis in the pancreas. Yet we accept that not all patients with pancreatic carcinoma have chronic pancreatitis or vice versa because it is well appreciated that the development of pancreatic cancer is multi-factorial with defects in many key pathways being involved.

The various ECM components as highlighted in figure 1 also have a pro-tumourigenic role in their own right. Fibrous proteins such as fibronectin and laminin stimulate reactive oxygen species (ROS) production through NAPDH oxidase, resulting in increased pancreatic cancer cell survival.[87] Matricellular proteins secreted from both stromal and cancer cells have also been shown to have a key role in facilitating tumour progression through downstream signaling that results in increased proliferation and metastasis of cancer cells. They have also been shown to directly impact on aberrant ECM remodelling, further promoting the pro-tumourigenic environment in the stroma.[88]

There is a large amount of evidence demonstrating the pro-tumourigenic effect of the stroma in pancreatic cancer, however as already mentioned the components microenvironment also exert a protective effect. An example of this is the secretory protein osteopontin (OPN), which is secreted from stromal macrophages and has been shown to contribute to tumourigenesis through MMP activation, and direct effects on cancer cell migration and invasion through apoptotic preventative mechanisms in breast and kidney

animal models.[89, 90] Furthermore over expression in resected hepatocellular carcinoma specimens has been identified as a biomarker of poor prognosis.[91] However, in the context of pancreatic cancer OPN expression has been shown to impart a significant survival advantage independent of tumour stage in resectable tumours. [92]This supports the complex heterogeneic nature of PDAC, and emphasises the degree of caution required when considering therapeutic targeting of the stromal elements. Additional tumour protective elements have been noted in both scientific and clinical studies, and these will be further discussed at relevant intervals throughout this review as we consider the most appropriate therapeutic approach.

## 1.3 Pancreatic Stellate Cells

Stellate cells have been found in a variety of organ locations, including liver, kidney, intestine and spleen.[93] Pancreatic stellate cells were first isolated in 1998 [94] and since then they have been shown to have a key role in health by maintaining tissue architecture through the regulation of ECM protein synthesis and degradation. In normal tissue PSCs exist in a quiescent state, comprising 4-7% of pancreatic parenchyma.[76] These cells contain an abundance of vitamin A lipid droplets within their cytoplasm and are therefore considered an adipogenic phenotype. In the normal pancreas PSCs become activated in response to any form of insult, triggering fibrosis through various stromal interactions and thus allowing the wound healing process to occur. On resolution the PSCs return to their quiescent state until they are called upon again in their damage limitation role.

PSCs have various other roles in normal pancreas functioning. Quiescent PSCs have a positive effect on epithelial integrity through the integrin β1-dependent maintenance of the basement membrane, thus demonstrating a role in acinar functionality.[95] An additional role in exocrine function has been demonstrated, whereby the gastrointestinal hormone cholecystokinin (CCK) induces acetylcholine secretion by PSCs, which in turn stimulates amylase secretion by acinar cells.[96] Furthermore CCK has been shown to have direct activating effect on PSCs, and induces collagen synthesis.[97]

The exact source of activated PSCs (myofibroblast phenotype) is still a matter of much debate; however a consensus exists that in the context of benign inflammation or malignancy, the surrounding cancer, immune or endothelial cells release various growth

factors and inflammatory cytokines that in turn activate these PSCs through paracrine signaling networks.[81]

When studying PSCs it is important to realise the inherent differences between and activated and quiescent phenotype, particularly for the purposes of cell isolation and culture, and these are listed in Table 1. A further consideration to take into account when culturing PSCs is the fact plastic itself stimulates activation and thus does not replicate the normal stromal microenvironment. A study comparing culture on plastic, matrigel (a basement membrane-like compound that mimics ECM in health) and collagen I (which mimics the ECM in diseased conditions) revealed significant differences in PSC gene expression patterns.[98]

The true hallmark of an activated PSC is αSMA expression, as demonstrated in Table 3. As such a prognostic marker has been developed termed the Activated Stroma Index (ASI), which is the ratio of αSMA to collagen.[99] If the ASI is high, this therefore indicates increased PSC activity and thus increased stromal interactions. The ASI has been identified as an independent prognostic marker in multivariate survival analysis when compared with the nodal status of the pancreatic cancer; however no correlation was found with tumour size or grade. Nonetheless this may represent a potentially useful prognostic tool by assessing the degree of stromal activity that is not precisely seen on radiological imaging.

Table 3 – The different characteristics of an activated as compared to quiescent PSC

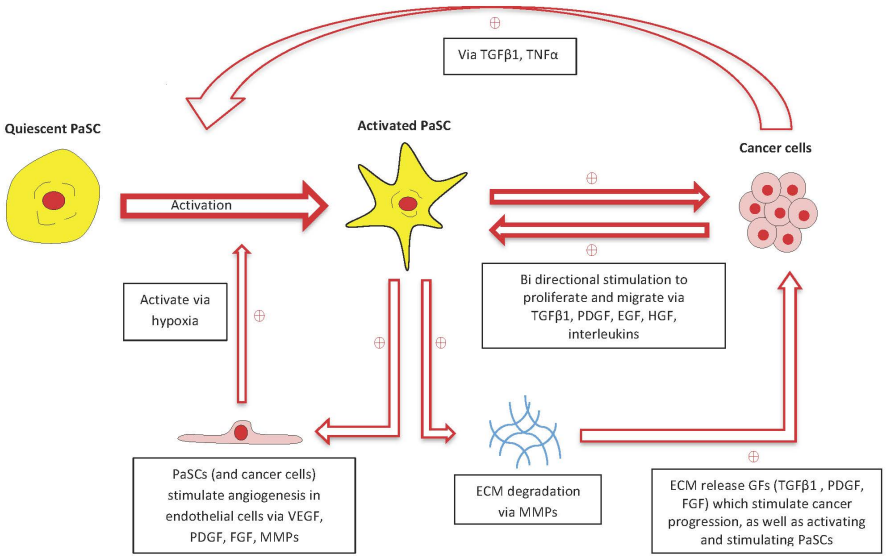
| **Characterisation feature**[2] | Activated PSC | Quiescent PSC |
|---|---|---|
| ***Appearance*** | Star shaped | Spindle shaped and smaller |
| ***αSMA expression*** | Positive (in >90%) | Negative |
| ***Desmin expression*** | Positive in 20-40% | Negative |
| ***Vimentin expression*** | Positive | Positive |
| ***GFAP expression*** | Positive | Negative |
| ***Adherence in tissue culture*** | +++ | + |
| ***ECM protein secretion*** | +++ | + |
| ***Receptor expression*** | +++ | + |
| ***Density*** | +++ | + |

### 1.3.1 Tumourigenic interactions

Recent evidence demonstrates that of all the components present within the stromal microenvironment, PSCs have the most influential part to play in both fibrogenesis and carcinogenesis. Figure 2 (adapted from Erkan et al 2012 [76]) demonstrates potential pathways that have been proven or postulated, and it is clear from this diagram that a complex relationship exists between the tumour and stroma that heavily involves PSCs. However the question remains as to which of the various pathways exert the most influence on disease progression. Is it the direct interplay between cancer cell and PSC, and if so which pathway exerts the greatest effect? Is it the effect on the ECM, with irreversible and disorganised deposition promoting carcinogenesis? Or is tumour aggressiveness more related to the disruption in vasculature that ensues, contributing to the hypoxic environment that not only drives PDAC but also increases chemoresistance. The likelihood is these concepts combine together to promote cancer progression. An exciting prospect

exists whereby if the PSCs can be targeted, one can exert an influential effect on the key and complex interplay between the cancer cells, ECM and tumour vasculature. The pro-tumourigenic effects of PSCs will now be discussed with respect to the relevant "Hallmarks of Cancer".[100]

Figure 2 – The complex bi-directional interactions of PaSCs (PSCs) with both the adjacent cancer cells and stromal elements within the tumour microenvironment




Genome instability/mutation and sustained proliferative signaling

As previously discussed PDAC is an extremely heterogeneous cancer, and a number of mutations drive its aggressive nature, most commonly the KRAS oncogene (in approximately 90% of cases), and the tumour suppressor genes such as CDKN2A (85%), TP53 (80%) and SMAD4 (50%).[9] These mutations underpin cancer initiation, development and invasion, however a supportive microenvironment is required allow sustained proliferation. This can involve genetic alterations in various pathways that are inextricably associated with the PSC-cancer cell relationship. It has been demonstrated that

tumour-stromal interactions contribute to oncogenic signaling involving the hedgehog pathway, cyclo-oxygenases, the ECM protein SPARC and NFκB.[101]

Cancer cells possess an innate ability to undergo sustained and belligerent proliferation, overriding the body's defence mechanisms through the deregulation of various signaling pathways. A bidirectional relationship exists between PSCs and the cancer cells of PDAC involving a number of pathways as outlined in figure 2. Firstly cancer cells secrete growth factors that stimulate PSC activation. These include TGF-β, HGF, FGF and EGF.[76] TGFβ has a complex role in PDAC and genetic analysis has revealed a mutation in at least one of the TGFβ family members in 100% of pancreatic cancers.[14] Both *in vitro* and *in vivo* co-culture studies have demonstrated that TGF-β expression induces proliferation of fibroblasts, and results in a more significant DR with excessive ECM protein deposition.[102, 103]

Sonic hedgehog (SHH) signaling has also been found to have a key role in both inflammation and malignancy through its effect on the DR. SHH has recently been found in precursor and primary pancreatic lesions, whilst being absent in the normal pancreas, thus implicating this pathway in the initiation and progression of pancreatic cancer.[104] It has been shown that SHH stimulates PSCs by a paracrine mechanism, and furthermore promotes EMT, proliferation and invasive capacity.[105] However attempts to utilise SHH inhibition as a treatment approach have yielded unfavourable results, potentially through PSC depletion; these findings will be discussed further in the therapeutics section.

Furthermore additional mutations may be contributing to an increase in tumour invasiveness; in a cancer cell-fibroblast co-culture it was found that the up-regulated genes

COX-2, HAS2 and MMP-1 were all associated with invasive potential.[106] It is certain more in depth studies are required to characterise the genetic molecular events that underpin the disruption and facilitation of the various signaling pathways between PSCs and cancer cells.

Invasion and metastasis

The process of invasion and metastasis has been sequentially broken down into the cellular processes that occur as cancer cells move into the local blood vessels and lymphatics, then extravasate into distant tissues and colonise, with progression of micro-metastases into macroscopic tumours. Studies have demonstrated that when cancer cells and PSCs combine, there is increased activity; a co-culture study in an *in vivo* subcutaneous mouse model showed the combination of cancer cells and PSCs resulted in increased tumour size compared to cancer cells alone.[107] With respect to dissemination, epithelial mesenchymal transition (EMT) has been importantly linked to this process. EMT allows cells to dissociate from their origin, losing their epithelial features, and is thereafter heavily linked with tumour proliferation and metastases [108]; PSCs have been shown to have a role in this key developmental regulatory programme. *In vivo* orthotopic models have shown when pancreatic cancer cell lines are combined with PSCs there is a more pronounced stromal reaction, increased invasion, and increased metastasis.[109]This is supported by the fact PSCs have been shown to induce EMT in cancer cells themselves, resulting in dissociation and increased migration, further suggesting a role in metastasis.[110] The presence of αSMA positive cells (a hallmark of the activated phenotype) and even PSCs themselves in metastases also suggests a role in facilitating seeding and proliferation of cancer cells at distant sites, and this concept certainly necessitates more investigation.[109, 111] PDGF, secreted from cancer cells, has a potential

role in this migratory process as it has been shown to have a chemotactic effect on PSCs.[112] A further effect of PSCs is their ability to suppress hydrogen peroxide induced apoptosis, and therefore prolongs pancreatic cancer cell survival.[109]

Tumour promoting inflammation

It has become accepted that infiltrating immune cells are present in all neoplastic lesions, in both a tumour preventative and promoting capacity. In the setting of a chronic non-healing wound, inflammatory cells release a host of growth factors, chemokines and cytokines with an overriding pro-tumourigenic effect. In PDAC it has been shown that leucocyte infiltration, even at the earliest stages, orchestrates an immune reaction that is immunosuppressive.[113] As such therapeutic approaches in the form of immunotherapy have been examined, with CD40 agonists being shown to induce PDAC tumour regression through T cell mediated pathways.[114]

With respect to immune cells and PSCs, a bidirectional relationship again exists, whereby as part of the inflammatory response, PSCs become activated and in turn stimulate the recruitment and localisation of macrophages, neutrophils, T cells and mast cells to the cancer stroma. In turn tumour associated macrophages secrete growth factors (PDGF, TGFB) and cytokines (IL6), which result in an increase in the DR through PSC activation and effects on other stromal constituents.[115] All inflammatory cells secrete a plethora of cytokines, in particular IL-6 which activates STAT3 (signal transducer and activator of transcription 3) to promote tumourigenesis in tissue with Kras mutations such as PDAC.[110] As well as recruiting copious numbers of inflammatory cells that further enhance the pool of growth factors and cytokines that promote the DR, PSCs also secrete galectin-1, which causes apoptosis of T cells and thus inhibits anti-tumour immunity.[116]

As mentioned the ECM provides a supportive environment for carcinogenesis, chiefly through the effect of collagen, resulting in reduced physical compliance through an induction of an inflammatory environment, and thereby promoting carcinogenic initiation. In the context of malignancy, it is observed that the stromal matrix, and not the tumour itself, produces the majority of collagen (in particular types 1 and 3), with the principle source being PSCs that transcribe and secrete the majority of the fibrous proteins constituting the ECM.[117] PSCs therefore potentially hold the key to developing targeted anti-stromal therapy for the treatment of both benign and malignant conditions of the pancreas. Collagen type 1 has been shown to increase the proliferation, migration and resistance to apoptosis of PDAC cancer cells, mediated via integrin receptor signaling.[118] Various matricellular proteins secreted by PSCs have also been implicated in increasing cancer cell invasiveness and migration, including SPARC[111] and periostin.[119] Overall the excessive ECM also creates a dense fibrotic paratumoural environment that is hypovascular and hypoxic, meaning there is reduced tumour perfusion of therapeutics, such as gemcitabine.[74] This is due to anomalous collagen formation and associated proteoglycan interactions in the ECM causing a physiological barrier to drugs.[120] It has even been shown that ECM proteins confer resistance in their own right.[121]

As well as excessive ECM deposition resulting in protumourigenic effects, ECM turnover and associated degradation also has a key role in PSC and cancer cell activity, with MMPs and proteases being key players in this process. Co-culture experiments have shown cancer cells exert a paracrine effect on adjacent PSCs resulting in MMP secretion.[122] MMPs stimulate the degradation of collagens type I, III and IV[123]; however the effect is most marked on type IV, resulting in basement membrane disruption and thereby promoting tumour progression.[124] As a result of the ECM degradation a host of growth factors are

released, including TGFB, PDGF and FGF.[76] Once again this represents a self-sustaining process whereby these growth factors affect cancer cell activity, as well as stimulating PSC activation, further contributing to an over-active and protumourigenic ECM.

Angiogenesis

The main vascular cells in the stromal microenvironment are fully differentiated endothelial cells that are stimulated by VEGF. In order for tumours to grow they require new blood vessels and VEGF, along with other growth factors such as PDGF and FGF, which stimulate angiogenesis allowing tumour progression.[125] These new blood vessels tend to be disorganised and haemorrhagic, and in combination with the associated fibrotic stroma this results in a suboptimal vascular supply and overriding hypoxic conditions, which in turn stimulates further VEGF secretion.[125] Therefore VEGF over-expression is linked with both poor prognosis and increased metastatic potential.[8] VEGF is secreted by PSCs, as well as fibroblasts, the ECM and cancer cells[126]; therefore anti-PSC therapies designed to cut off production at the source, rather than the current tactic of targeting VEGF receptors, represents a novel approach.

The desmoplastic reaction itself also has a key role in supporting tumour growth through distorted blood supply and an overriding hypoxic environment. A combination of reduced blood flow and increased metabolic activity in the surrounding stroma results in hypoxia that PDAC thrives in, contributing to the aggressiveness of disease.[127] It is already well known that hypoxia is strongly linked with both increased tumour growth and metastasis in PDAC.[70] The DR, which can now clearly be seen to be chiefly regulated and stimulated by PSCs, leads to an increase in ECM proteins (particularly collagen) and an increase in interstitial fluid pressure (IFP), resulting in this disorganised vasculature which supports

tumourigenesis.[79] Furthermore genetic factors are implicated, as evidence suggests hypoxia stabilises transcription factor HIF-1α and therefore up-regulates genes including glucose transporters (Glut1), glycolytic enzymes (Aldolase A) and angiogenic factors; this in turn fuels the interplay between cancer cells and their associated fibroblasts.[128] This is supported by studies showing HIF1α expression is associated with chemoresistance and increased tumour invasion.[70]

A recent study has demonstrated that a further self-perpetuating loop exists, whereby PSCs promote hypoxia, and in turn this hypoxic environment further stimulates PSC activation. It has been shown that cancer-associated PSCs in hypoxia enhance the invasion of cancer cells more strongly than those in normoxia, with carbonic anhydrase 9 (CA9) being a useful hypoxic-regulated marker (as well as HIF-1 α).[129] As expected VEGF expression was increased (4.5 fold) in hypoxia as compared to normoxia, however the role of connective tissue growth factor (CTGF) was elucidated. CTGF, which is secreted by PSCs, is already known to be expressed under hypoxia, and has been shown to protect cancer cells from apoptosis in an autocrine manner and have a key role in the hypoxic environment, with knockdown experiments revealing CTGF secretion was increased in hypoxia, resulting in an increase in PSC-induced cancer cell invasion in hypoxia as compared to normoxia; these findings were confirmed in both *in vivo* and *in vitro* experiments.[130]

Deregulated metabolism

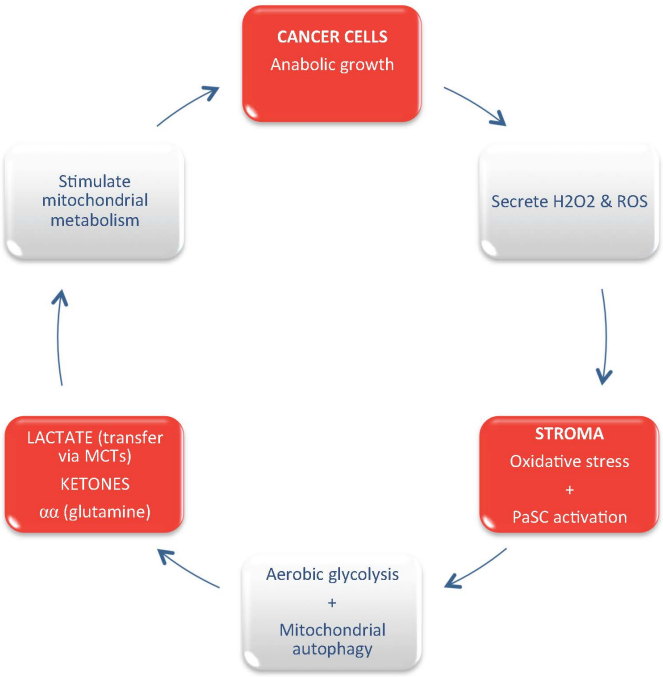
A topic that has received less attention with respect to the tumour microenvironment and fibroblasts in particular is the adjustment in energy metabolism that occurs. This may be because traditionally the Warburg Effect has been widely accepted as the mechanism that drives cancer cell metabolism and subsequent anabolic growth. The Warburg effect was a

term first coined in the 1920s and states that cancer cells undergo a metabolic shift from producing energy by mitochondrial OXPHOS to aerobic glycolysis, which involves the conversion of glucose into lactate, albeit with less ATP production than oxidative metabolism. It is based on the principle that "the prime cause of cancer is the replacement of respiration of oxygen in the normal body cells by fermentation of sugar", thereby generating energy for cancer cell proliferation.[131] However a concept published around the time of the commencement of this project, termed the "reverse Warburg effect", challenged the way we look at cancer cell behaviour with respect to lactate shuttling mechanisms with neighboring fibroblasts, and stimulated my interest to investigate this further in the context of PDAC. The theory was described in the breast cancer microenvironment, which like PDAC is an epithelial adenocarcinoma with an abundance of stromal cancer associated fibroblasts (CAFs), which resemble the activated PSC form. In brief it is theorized that cancer cells commandeer neighbouring fibroblasts, stimulating glycolysis with subsequent lactate production which the cancer cells hijack to use for oxidative metabolism, producing larger amounts of ATP and thus promoting anabolic growth (figure 3). [132, 133] Essentially it was proposed that CAFs are acting as an energy substrate factory and in turn feeding cancer cells, thus causing a perpetuating cycle of increased activity and proliferation. Given the similarities between breast cancer and PDAC, with both being epithelial tumours with a CAF-rich microenvironment, it could be hypothesised that PSCs may also be implicated in the movement of lactate between the stroma and epithelial tumour, and interrupting this food chain may induce a tumour suppressive effect.

Autophagy is another novel concept in which the stroma, and therefore PSCs, has a significant part to play. This is a homeostatic process present during metabolic stress (such

as hypoxia or nutrient deprivation) whereby ATP and metabolic by-products are recycled.[134] In the context of cancer, autophagy can be tumour-protective by helping to maintain essential cellular functions within the hypoxic environment, which is such a key feature of PDAC. The increased expression of HIF1α and oxidative stress also promotes autophagy. [79-80] Therefore it can be seen that autophagy facilitates the energy transfer processes between the tumour and stroma resulting in cancer growth. Furthermore loss of caveolin 1 (cav1) has been shown to be indicative of autophagy. [81] During the process of breast fibroblast activation, triggered by oxidative stress from hydrogen peroxide secreting cancer cells, there is a loss of cav1 and activation of MCT4, distinguishing these cells as CAFs. [81] This alteration in transporter expression is supported by oncogenetic activation, including RAS and NFKB. [82] So underlying genetic mutations, which traditionally were believed to act chiefly on cancer cells in allowing them to proliferate, are instead influencing CAFs to a greater degree, and thus reprogramming the microenvironment to be pro-tumourigenic by providing cancer cells with energy as well as activating CAFs to enhance the DR. Despite these studies having not been performed on pancreatic cancer, we can theorise that a form of metabolic symbiosis exists between PSCs and cancer cells which potentially revolutionises the way we think about cancer treatment, and represents an exciting avenue of investigation. This concept and others in relation to potential metabolic reprogramming in PDAC will be further discussed.

Figure 3.1 – The Reverse Warburg Effect – Cancer cells activate the surrounding stroma, resulting in aerobic glycolysis within neighbouring fibroblasts. The resultant nutrients are hijacked by the cancer cells to allow perpetuating anabolic growth.




### 1.3.2 Therapeutic approaches towards PSCs

As previously discussed, one of the main difficulties in treating PDAC is the poor response to chemoradiotherapy. It has been observed in a mouse model experiment that the pancreatic vasculature rarely comes close to the adenomatous component of the tumour, resulting in poor vascular perfusion and hence reduced effective delivery of chemotherapeutics.[74] So essentially it may be that current chemotherapeutics such as gemcitabine are being inappropriately sequestered in the surrounding stroma and not reaching the tumour itself, placing further emphasis on the importance of targeted stromal therapies to improve para-tumoural vasculature leading to more optimal delivery of chemotherapeutics. Therefore much research has concentrated on discovering novel or existing drugs which may play a role in combination with chemotherapy regimes in

generating a personalised multi-directional attack on carcinogenic pathways, which should now include the stroma and in particular PSCs.

However given the potential tumour-protective effects that the stroma and PSCs may incur, a degree of caution is required when deciding which processes and pathways may be targeted without incurring detrimental effects. Furthermore one must consider the impact on tumour blood supply; it is possible that reducing some elements of the DR within the stroma may impact on angiogenesis, resulting in improved organisation of para-tumoural vasculature and a resultant increase in metastatic spread. These considerations will be taken into account as the various methods of approach are discussed.

Target PSCs directly

The principles behind the targeting of PSCs, and a resultant suppression of the DR, can result in a multitude of tumour effects. Considering PSCs as the major player in the DR that facilitates cancer progression, they represent an ideal direct target to turn off stromal activity at the source and hence reduce cancer cell proliferation, invasion and dissemination. This has been approached in a variety of ways, however most successfully by inducing quiescence with vitamin A derivatives (all-trans retinoic acid), which has been shown to reduce tumour growth, cancer cell proliferation and invasion.[135] Retinoic acid signaling has been shown to act in conjunction with Histone De-acetylase (HDAC), and a recent study has demonstrated that HDAC inhibitors acted on both PSCs and cancer cells but not cancer cells alone in terms of reducing cancer cell invasion.[136] Alternatively one can eliminate PSCs with Vitamin E derivatives (tocotrienols), which have been shown to induce apoptosis of activated (but not quiescent) PSCs through the mitochondrial death pathway.[137]

Micro-RNAs act as negative regulators of gene expression at the transcriptional level, and it has been recently demonstrated that PSCs induced miR-210 up-regulation in pancreatic cancer cells through ERK- and PI3K/Akt-dependent pathways.[138] Subsequent inhibition of these pathways and resultant reduced miR-210 expression resulted in decreased migration and EMT, decreased the expression of vimentin and snai-1, and increased the membrane-associated expression of β-catenin in Panc-1 cells co-cultured with PSCs. MiRNAs have also been shown to have a role in PSC activation, whereby the expression profile is dramatically altered upon activation (42 upregulated >2 fold and 42 downregulated <0.5 fold).[139] Further studies into the role of micro-RNAs and the potential for targeting thus represent a novel and effective therapeutic approach.

Pirfenidone is an anti-inflammatory drug that targets fibroblasts, and is starting to develop an established role in the treatment of idiopathic pulmonary fibrosis as a result of trials suggesting significant clinical benefit.[140] A recent study has demonstrated efficacy in through the inhibition of the DR in pancreatic cancer, whereby *in vitro* pirfenidone treatment of primary PSCs isolated from human pancreatic tissue inhibited the proliferation, invasiveness and migration of PSCs in a dose dependent manner, whilst also reducing the protumourigenic effects of PSC supernatants through the reduction in growth factor and ECM component production (PDGF, HGF, collagen type 1, fibronectin and periostin).[141] A mouse model also demonstrated that oral administration of pirfenidone suppressed tumour growth, reduced the number of peritoneal disseminated nodules, and reduced the incidence of liver metastasis in mice co-implanted with cancer cells and PSCs. Combination with gemcitabine more effectively suppressed growth as compared to gemcitabine alone. Given the safety of the drug demonstrated in IPF, these findings suggest a RCT would be of great interest to observe potential clinical benefit.

However as previously mentioned, one must be wary of the potential protective effects of the stroma, and PSCs in particular. This is demonstrated by a recent study involving a transgenic PKT mouse model, whereby an approximate 80% depletion in αSMA myofibroblasts led to the development of highly differentiated PDAC tumours with significantly reduced survival as compared to controls.[142] This was associated with reduced collagen deposition, reduced ECM compliancy, enhanced hypoxia, EMT and cancer stem cells. A detrimental effect on immune surveillance was also observed; a reduction in effector T cells and increase in regulatory T cells correlated with disease progression, with an increase in expression of cytotoxic T lymphocyte associated protein 4 (CTLA4). The authors then elegantly displayed a potential therapeutic approach in the context of diminished αSMA myofibroblasts, whereby treatment with CTLA4-blocking antibodies reduced the proportion of tumour cells and significantly prolonged survival. This study perfectly highlights the need for further work in discovering the most appropriate methods of approaching direct PSC targeting with a degree of caution to avoid potentially detrimental results.

Target signaling pathways

Much work has been done investigating the antagonism of the TGF-β pathway resulting in reduced PSC activation and reduced EMT. Direct TGF- β antagonist with SR2F has been shown to reduce ECM deposition.[143] A multi-institutional phase 1b/2 clinical trial (NCT01373164) of the TGF-βR1 inhibitor LY2157299 $H_2O$ (in combination with gemcitabine) in patients with advanced or metastatic un-resectable PDAC revealed improved OS, with 4 proteins identified as predictors of response. However one must be cautious in the global inhibition of the TGF-β pathway, given that certain members of the

family have a key role in “normal” tissue functioning with respect to growth and cell cycle arrest, and inhibition of these elements needs to be avoided.

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and are known to have anti-fibrogenic functions. An *in vitro* study, in which isolated mouse and human PSCs were cultured and pretreated with BMP2 followed by TGF-β treatment, revealed that BMP2 pretreatment inhibited TGF-β-induced α-SMA, fibronectin, and collagen type Ia expression. Knockdown studies revealed this anti-fibrogenic effect is regulated through the Smad1 signaling pathway.[144] This is in contrast to previous studies demonstrating BMP induces EMT in pancreatic cancer cells through a SMAD-dependent mechanism, resulting in increased MMP activity and increased cancer invasiveness.[145] Given this conflicting evidence of an anti-PSC but also pro-cancer cell effect, more research is required to determine whether targeting BMP with therapeutics represents a feasible approach.

Alternatively PPARγ may be targeted, which has active involvement in the TGF-β pathway as well as trans-differentiation of fibroblasts. PPARγ agonist, for example thiazolidinediones such as rosiglitazone, have shown potential in a pulmonary study.[146] A potentially more straight forward treatment therapy is via COX-2 inhibition; activated PSCs express COX-2 when stimulated with TGF- β, and it has been shown that celecoxib reduces αSMA and collagen expression in PSCs.[147] Other growth factor pathways investigated for therapeutic targeting include PDGF, with PDGF antagonist trapidil being shown to reduce PSC proliferation in vitro.[148]

Targeting the relationship between PSCs and cancer stem cells (CSCs) represents another therapeutic approach, especially as CSCs have a key role in chemoresistance. Nodal/activins are secreted proteins of the TGFβ family involved in a variety of developmental processes. It has been shown that PSCs secrete nodal/activin and in turn create a paracrine niche for PDAC stem cells, promoting their self-renewal capacity and tumour invasiveness. A study of mice transplanted with human PDAC tumours demonstrated that subsequent nodal/activin inhibition increased chemosensitivity of the CSCs and led to increased PFS.[149] Interestingly this is in contrast to previous mouse model studies involving xenografting of isolated cells, which results in a less dense stromal reaction.[150] This emphasises the importance of representative animal models when conducting studies on the microenvironment with the aim of discovering clinically relevant therapeutics.

Sonic Hedgehog (SHH) signaling had originally shown potential promise as a therapeutic target in animal studies; the use of IP-926, an inhibitor of Smoothened (one of the two transmembrane receptors in the Hh pathway), resulted in PSC deactivation, reduced collagen formation, improved vascularity and more effective delivery of chemotherapeutics, however this effect was found to be transient.[74] Combination treatment with CSC targeted therapy may incur improved response, whereby the use of a smoothened inhibitor, nodal/activin inhibitor and gemcitabine resulted in significant PSC apoptosis, stromal depletion and improved survival.[149] However a subsequent study has demonstrated SHH loss (through both genetic and pharmacological targeting) increases pancreatic tumourigenesis and vascularity, and significantly reduces survival, with a concurrent significant reduction in αSMA myofibroblasts.[151] The latter finding emphasises the fact that simply depleting PSCs is not necessarily beneficial. However this

work did reveal an interesting knock-on effect of SHH loss, whereby treatment with VEGFR-2-blocking antibodies significantly improved overall survival. Whilst anti-angiogenesis therapy has not typically incurred a survival benefit in PDAC, studies have shown that some patients do respond to bevacizumab.[70] Therefore it may be that a subset of patients with poorly differentiated well vascularised tumours with SHH loss may benefit from angiogenesis inhibitors, and this certainly requires further investigation.

Given the conflicting results from the aforementioned *in vitro* and animal studies, the true *in vivo* effect of SHH inhibition warranted investigation and thus went forward to clinical trial, however yielded unfavourable results. A trial of saridegib (IPI-926 inhibitor) in combination with FOLFIRINOX in patients with previously untreated locally advanced or metastatic PDAC initially showed promising results with associated tumour shrinkage.[152] However on progression to a phase 2b trial (n=122), this study was prematurely terminated due to increased mortality in the treatment group. Another study of saridegib in combination with gemcitabine in patients with metastatic PDAC again showed no clinical benefit. [153] And furthermore an open-label, single arm, multi-center (USA) Phase II trial clinical trial (NCT01088815) investigating the SHH inhibitor GDC-0449 in combination with chemotherapy in patients with previously untreated metastatic PDAC revealed no difference in OS. As such these studies all suggest this approach most likely does not translate to significant patient benefit.

As previously discussed CTGF has a role in the hypoxic microenvironment, resulting in an increased DR and tumour invasiveness. A phase 2 open-label, single-arm, dose-escalation study (NCT01181245) of the CTGF monoclonal antibody FG-3019 in combination with gemcitabine and erlotinib is underway for patients with locally advanced or metastatic

PDAC (n=75). Preliminary results suggest OS improves with increasing exposure to FG-3019, and treatment was well tolerated.[154] The study group plan to conduct a phase 2 RCT of gemcitabine and abraxane +/- FG-3019 in patients with marginally inoperable PDAC to determine whether this treatment regime may be used as a conversion therapy.

Rhein is a natural anthraquinone compound found in rhubarb extracts which has been used as a laxative since ancient times in the Chinese population.[155] Recent in vivo and in vitro experiments in mouse model of cerulein-induced CP and in a cellular model of immortalized rat PSCs revealed Rhein treatment induced an anti-fibrogenic affect through a number of PSC-implicated pathways[156] Anthraquinone compounds are already known to exert anti-inflammatory effects through TNF-α and IL-1β inhibition.[157] However this study revealed an attenuation of PSC activation through suppression of TGF-β-induced upregulation of αSMA, fibronectin and collagen. Furthermore rhein was observed to suppress the TGF-β-induced trans-activation of NF-κB and SHH signaling, suggesting the therapeutic effect causes an overriding reduction in PSC activation and ECM production through the suppression of the SHH/GLI1 pathways. Further studies on human tissue, as well as observing the results in the context of cancer cells, would be of great interest.

The systemic renin-angiotensin system (RAS) plays an important endocrine role in the regulation of blood pressure and homeostasis, however local RASs have been recognised in a number of organs including the pancreas, exerting an acinar and ductal effect resulting in an inhibitory impact on the microcirculation, as well as the generation of ROS, ultimately promoting inflammation.[158] Angiotensin II is has been seen to increase the expression of various growth factors such as FGF, TGF-β and VEGF, all of which are implicated in PSC activation. Furthermore angiotensin II has been demonstrated to stimulate proliferation,

migration and ECM production in PSCs.[159] Clinical studies have supported the anti-tumourigenic effect of RAS inhibition; a retrospective study 155 patients with pancreatic cancer revealed treatment with angiotensin inhibitors was a significant prognostic factor for both PFS ($P$=0.032) and OS ($P$=0.014) in multivariate analysis.[160] The question therefore arises as to whether PSCs are implicated in the local RAS. In a subcutaneous mouse model study, olmesartan, an angiotensin II type I receptor blocker, has been shown to inhibit tumour growth with associated attenuation of PSC activity as evidenced by reduced αSMA expression and collagen deposition. Furthermore olmesartan decreased cell growth and type I collagen production in PSCs *in vitro*.[161]

Target ECM components

Whilst direct targeting of pathways associated with PSCs seems to have the most potential in providing a prognostic benefit, a variety of approaches have been taken to target the other components of the stromal microenvironment that play an important role in ongoing PSC activation. Any therapeutics could then be used in combination to develop a multi-directional attack on various aspects of the DR. Metalloproteinase (MMP) inhibition shows the most promise due to its key role in ECM degradation; however given the multitude of different MMP types the difficulty is finding the protease which exerts the greatest effect. As such trials of MMP inhibitors tanomastat/marimistat have so far revealed no significant benefit [162, 163]; however it is hoped with more selective targeting of specific MMPs a positive effect will be elicited.

Given the key role of collagen in providing a pro-tumourigenic environment as well as a physiological barrier to chemotherapeutics, collagenases pose an interesting approach however no agents are currently clinically available. Alternatively hyaluronidase is a

potential approach as it has been shown to enhance tumour sensitivity to chemotherapeutics, even in tumours deemed chemoresistant.[164] In the context of PDAC it has been shown to both reduce tumour volume, collagen content and the number of activated PSCs, and thus presents a promising adjunctive therapy.[165] PEGPH20 is a PEGylated version of human recombinant PH20 hyaluronidase, and a phase 1b/2 multicentre, international RCT (Halo-109-201 - NCT01453153) of an estimated 147 patients with stage IV previously untreated PDAC revealed the addition of PEGPH20 to gemcitabine was especially beneficial in patients with HA-high tumours, with higher response and longer PFS.

Transgelin is an actin stress fibre-associated protein of the calponin family, and recent studies have shown levels are significantly increased in activated as compared to quiescent PSCs, with increased expression in the stroma of human chronic pancreatitis sections, whilst being absent in the acinar cells.[98] Subsequent silencing of transgelin expression reduced PSC proliferation, as well as reducing PDGF-induced migration, thus representing a potential therapeutic target to regulate PSC activity.

Target the metabolic symbiosis

As previously mentioned, if a metabolic relationship exists between PDAC and PSCs, then targeting this symbiosis could prove beneficial by shutting down the energy transfer that is fuelling anabolic tumour growth. In relevance to the aforementioned reverse Warburg effect, it has been demonstrated that anti-oxidants such as N-acetylcysteine prevent the activation of breast cancer associated fibroblasts through Cav-1 and MCT expression.[166] Anti-oxidative approaches have also been investigated with respect to PSCs, whereby it has been observed that high glucose levels induce oxidative stress in rat PSCs with resultant

activation, and subsequent treatment with antioxidants attenuated these changes.[167] Given the similarities within the breast and pancreatic tumour microenvironment, these anti-oxidant therapies may be an intriguing avenue of investigation. However detailed knowledge of the metabolic relationship between PDAC and PSCs is lacking, meaning it is not clear whether targeting oxidative or glycolytic processes is the most effective form of attack. Therefore in this project I aimed to initially investigate the relationship between PDAC and PSCs to determine the most appropriate means of disrupting any potential symbiosis, prior to embarking on therapeutic experimentation. To help guide this process I wished to examine the underlying science of cancer metabolism as well as current evidence of metabolic reprogramming within the PDAC microenvironment, which will now be discussed.

## 1.4 Cancer metabolism

With the development of advanced biological techniques in the 1970s, molecular genetics has come to dominate cancer research in the modern age, diverting attention away from the importance of tumour metabolism. As such Sidney Weinhouse famously stated in 1976 that "since our perspectives have broadened over the years, the burning issues of glycolysis and respiration in cancer now flicker only dimly".[168] It was traditionally considered that alterations in metabolic processes was an indirect consequence of cancer, and therefore not an attractive target for therapeutic intervention, paling in significance to the oncogenic activation of proliferative and survival signaling which was seen as the key initiator and driver of tumourigenesis. [100] Whilst there is no doubt mutations in oncogenes and tumour suppressors is essential in permitting cancer cells to evade growth suppression and cell death, increasing evidence has demonstrated how this altered genetic landscape also leads to metabolic reprogramming of both the tumour and its microenvironment. This discovery was heralded by the fact various oncogenes and tumour suppressors have been shown to regulate metabolic processes [169], whilst the efficacy of certain therapies have been directly linked to their effect on cancer metabolism. [170] Therefore metabolic reprogramming of malignant tumours is being encouraged to be viewed as one of the core hallmarks of cancer, and as such an increasing amount of scientific research is now geared towards the mantra of potentially shutting down tumour metabolism and thus starving tumours of energy, as part of a multi-pronged attack to target genetic heterogeneity and sensitize tumours to traditional chemotherapeutic agent

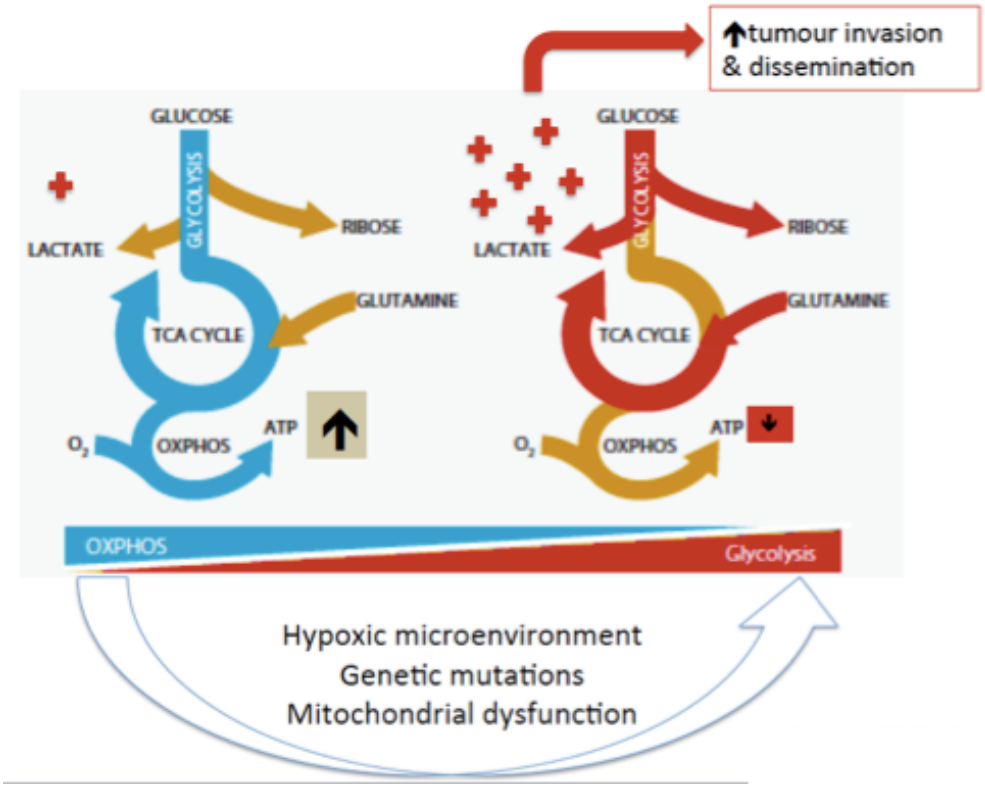
### 1.4.1 Metabolic reprogramming in PDAC

When considering normal cell metabolism, non-proliferating cells depend on oxidative phosphorylation to maintain integrity through efficient ATP production. Glucose is taken up by the cell and undergoes glycolysis to produce pyruvate which is then oxidized to CO2 by the tricarboxylic acid (TCA) cycle in mitochondria, whereby an electrochemical gradient is generated across the electron transport chain (ETC) thus facilitating ATP production. When cells are in a quiescent state, in the absence of growth factor signaling, they undergo autophagy, a form of nutrient recycling to allow energy production under starved conditions. This results in the catabolism of amino acids and lipids through OXPHOS in the TCA cycle provided there is sufficient oxygen levels, thus maximizing ATP production.[171] In contrast proliferating cells that receive instructional growth factor signaling are directed to increase uptake of nutrients such as glucose and glutamine.[172] The resultant increased biomass, in the form of lipid, protein and nucleotide synthesis, supports increased anabolic growth, with excess carbon secreted as lactate. Furthermore these proliferating cells adopt strategies to maintain the ADP:ATP ratio (through altering ATP production/consumption) and therefore maintaining glycolytic flux.

The aforementioned enigma that is the “Warburg effect” was first observed in the 1920s, describing how most cancer cells, regardless of oxygenation levels, utilise aerobic glycolysis and lactate fermentation for ATP production, rather than mitochondrial OXPHOS (figure 3.2). [131] The increase in glycolytic flux has been shown to be an extremely common cancer phenotype[100], with an increase in glycolytic gene expression observed in over 70% of malignancies.[173] The Warburg effect was always somewhat of a paradox, given glycolysis only produces two molecules of ATP per glucose molecule, as opposed to 38 by OXPHOS. Furthermore it was historically presumed to be due to mitochondrial

dysfunction, disabling the ability of cells to oxidise glucose carbon to CO2. However advances in cancer metabolism research technology has shown that in fact the cancer cell, and in particular the mitochondria, are susceptible to metabolic reprogramming via oncogenes and tumour suppressors, which support macromolecule synthesis (biomass) and resulting anabolic growth. This emphasis on increased biomass does not maximize ATP production, however emphasises how cancer cells do use mitochondria but in a different way, to maximize proliferation within a potentially hostile and hypoxic microenvironment. Given the close association of PDAC to its microenvironment, and altered pooling of metabolites and macromolecules, it is logical that stromal cells will also undergo a change in metabolic processes in line with the altered genetic landscape.

Figure 3.2 – The Warburg Effect, demonstrating how cancer cells switch from oxidative to glycolytic mechanisms of producing energy in the form of ATP, with a subsequent increase in protumourigenic lactate acidification



Whilst the exact metabolic mechanism underlying the Warburg effect is still not entirely clear, regardless of reduced energy production the switch to aerobic glycolysis is advantageous in a number of ways through the alteration of metabolic pathways which allows cells to survive and proliferate despite oxygen fluctuations within the tumour microenvironment. The beneficial nature of the Warburg effect is potentially most significant in PDAC compared to other maligancies for a number of reasons. Given the profoundly hypoxic PDAC tumour centre, and fluctuating levels within the tumour periphery due to the desmoplastic reaction within the stroma, this glycolytic switch allows PDAC cells to continue to thrive independent of inconstant oxygen diffusion that would usually have a negative impact on cells normally reliant on OXPHOS for ATP. [174]. Furthermore switching to glycolytic processes drives anabolic growth in cancer cells by exporting acetyl coenzyme A (acetylCoA) from the mitochondria and making it available for biomass synthesis such as fatty acids and cholesterol.[169] With respect to PDAC it has been shown that increased expression of fatty acid synthase, which catalyses the conversion of acetylCoA to long chain fatty acids, correlates with an advanced stage of disease [175]. Lipids have also been shown to have a profound effect on PDAC, with cell growth being stimulated via increased intracellular signaling activating growth factor receptors required for proliferation.[176]

This overall increase in anabolism results in large amounts of lactate, the metabolic end-product of aerobic glycolysis, being generated by PDAC cells, producing an acidic microenvironment that promotes invasiveness, metastasis and chemoresistance. [177] This acidification also results in evasion of the immune response, whereby the decline in the intracellular:extracellular lactate gradient means T lymphocytes, which rely on

glycolysis, suffer a dramatically reduced metabolic rate, significantly increasing immune suppression in PDAC and driving invasiveness and metastasis. [178]

Cancer cells typically demonstrate an addiction to the amino acid glutamine (Gln), which is both converted to α-ketoglutarate via glutamate to provide a carbon source for the TCA cycle, as well as providing a source of nitrogen for nucleotide and non-essential amino acid (NEAA) synthesis. [179] Indeed PDAC cells have been shown to be profoundly sensitive to Gln deprivation, indicating a key role in PDAC growth.[180] However Gln metabolism in PDAC differs from the above description, whereby it is redirected through a distinct pathway to fuel the TCA cycle; Gln-derived aspartate is converted from oxaloacetate (OAA) to malate and then pyruvate that increases the NADPH/NADP+ ratio, thus maintaining the cellular redox state and supporting growth.[181] KRAS is inextricably linked with this reprogramming process, through both transcriptional upregulation and suppression of metabolic enzymes essential to this pathway. Therefore the demonstrated dependence of PDAC on this pathway, which is dispensable in other cell types, suggests inducing glutamine deprivation by targeting specific component enzymes represents an ideal therapeutic approach.

Given the number of oncogenes and tumour suppressors linked to both PDAC and metabolic reprogramming, it seems commonsensical that developing means to attack this process will be efficacious. Table 4 details the role of KRAS, p53, Myc, HIF1a and the PI3K/Akt/mTORC1 pathway in metabolic reprogramming, all of which have a key role in PDAC tumourigenesis.

Table 4 – The most common mutations in PDAC and their associations with tumour metabolism

| **PDAC mutation** | **Metabolic effect in cancer** |
|---|---|
| KRAS | Increases glycolytic metabolism and glucose transporter Glut1 expression [182] |
| p53 loss | Enhances glycolysis and anabolic synthesis from glycolysis intermediates [183] |
| HIF1a | Inhibits mitochondrial metabolism of carbon by diverting pyruvate into lactate through PDH inhibition [184] |
| Myc | Promotes aerobic glycolysis and mitochondrial biogenesis [185], with Myc-driven malignancies associated with a dependence on reprogramming of glutamine metabolism [186] |
| PI3k/Akt/mTORC1 | Promotes anabolic metabolism through mitochondrial reprogramming; PI3k/Akt pathway activation increases glucose uptake and glycolysis (Glut1 and hexokinase induction)[187], whilst downstream activation of mTORC1 enhances protein synthesis associated with promotion of mitochondrial biogenesis [188] |

Overall this discussed physiological pro-glycolytic switch results in altered ionic and metabolic gradients within the microenvironment, with shifting of metabolites and lactate inducing prosurvival pathways and encouraging cancer cell proliferation. [100] A number of questions remain un-answered with respect to metabolic reprogramming in PDAC; how do PSCs impact on PDAC metabolism? What is the most appropriate means of targeting metabolism in PDAC; should this be directed towards specific glycolytic pathways within the tumour? Alternatively, given the altered pooling of glycolytic intermediates and

byproducts with the tumour stroma, should the method of attack be geared towards the effect on the microenvironment, for example reducing pro-tumourigenic acidity? Previously investigated approaches to targeting metabolism in PDAC will now be reviewed.

### 1.4.2 Targeting glycolysis

Therapeutic approaches towards glycolysis have been extensively investigated, with a variety of potential targets within the glycolytic pathways. The reduced ATP production in aerobic glycolysis is compensated via increased glucose uptake through a family of 14 glucose transporters (GLUTs), and unsurprisingly they have a strong association with cancer progression, with GLUT1 and GLUT3 considered the key transporters given they have the highest affinity for glucose. [189]PDAC tumours have been shown to have significantly increased glucose uptake [190], whilst GLUT1 expression strongly correlates with poor prognosis in PDAC, and more advanced stages of PanIN lesions. [191] The fact GLUT expression permits upregulation of glucose metabolism, inhibitors have been developed in efforts to starve cancer cells of their glycolytic fuel. Promising results have recently been demonstrated with the specific GLUT1 inhibitor WZB117, with in vitro treatment inhibiting the self-renewal and tumour initiating capacity of PDAC cancer stem cells (CSCs), which have a key role in tumourigenesis and have been shown to be highly glycolytic. In vivo treatment also suppressed tumour initiation with minimal side effects.[192] Concerns remain regarding the side effect profile of systemic GLUT inhibition in patients, however promising results from more sensitive and less toxic compounds suggest significant potential for this approach.

Targeting glycolytic enzymes is a promising form of attack given the high expression patterns and links to poor prognosis in PDAC. Hexokinase2 (HK2), which is

transcriptionally regulated by HIF1a, primes glucose for catabolism and is linked with increased expression in a range of cancers. [193] HK2 has recently been linked with reduced OS in PDAC, with increased expression in metastases confirming its association with aggressive tumour biology.[194] Subsequent knockdown reduced lactate production and invasion in vitro, and reduced tumour growth and lung metastasis incidence in cell line xenografts.

Phosphofructokinase-1 (PFK-1) catalyses fructose-6-phosphate phosphorylation in a rate limiting step of the glycolytic pathway, and becomes unregulated in the presence of Ras activation[195], therefore represents potential in PDAC. Inhibition of PFK-2, which in turn reduces levels of fructose-2,6-bisphosphate (an activator of PFK-1), has demonstrated antineoplastic effects, however issues remain with regards to solubility and high pre-clinical doses. Nonetheless nanoparticle delivery may negate this concern, and significant apoptotic effects in pre-clinical models offer some encouragement.[196]

Approaches towards the final steps of glycolysis in the form of pyruvate conversion have also shown promise. PKM2, one of the pyruvate kinase (PK) isoforms, is commonly expressed in tumours and associated with the Warburg effect and carcinogenesis. It even has potential as a biomarker, with a current trial investigating a link with raised plasma and saliva levels (NCT01130584). With respect to PDAC, increased PKM2 expression is linked with poor prognosis[197]. In vitro PKM2 knockdown reduces PDAC cell line proliferation, invasion and migration, with increased apoptosis.[198], whilst also enhancing sensitivity to gemcitabine.[199] The hope is these beneficial effects are translated into clinic, with the somatostatin structural analog TT-232 showing efficacy in

PKM2 inhibition with minimal adverse events, and therefore is currently in phase 2 trials for the treatment of renal cell carcinoma and melanoma (NCT0042278 and NCT00735332).

Inhibition of pyruvate dehydrogenase kinase (PDK), which shifts glucose from oxidative to glycolytic mechanisms and therefore eliminating excessive lactate acidification, has again shown promise. Dichloroacetate (DCA) is a cheap, oral drug that induces anticancer effects through PDK inhibition. Whilst evidence of efficacy in PDAC is lacking, it has shown promising results in lung, glioblastoma and breast cancer by switching metabolism to oxidative processes, thus increasing ROS production and sensitizing cells to apoptosis.[200] Clinical trial evidence is limited, however a phase 1 trial of glioblastoma patients confirmed mitochondrial depolarization, with moderate toxicity (NCT01111097) and results of other trials are awaited with interest.

Lastly lactate dehydrogenase (LDH), which catalyses the interconversion of pyruvate to lactate, also demonstrates strong associations with tumour initiation and progression however the exact mechanism is unclear. LDH is a tetramer consisting of 2 subunits, LDHA and LDHB, and can assemble into 5 different combinations (with LDH1 having four LDHB subunits, to LDH5 with four LDHA subunits). LDHB is ubiquitously expressed, however particularly found in heart muscle, whilst LDHA is predominantly found in skeletal muscle and highly glycolytic tissues.[201] Given the higher affinity of LDHA for pyruvate, it is more significantly associated with catabolism of pyruvate to lactate. This redox reaction both increases acidification and replenishes levels of NAD+, thus allowing glycolysis to become self sufficient, and given its transcriptional regulation by HIF1a it is strongly associated with highly hypoxic tumours such as PDAC.[202] Unsurprisingly LDHA expression correlates with poor prognosis. [197] In contrast there is limited evidence in the literature

regarding the prognostic relevance of LDHB expression in PDAC. With respect to therapeutic approaches, LDHA inhibition has been shown to inhibit in vivo progression of PDAC [203], however despite these promising results, it has proven a challenge to create a clinical applicable drug, hence the lack of clinical trials.

It is also possible to target the glutamine-addicted nature of PDAC as previously described. Solute carrier proteins (e.g. SLC1,5,6,7,35,38) are responsible for glutamine uptake, whilst glutaminase (GLS) drives conversion to glutamate and subsequent utilization in the TCA cycle, and expression of both is often upregulated in cancer.[204] Furthermore low expression of SLC's in breast cancer is shown to predict improved survival and response to chemotherapy.[205] Pharmacological modulation of glutamine metabolism has therefore been investigated, with a small molecule inhibitor of the glutamine transporter SLC1A5, gamma-1-glutamyl-p-nitroanilide, in pre-clinical studies, and the GLS inhibitor CB-839 presently in a phase 1 trial. Given the association with drug resistance there is also the potential to stratify patients towards personalized treatment plans based upon SLC expression profiles.[205]

### 1.4.3 Targeting tumour microenvironment metabolic factors

Rather than targeting specific mutations affecting the PDAC metabolic phenotype, or glycolysis itself, an alternative therapeutic approach would be to target the causes and consequences on the tumour microenvironment, thus identifying a more generic and globally applicable treatment for patients regardless of the genetic mutational landscape of the tumour which is invariably heterogeneous in PDAC. The pathology within the microenvironment is influenced by various physiological factors, such as oxygen transport,

vasculature, metabolites and pH, with significant regulation through oncogenes and tumour suppressors.

When considering these factors, there is no doubt that hypoxia is one of the most important characteristics that influence the metabolic phenotype towards an increase in glycolytic flux. It's relevance in PDAC is all the more significant given the profoundly hypoxic nature of the tumour stroma and associated desmoplastic reaction as previously discussed, with a strong correlation with poor prognosis and chemoresistance. We know that hypoxia develops as tumours grow away from local vasculature (beyond the oxygen diffusion limit) and stimulation of neoangiogenesis results in chaotic and immature vasculature with poor oxygen delivery.[206] Furthermore hypoxia leads to upregulation of HIF1a, with associated effects on metabolic reprogramming as discussed. As a result cancer cells maintain ATP production through glycolysis (the Pasteur effect) and this metabolic reprogramming is maintained even in the presence of adequate oxygenation (the Warburg effect).

Needless to say targeting hypoxia is not specifically disrupting cancer metabolism, as it will also impact on angiogenesis. Nonetheless some intriguing metabolic effects have been observed with respect to inhibiting hypoxia response pathways which allow cancer cells to overcome oxidative stress. For example topetecan, which inhibits HIF1a (as well as the primary mechanism of inducing genotoxic stress), results in decreased levels of GLUT1, as well as the expected reduction in angiogenesis through VEGF downregulation, resulting in reduced tumour xenograft growth.[207] PX-478, an orally available small molecule inhibitor of HIF1a, showed similar results with associated GLUT1 downregulation[208], with a phase 1 clinical trial of advanced solid tumours and lymphomas showing only mild toxicity (NCT00522652).

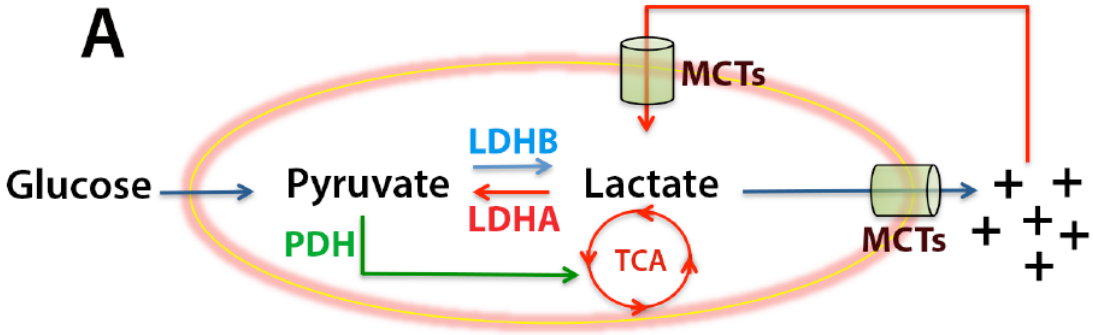
As mentioned the dependence on glycolysis leads to increasing amounts of H+ and lactate with resultant protumourigenic acidification of the microenvironment, and this acidosis also represents a viable therapeutic target. It is known that the microenvironment is more acidic (pH 6.5-6.9) as compared to normal tissue pH of 7.2-7.5.[209] This acidity is often primarily attributed to glycolytically generated lactate, often referred to as "lactic acid", which is transported by monocarboxylate transporters (MCTs). However it is important to note that acidification can occur without glycolysis, and instead originates from increased CO2, which upon hydration is converted to bicarbonate (HCO3), maintaining intracellular pH, and H+, which increases the extracellular pH and fuels the aggressive metabolic microenvironment. Carbonic anhydrase (CA) catalyses this reaction and helps maintain an appropriate acid base balance, however its role is more pronounced in the context of glycolytic tumours, whereby CAIX expression in PDAC has been shown to be an independent prognostic indicator and positively correlates with PDAC tumour size and staging, whilst silencing significantly inhibited invasion and metastasis.[210] Inhibitory approaches have included the use of sulfonamides, given CA's requirement of zinc for their activity, with indisulam being used in a number of clinical trials however issues have been encountered with regards to dose limiting toxicities and a lack of significant tumour response, and there is no evidence in PDAC. V-ATPase is another membrane bound transporter which moves protons into the extracellular space to prevent intra-cellular acidification, thus acidifying the surrounding stroma. [211]Whilst inhibition with bafilomycin has progressed since its discovery in the 1980s, evidence of clinical efficacy is limited again due to issues with toxicity.[212]

Lastly one may target lactate itself, by disrupting it's movement within the microenvironment, and ultimately neutralizing the pro-tumourigenic acidification effect. As such I wanted to look in more detail as to how lactate is produced and utilised in cancer, and more specifically PDAC, as well as how it is shuttled between different cell types via MCTs, whose role has not been clearly defined in PDAC.

### 1.4.4 Lactate metabolism

Lactate is a 3-carbon hydroxycarboxylic acid which exists in 2 isomeric forms, D-lactate and L-Lactate, the latter being the most commonly found form in the human body. It is produced by most tissues, with highest levels observed in muscles. In normoxic conditions, pyruvate produced by glycolysis enters the TCA cycle with resultant ATP production. However in anaerobic conditions pyruvate is instead converted to lactate by LDH (figure 3.3). As discussed, we know cancer cells undergo a metabolic switch (Warburg effect) whereby they preferentially convert pyruvate to lactate even in normoxic conditions, i.e. aerobic glycolysis, with resultant accumulation. This pathway, and associated increase in LDH activity, is of particular interest in PDAC, whereby patients with advanced disease and low serum LDH levels had significantly improved PFS and OS when treated with sorafenib.[213] There is limited basic science research into the intricate mechanisms of lactate metabolism in PDAC, possibly because metabolism in most cancers has historically been categorized under the umbrella of the Warburg effect. However it is increasingly clear that targeting lactate, either by encouraging tumour lactate uptake (thus decreasing accumulation) or reducing glycolysis can be an effective means of reducing tumour aggression.

Figure 3.3 – Lactate metabolism – in normoxia lactate is converted to pyruvate (facilitated by LDH) which is utilised in the TCA cycle to produce ATP, however the metabolic switch in hypoxia as part of the Warburg Effect leads to an increase in lactate production, which is then transported out the cells by MCTs, leading to acidification of the microenvironment. Alternatively lactate can also be taken up by cancer cells (again via MCTs) for energy production.




As well as increased lactate accumulation (through an increase in this glycolytic end-product), cancer cells also have the ability to take up lactate and use it for energy and amino acid production. This lactate utilization in cancer is dependent on oxygen availability (low in hypoxic PDAC tumours), lactate concentrations (often high in glycolytic PDAC), the presence of healthy mitochondria, and the availability of the appropriate lactate transporter (MCT).[214] A two compartmental theory of tumoural lactate has been proposed as a result of a glioma study, where separate metabolic pools of lactate/pyruvate were suggested, with one pool the result of cytosolic glycolysis, and the other pool from imported exogenous lactate that is then used for OXPHOS.[215]

When considering therapeutic approaches towards lactate metabolism, one can consider targeting preceding glycolytic pathways to prevent its formation, as previously discussed. Alternatively one may attempt to neutralize the acidifying impact of lactate accumulation. As already mentioned, attempts at reducing acidosis have had limited efficacy in drug discovery thus far, with either no significant response or issues with toxicity. On the contrary, inhibition of monocarboxylate transporters has been heralded as an exciting avenue of attack.

#### 1.4.4.1 Monocarboxylate transporters

Lactate homeostasis, in both normal and cancer cells, is regulated by monocarboxylate transporters (MCTs), which are part of the SLC 16 solute carrier family of transporter proteins consisting of 14 MCT members. Proton linked MCTs transport monocarboxylates other than lactate, including pyruvate and ketone bodies (e.g. acetoacetate, βhydroxybutyrate), and have an essential role in carbohydrate, fat and amino acid metabolism in normal cell.[216] Of the 14 family members, MCT1-4 are the most understood, with all being confirmed as proton-linked. MCT 8 (thyroid hormone transporter) and MCT10 (aromatic amino acid transporter) are both not proton linked, whilst the function of the other 8 MCTs is unknown. MCTs have a key metabolic role in regulating intracellular pH, particularly in highly glycolytic cells. In relation to the aforementioned sources of acidosis, other transporters such as V-ATPase, sodium proton exchangers and bicarbonate transporters also have a role in regulating pH, with inter-connected functions affecting each others activity, however MCTs are considered the most influential in cancer through their regulation of lactate movement.

Sodium coupled MCTs (SMCTs) are members of the SLC5A family and have only been recently discovered in the last decade. They transport similar substrates to MCTs, with a more prominent role in endothelial monocarboxylate transport in the gastrointestinal tract and kidney.[217] They demonstrate different expression patterns in human tissue, and have a less significant link with cancer. [218]

MCT1

MCT1 is known to bidirectionally transport lactate and short chain fatty acids, with influx or efflux depending on the intracellular:extracellular substrate concentrations and the pH gradient across the cell membrane.[216] In general MCT1 is more abundant in cells that tend towards lactate oxidation, as supported by the fact lactate-consuming cells such as SiHa exhibit high expression of MCT1.[219] Furthermore exercise studies suggest MCT1 is strongly associated with the TCA cycle, and therefore may have a more significant role in the influx of lactate. [220] Expression is reasonably ubiquitous as compared to the other MCTs, with increased levels seen in glioma, neuroblastoma, breast colorectal, gastric and cervical cancer. [201] Whilst there are no studies examining MCT1 expression in PDAC in the literature, the association between p53 loss and increased MCT1 expression suggests a potential link with PDAC.[221] There is however less association with hypoxia, with no correlation with HIF1a or the hypoxic marker EF5.[222]

MCT2

MCT2 has a higher monocarboxylate affinity than other MCTs, as demonstrated in table 5. MCT2 expression has been shown to be lower than MCT1 and 4 in human tissue, however closely linked with MCT1 when present, suggesting a role relating to environments which depend on a higher substrate affinity to maintain lactate homeostasis.[216] A recent study

confirmed MCT2 expression in PDAC, whilst revealing that the accessory protein EMBIGIN has a role in EMT in cancer progression, suggesting MCT2 inhibition may be another attractive treatment option.[223]

Table 5 – Comparisons of the affinity of MCTs for lactate and pyruvate (lower Km=higher affinity)

| Km (mM) | MCT1 | MCT2 | MCT4 |
|---|---|---|---|
| Lactate | 3.5 | 0.74 | 28 |
| Pyruvate | 1 | 0.1 | 150 |

MCT4

In contrast to MCT1, MCT4 is restricted to more glycolytic cells, and potentially have a more significant role in hypoxic tumour microenvironments, as supported by the fact HIF1a upregulates MCT4 mRNA and protein expression, whilst having no effect on MCT1. [222] Given the link with HIF1a MCT4 is strongly associated with hypoxic tumours, and is highly expressed renal cell carcinoma, cervical and prostate cancer.[201] A recent study revealed MCT4 defines a glycolytic subtype of PDAC that confers a poor prognosis. [224] As seen in table 5 MCT4 has less affinity for monocarboxylates as compared to MCT1/2, with a particularly high Km for pyruvate, revealing how MCT4 avoids pyruvate loss from cells therefore allowing removal of reduced NADH from glycolysis.[216]

CD147

MCT1 and 4 require the associated chaperone CD147 (also known as BASIGIN, EMMPRIN, OX-47) for expression and function of MCT1 and 4. Therefore their expression is also relevant in cancer, with normal tissue not demonstrating significant expression levels,

whilst increased levels have been seen in breast, oesophageal and endometrial cancer, and high expression associated with increased recurrence and reduced OS in breast cancer.[225] It has also been linked with chemoresistance in HCC.[226] CD147 also has a role in the tumour microenvironment, whereby it has been shown to induce MMPs (which breakdown ECM proteins) in adjacent CAFs, whilst stimulating VEGF via the PI3k.Akt pathway.[227] As such inhibition of CD147 has been investigated as a treatment approach, with knockdown resulting in reduced invasiveness, reduced MMP production, lower levels of chemoresistance, and increased apoptosis. [228] Human anti-CD147 antibodies have also been used to induce antibody-dependent cell-mediated cytotoxicity, whilst the aforementioned pCMBS exerts its inhibitory effect on MCT1 and 4 through disruption of their association with CD147.[229] As with MCTs, the issue with CD147 inhibition is the off target effects (such as towards MMPs and other immunoglobulin family members), as well as the fact it is fairly ubiquitously expressed, emphasizing the need to target CD147-MCT interactions in a more specific and selective manner depending on the tumours particular metabolic profile.

## 1.5 Introductory conclusions

Research into cancer metabolism remains a challenging field of investigation, primarily due to the fact it is difficult to distinguish exactly which compartment the metabolites originate from, whether it be the cytosol or mitochondria. The increasingly evident and key role of components within the tumour stroma also provides additional sources of metabolite pooling. Whilst measuring levels of individual metabolites is relatively simple, this does not account for metabolic flux, i.e. the fluctuations in movement of metabolites between compartments. With respect to tissue culture experimentation, these challenges are further confounded by the fact media is often incompletely defined. Lastly, and using lactate metabolism as an example, the non-cell autonomous effects of tumour metabolism is difficult to account for, such as the symbiosis between hypoxic and normoxic tumour cells, or with neighbouring stromal cells such as CAFs, endothelial and immune cells.

Nonetheless tumour metabolism has been demonstrated as an increasingly important hallmark of cancer in recent years, and an ideal target for therapeutic intervention. The potential impact of this in PDAC is profound, given the genomic and stromal complexities of the disease whereby current chemotherapeutics prove relatively ineffective compared to other tumour types. Metabolic reprogramming has been reported in a wide variety of tumours, however it is noticeable that there is a distinct lack of studies and therefore knowledge regarding tumour metabolism in PDAC as a whole. Furthermore given the influential PSC-rich stroma, very little work has investigated any metabolic role PSCs may have in PDAC. Given the limitations mentioned above, this project therefore aims to breakdown the relationship between PDAC cells and PSCs in a tissue culture setting, in an attempt to delineate metabolic flux between these cell types and define potentially

efficacious means of targeting glycolysis or lactate metabolism. This will be further supported by immunohistochemistry on resected human tissue to elucidate prognostic relevance and enhance the translational impact of the *in vitro* results.

In conclusion, the hypothesis at the start of my studies was that PSCs exert a metabolic effect on PDAC, and by therapeutically targeting this metabolic synergy one may be able to exert a prognostically beneficial effect, either through the impact on the cancer cells or the tumour microenvironment.

# 1.6 Aims

The specific aims are therefore as follows:

| Question | Methodology |
|---|---|
| What is the optimal method for isolating PSCs from both animals and humans? | • Source from both freshly resected tissue and acinar tissue from islet cell isolations<br>• Optimise isolation techniques including using density gradient method and outgrowth |
| What is the normal metabolic relationship between PSCs and PDAC? | • Effect of in vitro transwell coculture of PSCs and PDAC cell lines on MCT expression and lactate levels<br>• Seahorse analysis to examine alterations in metabolic phenotype following coculture |
| What is the expression profile of metabolic markers in PDAC, and how does this relate to patient outcomes? | • Examine expression of IHC stains in both tumour and stroma, with comparative ratios between the two compartments<br>• Correlate expression with patient survival using multivariate analysis to account for prognostic indicators |
| What is the most appropriate means of targeting a PDAC-PSC metabolic relationship? | • Repeat experiments utilising a variety of inhibitory approaches including:<br>o Anti-oxidant therapy<br>o Specific MCT1 inhibition with AZD3965<br>o Pan-LDH inhibition with Galloflavin |

| | |
|---|---|
| | • Measure the following outcome measures:<br>o Gene and protein expression<br>o Metabolic activity (MTT), apoptosis (Caspase-3) and proliferation (Brdu)<br>o Lactate concentration (intra-cellular and extra-cellular)<br>o Alteration in metabolic phenotype (seahorse analysis) |
| Does combination treatment enhance efficacy? | • Investigate the combination effect of the following drugs/reagents with MCT1 inhibition<br>o Gemcitabine (chemotherapeutic agent)<br>o Galloflavin (LDH inhibitor)<br>o Metformin (Inhibitory action on respiratory complex 1)<br>o N-Acetylcysteine (Anti-oxidant – ROS scavenger) |

# Chapter 2

# Materials and methods

## 2.1 Ethics and tissue acquisition

*Ethics*

This project involved the use of human pancreatic tissue and ethical considerations were addressed. Ethics approval was granted for retrospective acquisition of pre-2006 sections of resected PDAC tumours through the Newcastle Biomedicine Biobank (REC ref 12/NE/0395) for subsequent IHC staining and analysis. Approval was also granted for the acquisition of fresh pancreatic tissue through the Newcastle Hepatopancreaticobiliary (HPB) Research Tissue Bank (REC ref 10/H0906/41). This allowed isolation of PSCs from tissue not required for diagnostic purposes (i.e. no interference with procedure itself or resection margins). Procedures were performed at the HPB unit, Freeman Hospital, with written informed consent from all patients.

*Tissue acquisition*

The above ethics approvals allowed acquisition of tissue for PSC isolation and IHC analysis. The protocol for tissue collection in each instance was as follows:

1. PSCs for isolation

- A 1-2cm$^2$ piece of fresh non-tumoural pancreatic tissue was obtained from a pancreatic resection at the Freeman Hospital for PSC isolation (see 3.3.2) – this did not effect the procedure itself, nor impact on resection margin analysis

- Tissue was taken from the specimen once it had been resected and removed from the patient, then placed in HBSS+ solution and immediately transported to the laboratory for isolation within 1 hour of resection to ensure maintenance of cell viability
- Tissue was attained through one of 3 procedures - Distal pancreatectomy, Whipple's procedure or benign disease resections e.g. Freys procedure
- Anonymised records were maintained to ensure tracking of subsequent outgrown cells

2. Sections for IHC analysis

- Core biopsies were taken from patients undergoing resection for PDAC (confirmed on EUS biopsy or high suspicion on radiological imaging)
- Procedures include either Whipple's or distal pancreatectomy
- Specimens were fixed in formalin and transported to the laboratory for IHC
- Tissue was stored and catalogued in an online anonymised database

Blocks of PDAC tissue were also obtained from pre-2006 Whipple's procedures. These were validated as being histologically confirmed PDAC tumours through review of pathology reports. The sections were then cut and IHC performed (see 3.8)

3. Rat PSC acquisition

Animals were housed in pathogen-free conditions, and kept under standard conditions with a 12-hour day/night cycle and access to food and water ad libitum. Animal experiments were approved by the Newcastle Ethical Review Committee and all work performed under a UK Home Office license in accordance with the ARRIVE guidelines.

## 2.1 PDAC database

Patient details were provided on the aforementioned pre-2006 PDAC sections, and a database generated of all relevant demographical data and overall survival. Furthermore data on prognostic indicators was collected as demonstrated in table 6 with associated relevance to PDAC. It is worth noting that the 7th edition of the AJCC/UICC TNM staging system was used as this were the classification guideline in place at the time of histopathological reporting of the resection specimens. [6]

Table 6 – List of prognostic indicators collected for the patient database, with their associated relevance to PDAC

| **Prognostic indicator** | **Relevance in PDAC** |
|---|---|
| ASA score | A higher ASA score indicates a worse performance status, which can increase the risk of morbidity and mortality post-procedure |
| T status | T3 tumours potentially are more aggressive than T2 [6] |
| N status | A positive lymph node (N1) indicates a degree of dissemination and therefore poor prognosis [6] |
| Lymph node percentage positivity | Evidence exists that a higher percentage of positive lymph nodes correlates with reduced OS [230] |
| Pancreatic leak | Associated morbidity theoretically worsens prognosis [50] |

# 2.3 PSC isolation

### 2.3.1 Rat PSC isolation

Prior to ethics approval being obtained, preliminary experiments were performed on rat PSCs, the isolation of which will now be described. A Sprague-Dawley rat (250-300g) that was sacrificed for other experiments was used. Immediately after death the pancreas was resected, any attached adipose tissue removed, and the specimen placed in HBSS+. The tissue was minced and placed into enzyme solution containing 10mls collagenase P (0.5mg/ml), 10mls pronase (1mg/ml) and 5mls Deoxyribonuclease 0.1% (DNase). This was then transferred into a shaking incubator at 37 Celsius for 30 minutes. The digested tissue was filtered through a nylon mesh and DNase added. This cell suspension was then centrifuged at 1500rpm for 5minutes to obtain a pellet which was then resuspended with Optiprep (Sigma, Poole, UK) 12%v/v in HBSS. These were then placed into falcons with a buffering layer of HBSS (2ml) on top. This was centrifuged for 20 minutes at 2000rpm at 4°C. The stellate cells were collected from the band between the optiprep/HBSS interface. The cells were pelleted by centrifugation (at 500g, for 5minutes, at 4°C) and resuspended with HBSS+ in order to remove any residue of optiprep. The cell suspension was again centrifuged and the pellet re-suspended in Dulbeccos Modified Essential Medium (DMEM) with 16% fetal calf serum (FCS), supplemented with penicillin, streptomycin and gentamicin (PSG). The cells were seeded into a 75$cm^2$ culture flask and placed in a humidifying incubator at 37 degrees with 5% CO2. The following day the culture media was changed. Rat PSCs were then observed to grow out and subsequently passaged once a confluent monolayer developed.

### 2.3.2 Human PSC isolation

Human tissue was collected as already described. The isolation technique was initially identical to the rat technique. Results and subsequent attempted optimization of this technique, including the density gradient and outgrowth methods, will be further discussed in the relevant results chapter (4.1.2).

# 2.4 Tissue culture

### 2.4.1 PDAC & PSC cell lines

The Miapaca2, Panc1 and Bxpc3 PDAC cell lines were obtained from ATCC. The former two cell lines were chosen as they represent both ends of the spectrum with respect to phenotypic changes seen in PDAC. The latter was chosen as a KRAS wild-type, thus helping to determine the mechanistic effect of KRAS in any observed changes in experiment.

An immortalised and validated PSC cell line PS1 was obtained through collaboration with Prof Hermant Kocher (Barts, London).

### 2.4.2 Culture methods

Cells were passaged according to the following protocol. An initial wash in HBSS was performed to remove any residual serum, and the cells then exposed to TEDTA (5mls – 1x) in HBSS at 37 ºC for 5 minutes. Once the cells were detached from the flask, TEDTA was inactivated with 5ml of media. The detached cells were collected into falcons and centrifuged (1,400rpm, 4 minutes). The pellet was then resuspended and split back into the T75 flasks.

Cells were also cryopreserved for long term storage and use in future experiments. This involved making up freezing media consisting of 4ml FBS, 5ml media (10/16% FBS) and 1ml DMSO. 1ml of this solution was then used to resuspend a pellet of cells from a T75 flask, pipetted into a cryotube. This was initially placed in a -20 Celsius freezer for a few hours, and then transferred to -80. After a few days the tubes were then transferred and cataloged in a liquid nitrogen store.

### 2.4.3 Transwell co-culture

Transwell microporous inserts were obtained from Thermofisher Scientific, which allowed different cell types to be cultured in either a 6 or 24 well plate set up. The advantage of this system is it allowed cells to directly interact within the media, whilst also allowing one to source each cell type separately at the end of the treatment time course, and therefore determine how co-culture or certain treatments specifically affected each cell. Comparisons could then be made with solo controls to determine whether certain treatments were more or less efficacious on PDAC cells when in the presence of PSCs, and vice versa.

Cells would be seeded into a 6 or 24 well plate at a density of 150,000 or 30,000 cells per well respectively using an haemocytometer, with 2mls or 200ul of DMEM/10% FCS/PSG. The opposing cell type would then be seeded into the transwell insert at the same seeding density and media volumes. After 4 hours, once cells were adherent, the media was aspirated to remove any dead cells, and the same volume of fresh media applied. Cells were cultured, with solo controls for comparison, for the allotted time course prior to analysis. In general this was between 24-48 hours, as this allowed sufficient density of cells at commencement of the treatment, whilst the rapidly proliferating cell lines generally reached near confluence at 48 hours.

## 2.5 Tissue culture assays

### 2.5.1 MTT

Following completion of the treatment time course, media was removed and replaced with a fresh media solution containing a 1:10 dilution of thiazoyl blue (5mg/ml). Culture plates were then incubated for 3 hours at 37 $^{O}$C with 5% CO2. The media was then removed, 1-isopropanol added and placed on a shaker for 5 minutes. The cell solution was then transferred to a 96 well plate and absorbency measured on an ELISA plate reader (true absorbency = 570nm - 620nm readings).

### 2.5.2 BrdU

This is a calorimetric immunoassay for quantification of cell proliferation based on the measurement of BrdU incorporation during DNA synthesis (Roche, 11647229001). Following the treatment time course in a 24 well plate set up, media was removed and the BrdU labelling agent was applied to cells (200ul media/well, 1:100 reagent, final concentraion 10uM BrdU) over-night. The following day cells were fixed with FixDenat for 30mins at room temperature. The FixDenat solution was then removed and anti-BrdU-POD (monoclonal antibody from mouse-mouse hybrid cells conjugated with peroxidase) added at 200ul/well for 2 hours. This solution was then removed and wells were washed three times with PBS. Substrate solution (tetramethyl-benzidine) was then added to initiate the reaction, and media transferred to a 96 well plate after 5 minutes for analysis on an ELISA plate reader at 370nm (reference wavelength 492nm).

### 2.5.3 Caspase-3

The caspase 3 assay kit (colorimetric) (ab39401) was used to assay the activity of caspases (highly conserved cysteine proteases that play an essential role in apoptosis) that recognize the sequence DEVD. Following the treatment time course, protein was isolated by scraping cells into PBS, centrifuging at 1400rpm for 4 minutes, and resuspending cells in 50uL of chilled cell lysis buffer for 10mins. Cells were then centrifuged at 10,000G for 1 minute, and the supernatant transferred into a fresh tube for protein quantification; all samples were normalised to 100ug of protein.

The assay procedure was then carried out as follows in a 96 well plate. 50ul of x2 reaction buffer (containing 10mM DTT) and 5uL of 4mM DEVD-pNA was added to each well. Samples were then incubated for 2 hours at 37 degrees (5% CO2). Output was then measured on an ELISA plate reader at OD 400nm.

### 2.5.4 Lactate

Extra-cellular

1ml of media was collected following 48hours treatment and lactate levels (mmol/L) analysed using a calorimetric assay on Roche Cobas 702 analysers. When in the presence of transwell co-culture the media was pooled and 1ml of media collected.

Intra-cellular

The Trinity Biotech Lactate Reagent (735-11) and standard solution 40mg/dL (826-10) was used for the quantitative, enzymatic determination of lactate in cell culture media following completion of the treatment time course. 200ul of Lactate Reagent Solution was added to 20ul of cultured media. Lactate standard solution was used as a control. Samples

were incubated for 10 minutes at room temperature, and output measured on an ELISA plate at 540nm. Lactate concentration (mg.dl) was then calculated ((test absorbance/standard absorbance)* 40) and converted to mmol/l (*0.111)

# 2.6 Western blot

### 2.6.1 Protein extraction

Pellets from cultured cells were resuspended in Radio Immuno-Precipitation Assay (RIPA) lysis buffer, containing protease inhibitors (Roche Diagostics Ltd) and phosphatase inhibitors (Sigma-Aldrich). These were placed on ice for 20 minutes, with intermittent vortexing every 5 minutes. Samples were then sonicated for 8 minutes, and then centrifuged at 4 $^{0}$C for 15mins at 13000xg. The supernatant was aspirated and protein levels quantified using a modified Bradford assay according to the manufacturers instructions (DC Protein Assay, Bio-Rad Laboratories).

### 2.6.2 Western Blot protocol

30ug of each protein sample was made up to 25ul with RIPA buffer, then 5ul of loading buffer added (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris HCl). Samples were denatured by incubation at 95 $^{0}$C for 5 minutes and then separated by electrophoresis on 10% sodium dodecylsulfate–polyacrylamide gels. Gels were run for approximately 2 hours at 100 volts then transferred to nitrocellulose membranes. Membranes were first blocked to minimise non-specific binding using 5% Milk in Tris-buffered saline (TBS)/Tween 20 (TBS-T) solution. After 1 hour blocking, membranes were incubated with the primary antibody overnight at 4$^{0}$C. Table 7 details the primary antibodies used, including optimal concentrations, predicted bandwidth, source and suppliers.

The following day the membranes were washed three times with TBS-T solution before 1 hour incubation at room temperature with the appropriate HRP conjugated secondary

antibody at a 1:5000 dilution. Membranes were again washed with TBS-T before visualisation by a chemiluminescence reaction (Thermo-Scientific) followed by exposure to film for band production. GAPDH was used as the loading control antibody.

Table 7 – List of antibodies utilised in IHC analysis

| **Antibody** | **Concentration** | **Bandwidth** | **Source** | **Supplier (ref)** |
|---|---|---|---|---|
| αSMA | 1:1000 | 42 | M | Sigma (A5228) |
| Desmin | 1:1000 | 53 | R | Abcam (ab8470) |
| MCT1 | 1:1000 | 54 | M | Abcam (ab90582) |
| MCT4 | 1:500 | 43 | R | SantaCruz (50329) |
| CD147 | 1:1000 | 55 | R | Abcam (ab232967) |
| LDHA | 1:1000 | 36 | R | Abcam (ab47010) |
| LDHB | 1:5000 | 37 | M | Abcam (ab85319) |
| PDHX | 1:500 | 54 | R | Abcam (ab71325) |
| γH2AX | 1:1000 | 17 | R | Cell signaling (9718) |
| GAPDH | 1:1000 | 37 | R | Abcam (ab9485) |
| βActin | 1:1000 | 42 | M | Abcam (ab8226) |

# 2.7 Genomic analysis

### 2.7.1 RNA isolation and cDNA Synthesis

The RNeasy kit (Qiagen) was used following the manufacturer's protocol to isolate whole RNA, with amounts quantified on nanodrop spectrophotometer for subsequent cDNA synthesis. RNA integrity was ensured by using an OD 260/280 cut off of no less than 1.8. Samples were stored at -80°C.

cDNA templates were prepared using Moloney murine leukemia virus reverse transcriptase (MMLV-RT). All reagents were from Promega (Southampton, UK). 1µg of RNA was diluted to 8ul in nuclease free water and incubated at 37 $^{O}$C for 30minutes with 1µl DNAse and 1µl DNase buffer. DNAse activity was stopped using 0.5M EGTA solution and incubated at room temperature for 2 minutes before adding 0.5 µl of random primers with 2ul of nuclease water. After a 5 minute incubation period at 70 $^{O}$C, samples were placed onto ice and a PCR mixture of 0.5 µl RNA synthase inhibitors, 1µl MMLV-RT, 1 µl dnTP oligonucleotides and 4 µl MMLV-RT buffer was added. Samples were then incubated for 60mins at 42 $^{O}$C, then diluted with RNase free water to 10ng/µln.

### 2.7.2 Real Time PCR

Real Time Polymerase Chain Reaction (RT-PCR) was performed to establish gene expression. The PCR reaction mix consisted of 1ul cDNA, 12.5 ul of PCR Master Mix (Promega), 1ul of each forward and reverse primer (table 8), and 9.5 ul of nuclease free water. The PCR reaction was performed on a thermal cycler using the following protocol; an initial 3 minutes at 94 $^{O}$C, followed by 35 cycles of denaturation for 20 seconds at 94 $^{O}$C, annealing at 50 $^{O}$C

for 30 seconds and elongation at 72 $^{O}$C for 30 seconds, followed by a final 7 minutes at 72$^{O}$C. Once amplification was completed, 3 µl of 10x loading buffer was added to samples before being loaded and subjected to electrophoresis on 1% agarose gels. Gels were run for one hour at 0.15A and results then visualised under UV light.

### 2.7.3 Quantitative RT-PCR

qRT-PCR was performed using an ABPI 7500 Fast system (Applied Biosciences). The PCR mix included 6.5 µl of SYBR Green Master Mix (Sigma), 10ng of cDNA (equivalent to 1ul of stock as produced above) and 0.1 µM of primers (table 8) made up to a 13 µl solution with nuclease free water. The program consisted of 40 cycles of denaturation for 10 seconds at 95 $^{O}$C, annealing at 50 $^{O}$C for 30 seconds and elongation at 72 $^{O}$C for 30 seconds, followed by a final dissociation curve cycle of 95 $^{O}$C for 15 seconds, 60 $^{O}$C for 60 seconds and 95 $^{O}$C for 30 seconds. Melt curve analysis was performed to validate results. Data was normalised to a control housekeeping gene (GAPDH), with fold change in mRNA expression calculated using the Δ Δ CT method.

Table 8 – List of primer sequences used for each gene examined in RT-PCR

| Product | Forward Primer Sequence | Reverse Primer Sequence |
|---|---|---|
| MCT1 | F 5'-TCCAGCTCTGACCATGATTG-3' | R 5'-AGTAGCAAGCCCCCAAGAAT-3' |
| MCT4 | F 5'-GGGGATGGATGATTGTAT-3' | R 5'-GCCCCAAGAATGGAGGTAG-3' |
| IL6 | F 5'–ACAGCCACTCACCTCTTCAG–3' | R 5'-CCATCTTTTTCAGCCATCTTT-3' |
| IL8 | F 5'-AGAGTGATTGAGAGTGGACC-3' | R 5'-ACTTCTCCACAACCCTCTG-3' |
| αSMA | F 5'-CTGTTCCAGCCATCCTTCAT-3' | R 5'-TCATGATGCTGCTGTTGTAGGTGGT-3' |
| GAPDH | F 5'-GAAGGTGAAGGTCGGCGTC-3' | R 5'-GAAGATGGTGATGGGATTTC-3' |

## 2.8 Immunohistochemistry

All IHC analysis was performed on 5um thick formalin-fixed paraffin-embedded tissue sections.

### 2.8.1 Staining protocol

IHC was performed on PDAC core biopsies retrieved from resected specimens. Sections were initially de-waxed in Clearene I and II (Leica Microsystems Ltd) for 5 minutes each, followed by immersion in 100% and 70% alcohol for 5 minutes each. To block endogenous peroxidases, slides were then placed in methanol/2% hydrogen peroxide (7ml H2O2 in 350ml methanol) for 15 minutes. Following a wash in PBS, antigen retrieval was then performed by immersing slides in Antigen unmasking solution and heating in the microwave for 5 minutes before being allowed to cool. Slides were then mounted into a sequenza and non-specific binding inhibited by exposing to Avidin (3 drops per slide, 20 minutes) then Biotin (3 drops per slide, 20 minutes) (Vector Laboratories). Slides were then blocked with 20% piggy serum for 30 minutes, before the primary antibody was added and left at 4 °C overnight. Primary antibodies used, including optimal concentrations, source and supplier are provided in table 9.

Table 9 – List of primary antibodies utilised in Western Blot protein analysis

| **Antibody** | **Concentration** | **Source** | **Supplier (ref)** |
|---|---|---|---|
| αSMA | 1:500 | R | Sigma (F3777) |
| MCT1 | 1:100 | R | Abcam (ab90582) |
| MCT4 | 1:50 | M | SantaCruz (sc-50329) |
| LDHB | 1:1000 | M | Abcam (ab85319) |

The following morning slides were washed in PBS prior to incubation with the appropriate secondary antibody for the respective primary. After a further PBS wash slides were exposed to the streptavidin biotin-peroxidase complex (Vector Laboratories) for 60 minutes, before the addition of 3,3'-daminobenzidine tetrahydrochloride (DAB) (Vector Laboratories). Counterstaining with Meyers Haematoxylin was then performed, prior to dehydrating with increasing concentrations of alcohol (50,70,100%) then Clearene I and II. Slides were then mounted onto Pertex (CellPath Ltd) and allowed to dry prior to visualization.

### 2.8.2 Aperio analysis

Following stain completion, all sections were analysed using the Aperio ScanScope (Aperio Technologies) system. This process will be described in further detail in the relevant chapter (5.2).

## 2.9 Seahorse technology

The Agilent Seahorse XF Analyzer provides the ability to obtain functional data from live cells in real time, using transient microchambers which enable sensitive and accurate measurements of cellular metabolism, simultaneously measuring outputs related to the two major energy pathways of a cell (oxidative phosphorylation and glycolysis). The principles and applications of Seahorse technology will be further discussed in the results chapter, however in brief the protocol involves preparing cells in specialized media with specific metabolite content, and then treating these cells within the machine with a choice of reagents which inhibit different parts of either the mitochondrial electron transport chain (ETC) or glycolytic pathways. Serial measurements of oxygen and pH levels are performed and the oxygen consumption rate (OCR – correlates to OXPHOS) and extracellular acidification rate (ECAR – correlates to glycolysis) of cells is calculated.

Following completion of the treatment time course, cells were seeded into the specialised 24 well seahorse plate at a density of 15,000 cells per well, using conditioned media from each condition, with 5 repeats per condition. The smart cartridge insert (for reagent injections) is hydrated with calibration buffer (1ml/well) over-night at 37 degrees (no CO2). On the following day specialised seahorse media (33.565mls) is freshly made with the addition of 31.5mg glucose (5mM), 350ul 200mM L-glutamine (2mM), 700ul FBS (2%) and 35ul 1M pyruvate (1mM). Wells are washed once with seahorse media, then 500ul fresh media added and the plate incubated for 1hr at 37 degrees (no CO2)

The smart cartridge is then loaded with the appropriate reagents. The reasoning behind reagent choice will be discussed in the results chapter. The injections utilised included

Oligomycin (1ug/ml), FCCP (1uM), Antimycin (2.5uM) combined with Rotenone (0.5uM) and 2DG (50mM). This cartridge is then loaded into XF24 analyser for calibration. Once completed the 24 well plate containing the cells is loaded into the machine and the run is commenced. Outputs are exported into an excel spreadsheet for tabulation and analysis.

## 2.10 Statistical methodology

The SPSS Statistics software package was used for all statistical analyses. In comparative analyses, e.g. from in vitro experimentation/comparing conditions or treatment effects, the appropriate statistical test was used depending on whether the data was normally distributed. For example, when comparing 2 conditions the students t-test or Mann-Whitney U test was performed if parametric or non-parametric respectively, whilst comparing more than 2 independent replicates the unpaired t-test was used. Survival analysis was performed using Kaplan Meier curves and subsequent log-rank tests to determine significance. In more extensive data-sets involving three or more independent groups, ANOVA was performed to determine any statistically significant difference between them. Spearman's rank order correlation was performed when analyzing the association between ordinal variables or continuous data that failed the assumptions for Pearson's correlation. A p value of $<0.05$ was considered statistically significant.

Data within this thesis is presented in the appropriate graphical format, in the majority as bar charts. All assays were performed in triplicate, and all results are expressed as means with associated standard error bars.

## 2.11 Declaration of experimental contribution

I can declare that the majority of these aforementioned experiments were performed by myself alone, following a period of training on the various tissue culture techniques/assays by staff at the Fibrosis Lab/Centre for Ageing and Vitality at Newcastle University. The only experiments where I required more assistance or did not perform certain aspects included:

- Harvesting of pancreata from rats for PSC isolation
    - Given I myself was not in possession of an animal licence to perform this procedure, this instead was performed by Fiona Oakley and colleagues at the Fibrosis Lab, Newcastle.
- IHC staining of the PDAC specimens
    - As described in the above methodology, I worked up the staining protocol initially, including the appropriate antibody concentrations, however thereafter in the interest of speed and ease I utilised the Ventana auto-stainer at the Newcastle Upon Tyne Hospitals Department of Histopathology, under the responsibility of Anna Long (deputy operations manager, Cellular Pathology).

# Chapter 3

# PSC isolation and characterization

## 3.1 Background

Research into pancreatic fibrosis was historically limited by the lack of *in vitro* and *in vivo* models. In contrast knowledge of the pathogenesis behind liver fibrosis was better elucidated following the discovery of the key role hepatic stellate cells (HSCs). Stellate cells are classed as vitamin A storing cells, and in the liver it was shown that under the influence of proinflammatory cytokines and oxidative stress they transform into an activated myofibroblast phenotype and become major effector cells in the fibrotic process.[231] Following the identification of vitamin A storing cells in the pancreas by Watari et al in 1982 [232], it was postulated that PSCs may have a similarly key role in fibrogenesis, and given the link between inflammation and cancer this also represented a potential avenue for improved chemotherapeutics.

Therefore efforts were then underway in the 1990s to optimize PSC isolation techniques, with the first documented successful attempt being made by Minota Apte and colleagues in 1998.[233] This was performed on a rat pancreas using a density gradient centrifugation method. In a similar fashion to HSCs, it was found that once cultured on plastic, these PSCs converted from a quiescent to activated form, exhibiting the hallmarks of positive αSMA staining after 48 hours. This group then went on to be leaders in the field of PSC research, establishing their key role in both fibrosis and cancer as previously described.

Soon after Apte et al's successful isolation, Bachem et al described the first isolation of PSCs from resected human tissue.[94] This was performed using the outgrowth method, with protocol details explained in the proceeding methodology. However in brief this involved placing small pieces of resected pancreatic tissue into culture dishes, with the observation that PSCs subsequently grew out into monolayers. As previously seen when cultured on plastic, these PSCs were observed to convert from their quiescent fat-storing phenotype into an activated myofibroblast form, expressing both αSMA (>90%) and desmin (20-40%), as well as strongly positive staining for collagen I & II, fibronectin and laminin. An alternative form of outgrowth method was also developed from pancreatic acinar tissue in rats by Kato et al.[234] They observed that periacinar fibroblastoid cells (PFCs) proliferate as a myofibroblast phenotype in culture, synthesizing ECM components and expressing the classical PSC expressional markers.

Vonlaufen and colleagues then more recently successfully isolated PSCs from resected human tissue using the density gradient method, with culture activation again demonstrating expression of αSMA, desmin, glial fibrilliary activated protein (GFAP) and the lipopolysachharide (LPS) receptors TLR4 and CD14.[235]

From the combined efforts of the research community, Apte and colleagues then published a definitive protocol for the isolation of rat and human PSCs using the density gradient method on the web resource Pancreapedia. [236] This protocol, in combination with our institutional experience of HSC isolation, was used in efforts to isolate human PSCs for this project. The fibrosis laboratory, led by Prof Derek Mann, Dr Jelena Mann and Prof Fiona Oakley, is an internationally renowned research group into the study of hepatic stellate cells, resulting in new therapeutic strategies being developed which have been used to

target ineffective and aberrant wound healing that is also potentially carcinogenic.[237-239] With extensive experience in HSC isolation, characterization and subsequent experimentation, the lab therefore represented the ideal setting to study PSCs.

## 3.2 Methodology

PSC isolation

Details of the tissue collection and PSC isolation protocols from both rat and human sources are detailed in chapter 3. The latter was a modified HSC protocol, as previously used successfully by one of the fibrosis lab P.I.'s (Prof Fiona Oakley) for PSC isolation from rats.

In addition to the density gradient method, the outgrowth method was also utilised for the isolation of human PSCs (hPSCs). In this technique, following tissue collection from resected specimens as previously described, small tissue blocks of no more than $1mm^3$ were seeded into a 10cm uncoated plate. 5 tissue blocks were used in each culture dish, and 16% DMEM media (supplemented with L-Glutamine, Penicillin, Streptomycin and Gentamicin. Dishes were the incubated at 37°C (5% CO2). After 18-24 hours, fresh culture media was applied, then in a further 24 hours the tissue blocks were transferred to a new culture dish.

One final method involved attempting to isolate hPSCs from discarded acinar tissue from a pancreatic islet cell isolation. In this process, pancreata from transplant retrievals are digested and islet cells extracted for use in autologous transplantation. On completion of the protocol, a large amount of acinar tissue is available and usually discarded. However we attempted to outgrow PSCs from these cells by resuspending and placing in T175 flasks with 10% supplemented DMEM media. The following day the media was changed, and cells observed for outgrowth.

Characterisation

PSCs were characterised mostly by western blot (see chapter 3.6) for protein expression. Markers of activation examined included αSMA, desmin, collagen type I, MMP13 and fibroblast activation protein (FAP). The latter is a relatively new marker of fibroblasts, which is thought to help in controlling fibroblast growth and interactions during development, tissue repair and epithelial carcinogenesis. [240] Cells were also examined for the typical spindle-like morphology seen in the activated phenotype.

## 3.3 Results

rPSC

PSCs were successfully isolated from sacrificed Sprague-Dawley rats, with the pancreata being resected from the rats whilst sedated. It has been reported that decapitation provides sufficient exsanguination and minimises pancreatic congestion of the pancreas, thus improving subsequent tissue digestion by proteases.[236] However by using our resection technique, combined with the Optiprep density gradient method, a significant band of cells was observed, and sufficient PSC yield generated. Cells generally became confluent after 7 days following a passage of 1:10. From one isolation these rPSCs usually reached 10 passages before they stopped growing. A total of 5 isolations were performed which allowed for enough cells to perform characterization and validation experiments.

Following one passage (i.e. cells in culture for >7 days), protein was extracted for western blot. Figure 4.1 demonstrates positive staining for αSMA, desmin, MMP13 and collagen type I. Human HSCs were used as positive controls.

rPSCs were then blotted for MCT1, MCT4 and CD147 (figure 4.2). It was observed they express all the above markers, however MCT4 was more strongly expressed than MCT1, suggesting a more glycolytic phenotype.

PSCs typically respond to doxorubicin through an increase in chemokine activity related to induction of the senescence secretory phenotype (SASP). Therefore to validate our rPSCs were behaving as an activated phenotype, they were subjected to 24 hours of treatment with doxorubicin at 50nM. mRNA expression analysis by qPCR of the chemokines IL6 and

IL8 confirmed a significant upregulation (17.8-fold and 22-fold respectively; $p<0.001$) as seen in figure 4.3, thus confirming their ability to respond to cytotoxic stress. Furthermore treatment induced protein expression of γH2AX, again confirming activation.

Figure 4.1 – Protein expression of fibroblast-relevant markers to validate rat PSCs, with human HSCs as controls

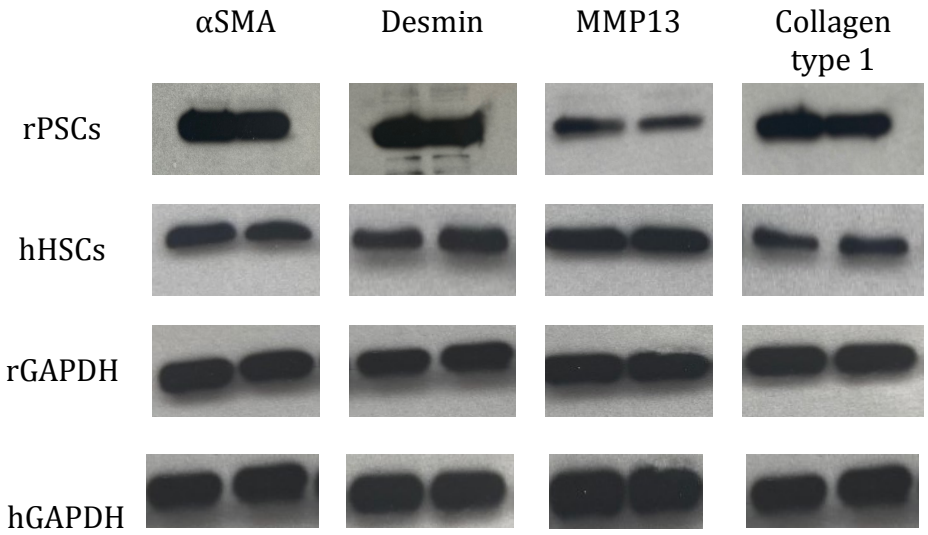



Figure 4.2 – Protein expression of MCT1, MCT4 and CD147 in isolated rat PSCs

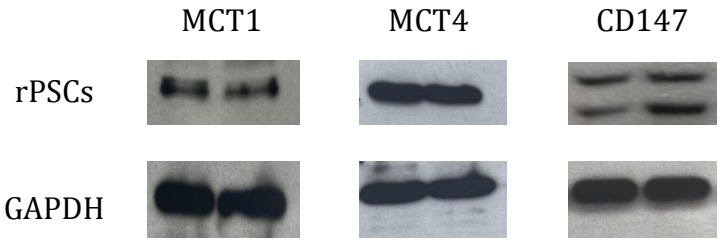
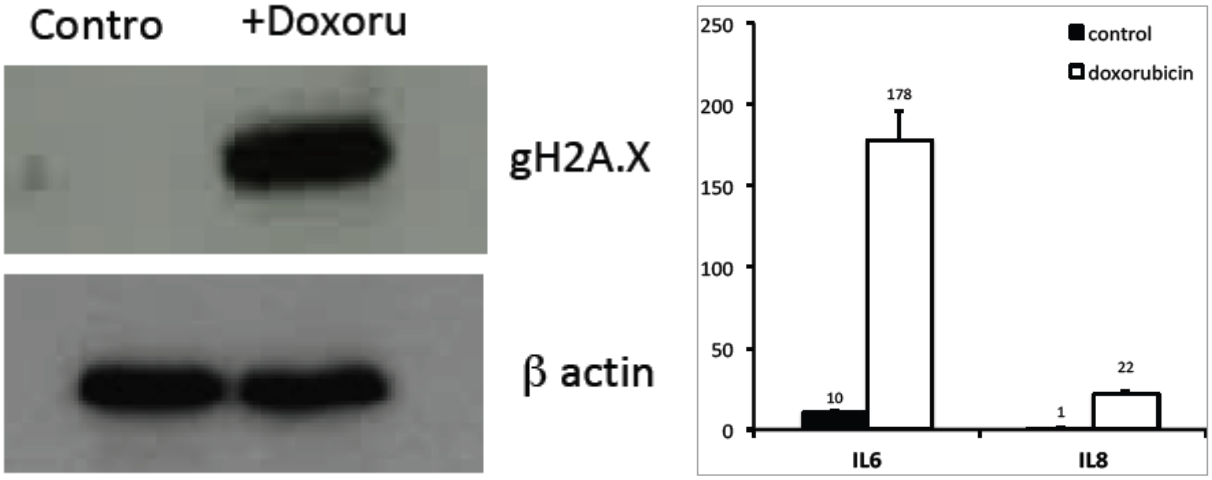


Figure 4.3 – Treatment of rPSCs with doxorubicin induced γH2AX expression and a significant upregulation in IL6 and IL8




hPSC

The original density gradient isolation protocol previously described failed to yield any viable hPSCs over 5 separate attempts from resected pancreatic tissue. In some instances no band was observed at the density gradient. Alternatively when cells were seen, these failed to adhere to the culture flasks, indicating non-viability.

Following 5 failed isolations, modifications were subsequently made to the human isolation as guided by the pancreapedia protocol.[236] The key changes made were as follows:

- The tissue specimen was placed into ice cold PBS supplemented with Penicillin and streptomycin, instead of HBSS+
- Alterations were made to the enzyme solution content/concentrations, whereby the Collagenase P concentration was increased from 0.5mh/ml to 1mg/ml, and protease was used instead of pronase

- Instead of merely placing the tissue into the enzyme solution, insulin needles were used to inject the specimen, including within any available duct lumens, thus maximizing digestion.
- The incubation timings during the digestion process was changed, using a 7 minutes incubation before and after the mincing process, rather than a prolonged 30 minute incubation just after mincing.

Whilst these protocol alterations created a thicker band of cells at the density gradient, they still failed to adhere to flasks. Attempts to rectify this issue was made by placing cells onto collagen-coated plates to facilitate adherence, however again this failed to allow hPSC outgrowth.

The density gradient method was initially used as, compared to outgrowth methods, it is reported to improve the quality and yield of PSCs. Furthermore outgrowth methods can often lead to bacterial infection. Nonetheless outgrowth was attempted on both tissue blocks (n=3), and from acinar tissue (n=2). Cells from the tissue blocks adhered to the culture dish, however these did not appear to be morphologically similar to fibroblasts, and they failed to reach confluency on 2 occasions, whilst an infection meant a flask had to be discarded on the third attempt. Only a scanty number of cells became adherent in the acinar tissue outgrowth, again failing to reach confluency.

Therefore, despite the limitations of an immortalized nature, a PSC cell line was obtained through a collaboration with Prof H.Kocher (Barts, London) for use in subsequent

experiments and thus avoiding any delay in commencing investigation into the metabolic relationship between PSCs and PDAC.

## 3.4 Discussion

Since the discovery of the potentially key role of PSCs within the tumour microenvironment of PDAC, significant progress has been made with regards to the multi-functional role they play.[76] By the time this interest in PSCs had come to the fore, the field of HSC research had already reached a high level of sophistication, with their key role in fibrogenesis being elucidated with the potential for therapeutic intervention in patients with various forms of liver disease (ALD, NAFLD etc.).[237-239] Indeed similarities seemed to exist between the fibrogenic mechanisms in both the liver and pancreas, whereby progressive wound healing can ultimately lead to irreversible damage, in the form of cirrhosis or CP respectively. Furthermore the links between these diseases and cancer development suggest the mediators of the key fibrogenic processes, including the respective stellate cells, play an important role in tumour initiation.

Therefore it was logical to utilise the expertise of the fibrosis lab in this project to study PSCs. The lab had previous experience in rPSC isolation, and indeed I successfully isolated these cells utilizing their protocol adapted from HSC isolation. However problems were encountered with respect to hPSC isolation, which is far from uncommon in the research literature, and is in contrast to the more consistent success in HSC isolation experienced in the fibrosis lab, and could be attributed to a number of factors. Firstly, it has been noted that stellate cells are 10 times less abundant in the rat pancreas than in the liver, meaning any attempts at isolation would always be more challenging.[241] Furthermore, the fact the liver and pancreas differ with respect to structure and tissue reactivity means cells may behave differently in a density gradient set up. The regenerative potential of hepatocytes is well documented, whereby the liver is able to restore functional mass in response to both

acute and chronic damage. However pancreatic tissue has limited regenerative potential, and in combination with its high enzymatic content, the organ is extremely prone to fluid extravasation and tissue oedema.[242] In addition, patients undergoing resection for malignant tumours were invariably noted to have densely calcified pancreatic tissue, either due to the fibrotic stromal reaction spreading away from the tumour into "healthy" tissue, or pre-existing CP. Alternatively patients undergoing resection for benign disease (e.g. Frey's procedure) have significantly inflamed tissue due to CP. Furthermore tissue specimens often had significant adiposity, often related to raised patient BMI; whilst every effort is made to remove fatty tissue, some intrinsic adiposity may remain. Taking these various aspects into account, in theory this lack of pancreatic tissue integrity means isolating a higher yield of PSCs is more challenging.

Another aspect to consider is the underlying pathology of the pancreatic tissue being resected, i.e. benign or malignant disease. Theoretically this could impart distinct differences in PSC physiological function that may persist following isolation and outgrowth, and therefore affect experimental consistency and translational impact, as well as making it difficult to make direct comparisons between studies. Indeed it has been shown that there are inherent differences in PSC expression patterns depending on the underlying pathology of the tissue source.[243] However in contrast Han et al demonstrated that outgrown PSCs from different donor pathologies did not demonstrate differences in soluble mediator secretory profiles (growth factors/chemokines/cytokines).[244] However they did observe these profiles evolved over time, and whilst they hypothesized this was due to high passage number, it was not clear whether differences in pathology was implicated.

Whilst my isolation attempts sourced tissue from a combination of benign and malignant conditions without success, in combination with the above points regarding tissue integrity, one would hypothesise that the most chances of success are from large pieces of less diseased/healthy tissue with low adiposity. In the context of my experimental set up, I would have sided on isolating PSCs from a benign source, whereby quiescent cells are isolated, activated through plastic exposure in culture, and subsequently converted into a CAF-phenotype through co-culture with PDAC cell lines.

The difficulty in PSC isolation has been shared within the research community, and extends to issues with PSC survival after isolation. Even once hPSCs are successfully isolated, further difficulty is encountered due to the limited passage limit, whereby primary isolated PSCs undergo senescence within a limited number of passages.[245] With this in mind, and given the potentially heterogeneous nature of primarily isolated PSCs, a number of groups have generated PSC cell lines.[245, 246] Whilst the immortalized nature of these cells means their rapid growth and proliferation do not entirely mimic primary cell phenotype, they do allow for both improved availability and sufficient cell number for experimental studies. Jesnowski et al were the first group to document the immortalization of outgrown human PSCs from resected chronic pancreatitis tissue via transfection methods, confirming the RLT-PSC cell line retained the activated phenotype with positive αSMA, desmin, vimentin and GFAP expression. [245] In efforts to delineate differences between benign and malignant associated PSCs, Rosendahl et al recently successfully generated conditionally immortalized human non-tumour (NPSC) and tumour-derived (TPSC) PSCs.[246] This was performed through transformation of primary isolated PSCs (outgrowth method) using the temperature sensitive SV40 large T antigen and human telomerase (hTERT). Once SV40LT was switched off, the cells regained their primary PSC

phenotype; whilst both NPSC and TPSC cells demonstrated characteristic markers of PSC activation, there were inherent differences in protumourigenic protein expression. However the cells isolated for NPSC generation were still generated from a malignant specimen, albeit distant to the resection margin, and given the extensive spread of desmoplasia seen in resected specimens, one may postulate that these cells may still harbor different physiological characteristics from PSCs in a normal non-diseased pancreas.

Following discussion with other institutions it appeared many scientists experienced similar frustrations in PSC isolation. This included Prof Hemant Kocher, Professor of liver and pancreas surgery at the Barts Cancer Institute in London. He had successfully developed a PSC cell line (PS1), which he kindly agreed to share with us through collaboration. Despite the aforementioned limitations of this being an immortalized and non-primary cell, in order to avoid any further delays in commencing experiments to examine the PSC-PDAC metabolic relationship, this line was used throughout this project.

**Summary**

- The validated protocol for HSC isolation was effective in isolating PSCs from rat pancreata
- rPSCs expressed MCT1 and MCT4, and responded to cytotoxic stress
- Despite numerous alterations to HSC/rPSC protocol, I was unable to isolate PSCs from resected human tissue – this may be due to the extent of pre-existing calcification/fibrosis within the specimens
- To ensure project progression, the decision was made to use an immortalized line of hPSCs obtained through a collaboration

# Chapter 4

# Investigating a metabolic symbiosis between PSCs and PDAC

## 4.1 Background

Despite the wealth of research attention towards the role of PSCs in the PDAC microenvironment, the impact on metabolic reprogramming has been seemingly over-looked. This may be due to the fact metabolomic research has only gathered pace in recent years, with increasing evidence that targeting metabolic pathways in PDAC is an attractive therapeutic tactic. However it is still unclear as to what approach will be most efficacious, whether that be anti-oxidative or anti-glycolytic, and which specific pathways or byproducts should be targeted.

However studies have simply concentrated on the tumoural component, despite the fact metabolite pooling will invariably affect other stromal elements such as PSCs. It has been shown that the proglycolytic switch in cancer causes altered metabolic gradients within the microenvironment, and the shifting of metabolites including lactate induce prosurvival pathways and encourage cancer cell proliferation. [100] The question is whether stromal cells, and specifically PSCs, have a role in promoting this protumourigenic acidified milieu; answering this question will not only improve our mechanistic understanding of how metabolic reprogramming occurs in PDAC tumours, but also guide towards the most effective means of attack.

With respect to the combination of lactate production and utilization within the tumour microenvironment, it is extremely challenging to understand exactly how lactate is being used, especially when you consider other stromal cells which may also be producing/utilizing lactate within the tumour microenvironment. Given the fact acidification (from lactate accumulation) confers a poor prognosis in a multitude of malignancies, the question remains as to how this is mechanistically explained. Is it due to the resultant utilization of the lactate by tumours that drives invasiveness and metastasis? Or rather does the acidification of the microenvironment, and effect on stromal cells, incur a more significant impact on tumour aggression? As previously discussed, studies in other forms of cancer have investigated a potential metabolic symbiosis between cancer cells and their CAFs, and in particular whether lactate shuttling between them in the form of the reverse Warburg effect may allow cancer survival despite the hypoxic and nutrient deficient microenvironment[132, 133, 166, 247-250]; however does this relationship exist in PDAC?

To simplify down to a specific PDAC-PSC relationship, I initially wanted to investigate how each cell type impacts on each other's metabolic tendencies. Utilising 3 different PDAC cell lines, with inherently different genomic and metabolic phenotypes, would mimic the heterogeneity of PDAC tumours, as well as help delineate mechanisms of effect. I particularly wanted to see how lactate was being used when PDAC and PSCs were cultured in combination, and by using a transwell system I could see the impact on each cell type individually, as well as how combination is affecting media content of metabolites. Furthermore any impact on MCT expression may help delineate how lactate is being used.

This chapter of results will initially concentrate on baseline validation of the transwell co-culture set up, examining the effects of PSCs on PDAC (and vice versa) with respect to proliferation and chemokine expression (which is typically raised due to PDAC-PSC interactivity). The impact of each cell type on metabolism will then be investigated, with particular reference to alterations in phenotype (seahorse), lactate and glutamine production (assays), and MCT expression (PCR/WB). Conclusions will then be drawn as to how PSCs affect PDAC metabolism, and ultimately guide the ideal means of therapeutic disruption of any pro-tumourigenic relationship with particular reference to glycolysis and lactate metabolism/shuttling.

## 4.2 Results

### 4.2.1 Baseline validation of co-culture system

The hallmark of PSC activity is αSMA expression, therefore initially I examined the effect of the different PDAC cell lines on αSMA expression by PCR (fig 5). This revealed as significant increase in expression by 6.3 (p=0.012) and 6.4 fold (p=0.016) respectively with Panc1 and Miapaca2 respectively, however intriguingly no significant increase with the Bxpc3 line, suggesting any relationship between PSCs and PDAC is KRAS dependent. Nonetheless the continued use of Bxpc3 was still warranted to delineate any KRAS-dependent effects of subsequent observations, either in co-culture or following drug treatments.

The impact of transwell co-culture on cellular activity of PSCs and the 3 PDAC cell lines was then examined using the MTT and BrdU assays over a 48-hour time course (Fig 6). Co-culture with all PDAC cell lines had no significant impact on PSC metabolic activity (MTT) or proliferation (BrdU). Co-culture with PSCs led to a significant increase in Miapaca2 metabolic activity (solo 0.758 O.D. vs co-culture 1.249 O.D.; p=0.031) and proliferation (solo 0.619 O.D. vs co-culture 0.938 O.D.; p=0.045). In contrast the addition of PSCs to Panc1 cells led to a significant reduction in metabolic activity (solo 1.8 O.D. vs co-culture 1.522 O.D.; p 0.041), however no effect on proliferation. The Bxpc3 cell line showed no significant change in metabolic activity or proliferation when co-cultured with PSCs.

As previously described, PDAC induces a proinflammatory microenvironment in vivo through interactions with PSCs, therefore we then examined whether this effect was mimicked *in vitro*. A significant upregulation in PSC expression of IL6 (Fig 7.1) was seen when co-cultured with Panc1 (2.2-fold increase, p=0.001), Miapaca2 (6.7 fold increase,

p<0.0001) and Bxpc3 (2.6 fold increase, p=0.001). PSC expression of IL8 also increased significantly when co-cultured with Panc1 (12.8-fold increase, p<0.0001), Miapaca2 (6.5 fold increase, p=0.0034) and Bxpc3 (19.6 fold increase, p=0.023).

In theory the transwell system functions by allowing secreted growth factors and metabolites to pool within the culture media and affect the opposing cell type, however it also allows for more dynamic interactions that require the presence of both cells. To determine whether the observed effect on chemokine expression is purely due to secreted factors, I then treated the PSC line with conditioned media from the PDAC cell lines. This revealed similar results (Figure 7.2), whereby treatment of PSCs with Panc1 and Miapaca2 supernatant led to a significant upregulation in IL6 (3.6 (p=0.0009) and 2.5-fold (p=0.048) respectively) and IL8 (4.9-fold (p=0.0001) and 10.1-fold (p=0.045) respectively), whilst a significant upregulation in IL10 of 1.89-fold was seen with Miapaca2 (p=0.039) albeit not with Panc1 (2.2-fold; p=0.24).

Chemokine expression in the PDAC lines when co-cultured with PSCs was also examined, whereby a significant increase in IL6 was seen in Panc1 (6.5 fold; p=0.006), Miapaca2 (4.6 fold; p=0.0036) and Bxpc3 (7.5 fold; p=0.0007). IL8 expression was also investigated, whereby a significant increase was seen in Bxpc3 when co-cultured with PSCs (7.0 fold; p=0.0055), however no significant change in Miapaca2 or Panc1. The reason for the latter was not clear, however in view of the effect on IL6, along with the supernatant results, and in particular the profound effect on PSCs, I therefore concluded that this transwell system was mimicking the pro-inflammatory *in vivo* effect of the PDAC-PSC relationship.

The heterogeneity of results with respect to both proliferation and interleukin expression highlights the limitations of *in vitro* work, with the complex and differing genetic mutational landscape of cell lines giving rise to conflicting results. Nonetheless this also reflects the heterogeneic nature of PDAC tumours, and therefore the challenging nature of investigating therapeutic approaches to this aggressive disease. Furthermore it emphasizes the fact that the pro-tumourigenic effect of PSCs on PDAC is due to a complex interaction with a variety of stromal components that then leads to an increase in tumour activity in vivo as described in chapter 1. Nonetheless, given the limitations of basic science techniques in cancer metabolism research, it is essential that both the metabolic contribution of each cell type, and their relationship with each other, is better delineating to further understanding of the metabolic reprogramming process, and potentially identify targets for therapy.

Fig 5 –PDAC co-culture results in a significant increase in PSC expression of αSMA in Miapaca2 and Panc1 lines, but not Bxpc3

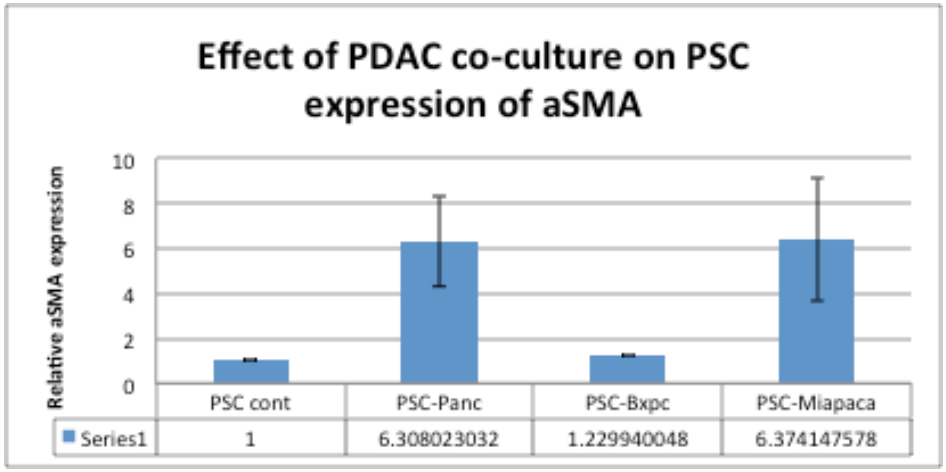
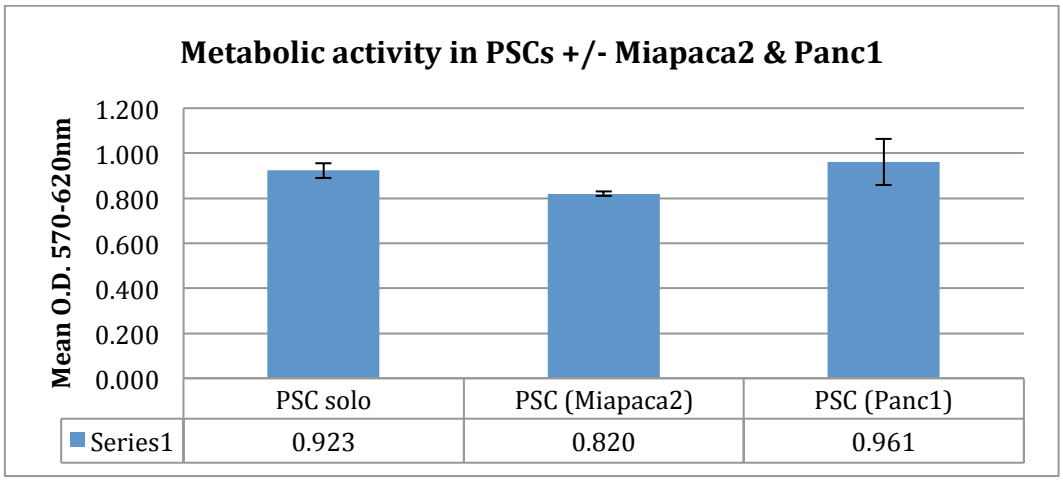
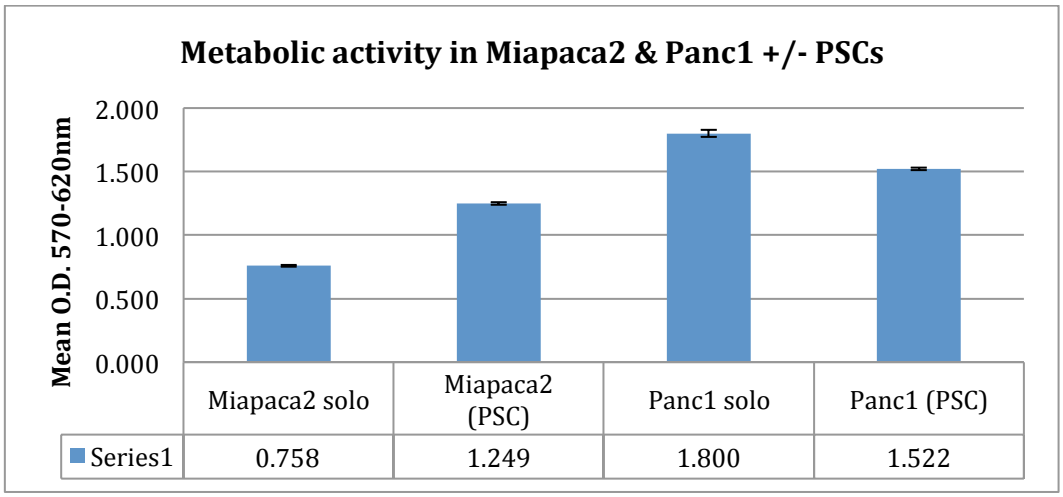



Figure 6 – The effect of co-culture of PSCs and PDAC cell lines on; 6.1 Metabolic activity (MTT assay) and 6.2 Proliferation (BrdU assay)

Fig 6.1




Metabolic activity in Miapaca2 & Panc1 +/- PSCs
Mean O.D. 570-620nm
2.000
1.500
1.000
0.500
0.000
Miapaca2 solo
Miapaca2 (PSC)
Panc1 solo
Panc1 (PSC)
Series1
0.758
1.249
1.800
1.522

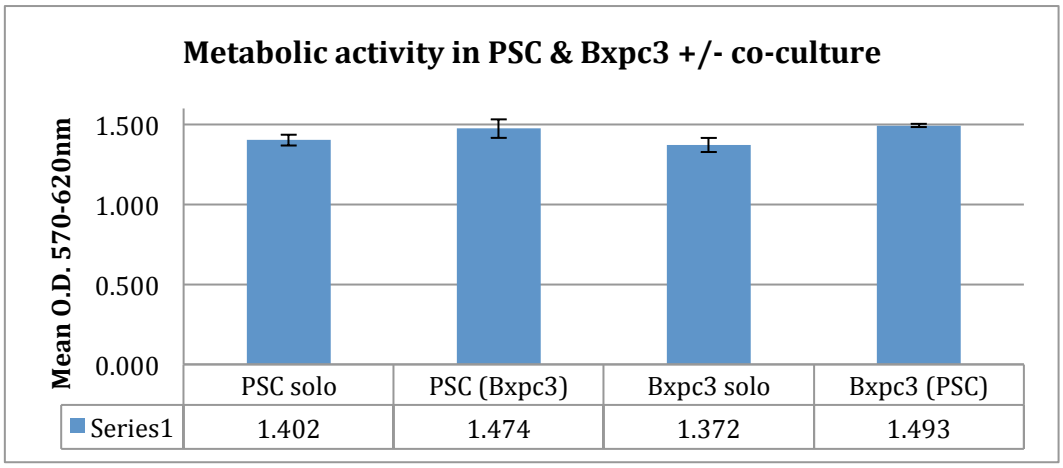
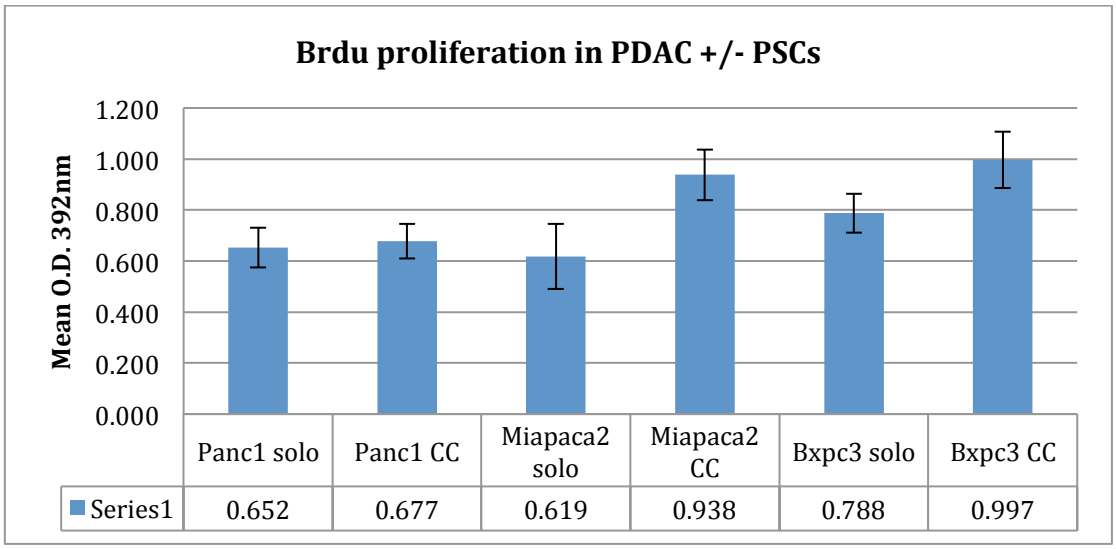

Metabolic activity in PSC & Bxpc3 +/- co-culture
Mean O.D. 570-620nm
1.500
1.000
0.500
0.000
PSC solo
PSC (Bxpc3)
Bxpc3 solo
Bxpc3 (PSC)
Series1
1.402
1.474
1.372
1.493

Fig 6.2


Brdu proliferation in PDAC +/- PSCs
Mean O.D. 392nm
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Panc1 solo
Panc1 CC
Miapaca2 solo
Miapaca2 CC
Bxpc3 solo
Bxpc3 CC
Series1
0.652
0.677
0.619
0.938
0.788
0.997

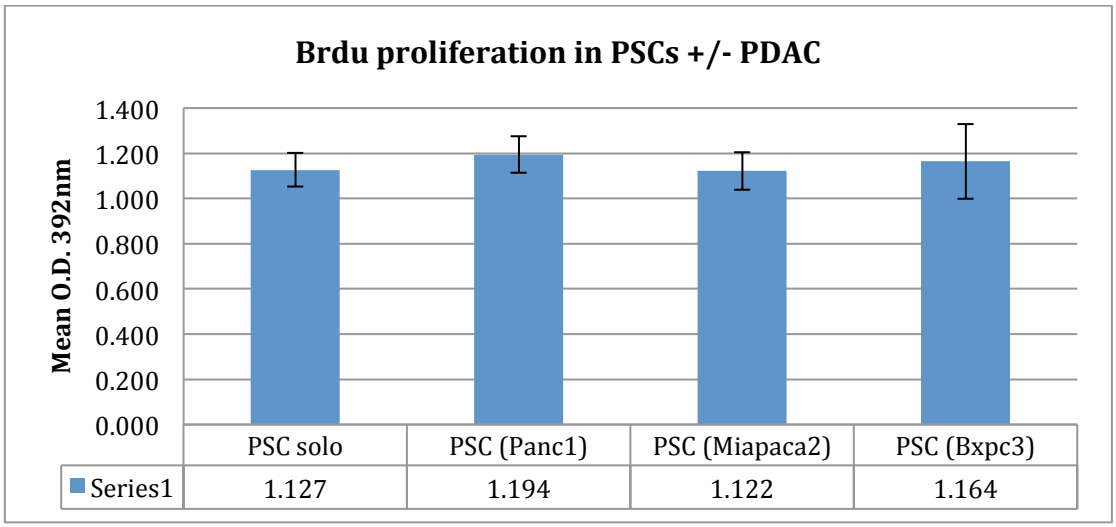
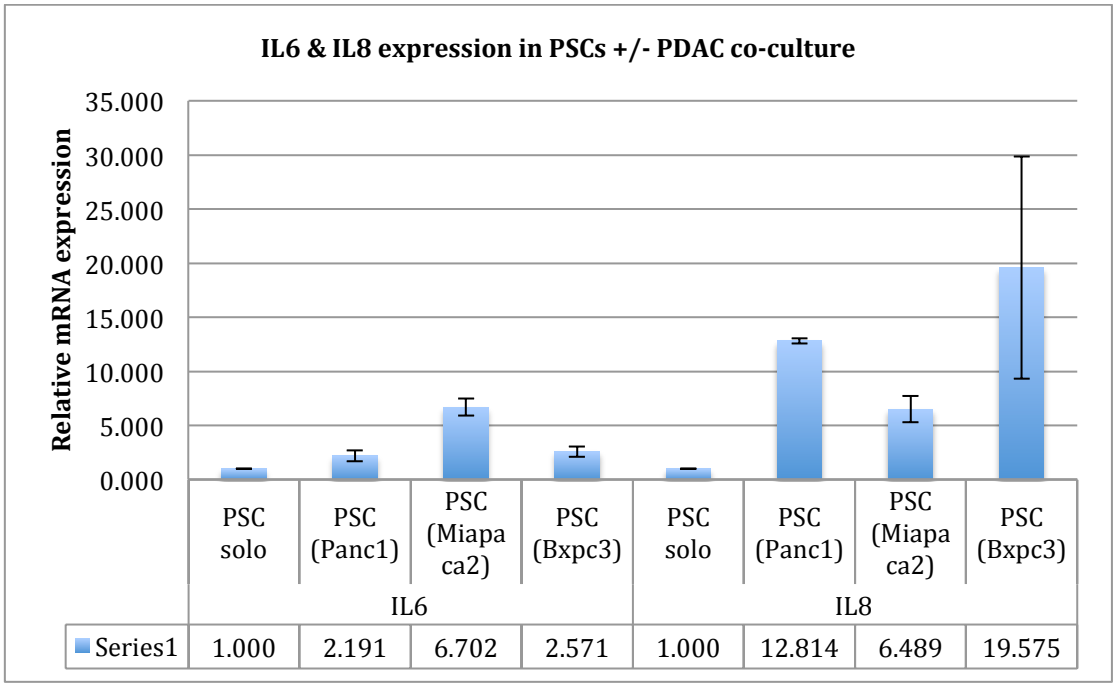
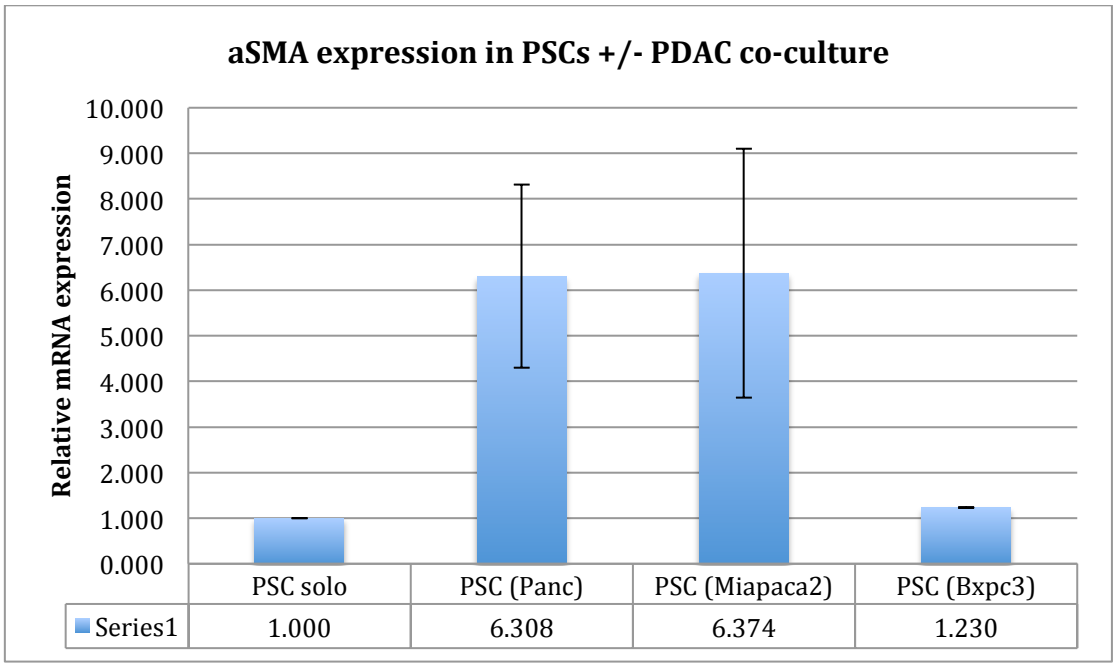

Brdu proliferation in PSCs +/- PDAC
Mean O.D. 392nm
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
PSC solo
PSC (Panc1)
PSC (Miapaca2)
PSC (Bxpc3)
Series1
1.127
1.194
1.122
1.164

Figure 7 – Pro-inflammatory gene expression in PDAC-PSC co-culture

Fig 7.1 - Transwell co-culture



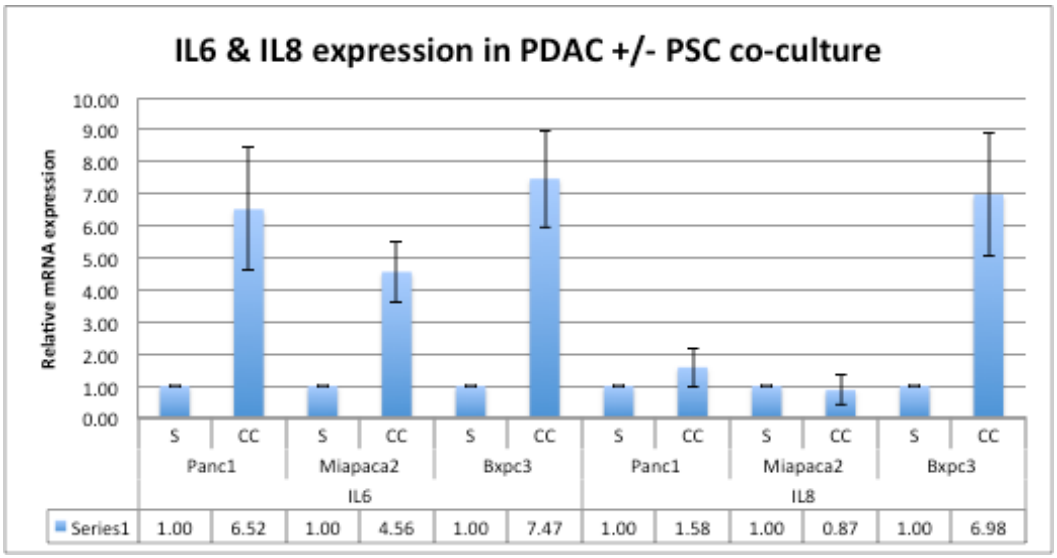
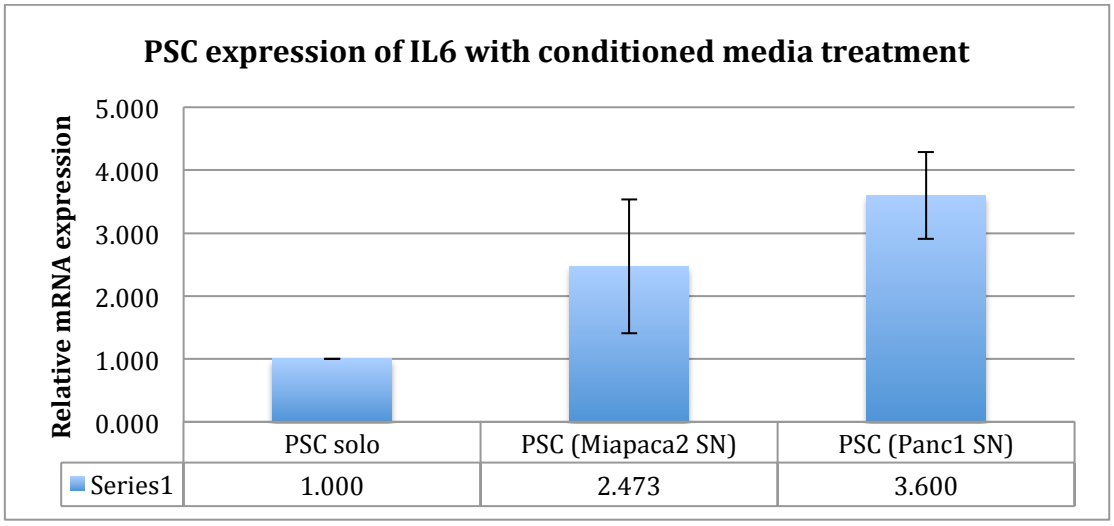
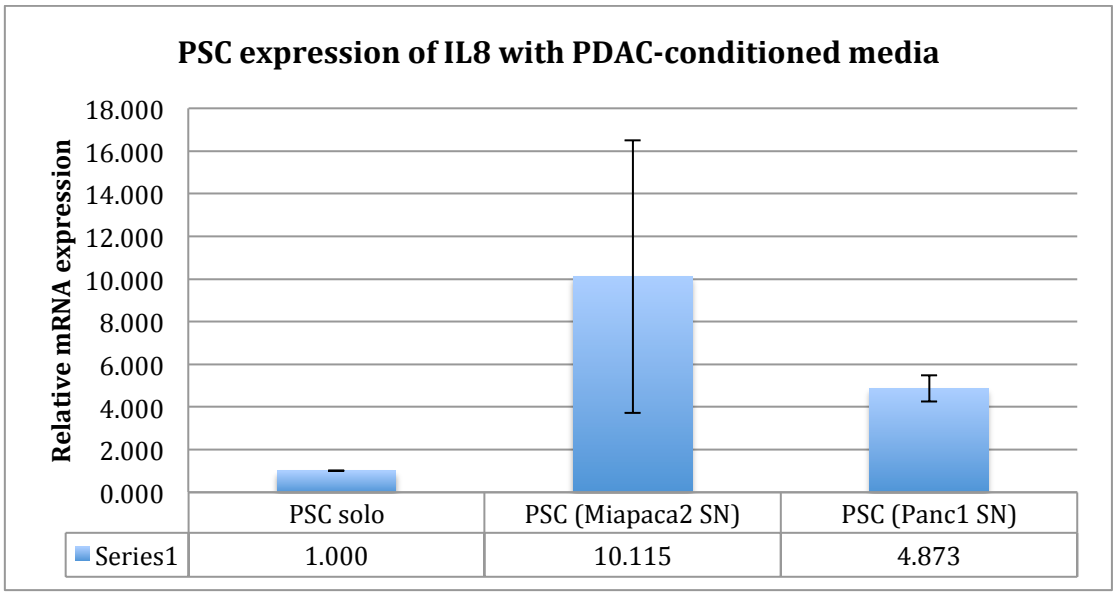
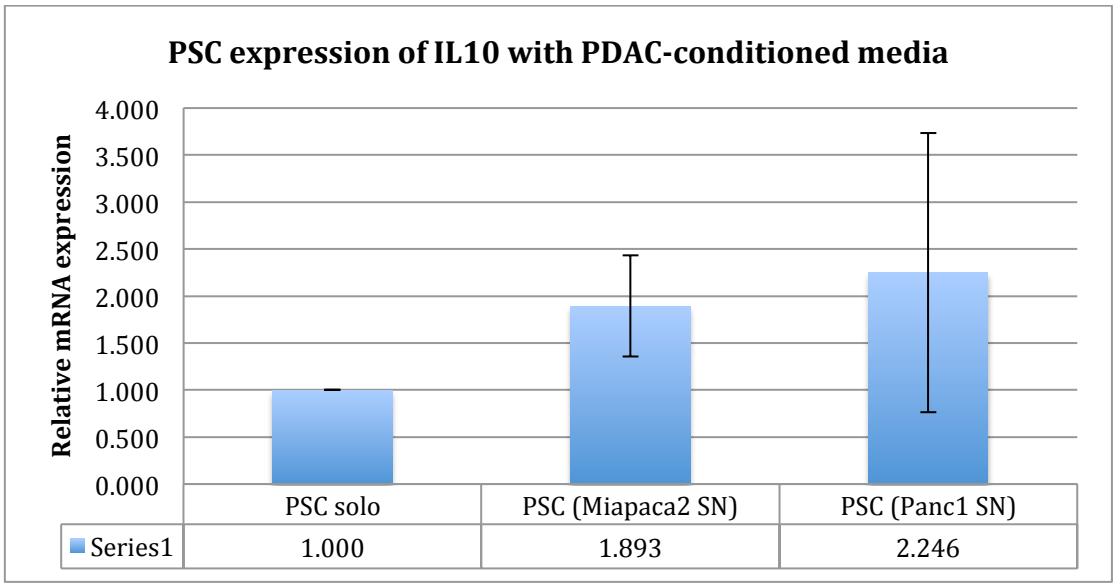



Fig 7.2 - Supernatant (conditioned media) co-culture






### 4.2.2 The metabolic relationship between PSCs and PDAC

As previously described, seahorse technology is an ideal method of determining the impact of treatments on metabolic phenotype. Therefore I next aimed to investigate the effect of co-culture treatment on PDAC and PSC metabolism using this technology, and provide a baseline relationship prior to in vitro therapeutic intervention. Results are expressed as a mean percentage change in metabolic parameters across the 3 separate experiments with each co-culture condition.

The protocol for seahorse analysis underwent various alterations in the early period of the project to optimize results. This included reducing the number of cells seeded into each well from 30,000 to 10,000; this prevented confluence of cells that potentially increases cellular stress and produces less reliable results. Furthermore when seeding the cells, the media supernatant from each solo or co-culture condition was used as the resuspension media when re-seeding onto the specialized seahorse plate, therefore allowing any secreted metabolites to maintain their potential effect. Lastly, in the initial stages 3 re-agents were used as part of the treatment algorithm (Oligomycin, FCCP at x2

concentrations, and Rotenone/Antimycin). In an effort to provide improved analysis of the glycolytic impact of co-culture, one of the FCCP concentrations was substituted for the glycolysis inhibitor 2-DG, which would allow more reliable information to be imparted on extra-cellular acidification rates.

Firstly a comparison was made between the four cell lines (PSC, Panc1, Miapaca2 and Bxpc3) to determine their baseline metabolic activity, and therefore how much each cell line relies on either glycolysis or OXPHOS for energy production. Results are expressed as a mean of 3 experiments for the PDAC cell lines, and a mean of 6 for PSCs. When studying differences between the PDAC cell lines, as demonstrated in figure 8, the rate of mitochondrial respiration was similar between Panc1 and Miapaca2; basal OCR was 351.1 and 254.1 pmmols/min respectively, whilst ATP production from OXPHOS was 507.9 and 329.2 pmols ATP/min respectively. However the KRAS-mutant Bxpc3 was found to be significantly more oxidative than both Panc1 and Miapaca2, with a basal OCR of 925.2 pmols/min, and OXPHOS ATP of 2180.3 pmols ATP/min. This is in keeping with the fact KRAS is heavily implicated in the metabolic switch to glycolysis as observed in the Warburg effect, and further supported by the glycolytic data; whilst there was no significant difference seen in acidification rates (ECAR), the Panc1 and Miapaca2 lines produced significantly more ATP by glycolysis (107.1 and 55.9 pmols ATP/min respectively) as compared to the Bxpc3 cell line (24.8 pmols ATP/min) (Figure 8.2).

On investigating the effect of Panc1 on the PSC metabolic phenotype (Fig 9.1), a significant increase in basal OCR (51.4%;p=0.01), maximal OCR (56.7%;p=0.0086) and OXPHOS ATP production (64.6%;p=0.021) was seen in the PSCs, however no inverse effect on glycolytic

parameters (ECAR or glycolytic ATP production). The Miapaca2 and Bxpc3 cell lines exerted no significant oxidative or glycolytic effect on the PSCs (Fig 9.1).

The most intriguing results were revealed regarding the metabolic effect of PSCs, whereby a significant increase in glycolytic parameters was observed in all PDAC cell lines (Fig 9.2). A significant increase in ECAR was seen in Panc1 (82.8%;$p<0.0001$), Miapaca2 (40.5%;$p=0.0024$) and Bxpc3 (45.7%;$p=0.049$), whilst a significant increase in glycolytic ATP production was seen in Panc1 (46.2%;$p=0.012$) and Bxpc3 (53.4%;$p=0.006$), however not in Miapaca2. Conversely, Miapaca2 showed a significant reduction in all oxidative parameters, with reductions in basal OCR (20.9%;$p=0.0001$), maximal OCR (24.3%;$p=0.0005$) and OXPHOS ATP production (54.0%;$p=0.0021$). However no significant effect on oxidation was seen in Panc1 and Bxpc3.

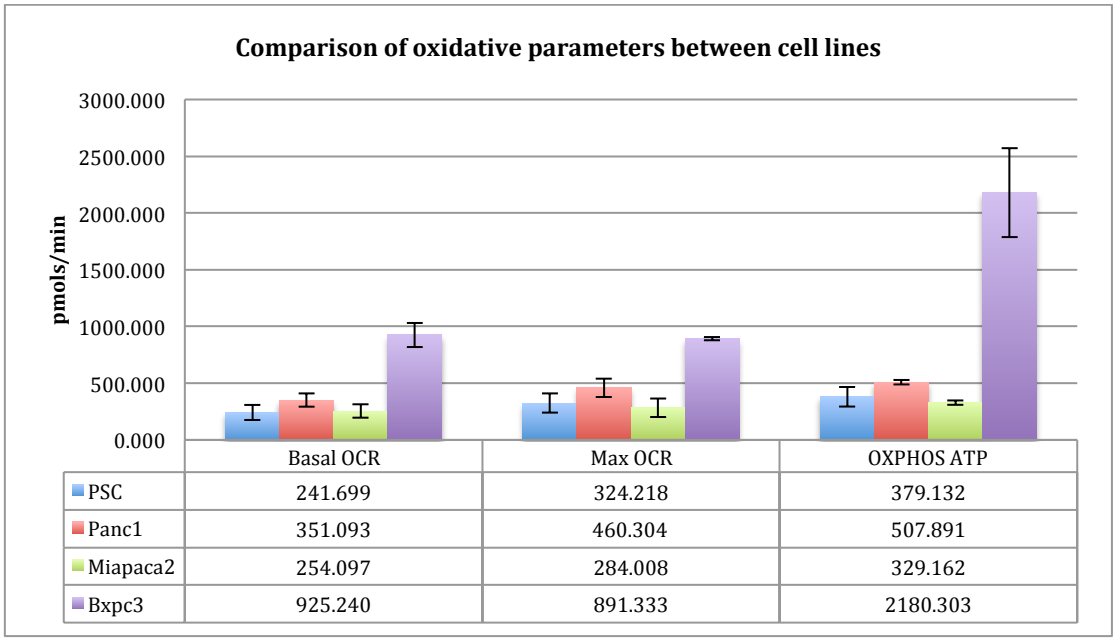
Therefore it is clear that PSCs exert a pro-glycolytic effect on PDAC, a phenomenon that has never previously been demonstrated, and potentially implicates PSCs within one of the factors which contribute to the Warburg effect. These experiments were performed following 48 hours of transwell co-culture, and I then aimed to determine whether this metabolic effect of PSCs is purely due to secreted metabolites or growth factors. Therefore the Panc1 and Miapaca2 cell lines were treated for 48 hours with conditioned media (CM) from PSCs, and the cells analysed for alterations in metabolic phenotype. As seen in fig 9.3, CM media treatment did not replicate the glycolytic effect of PSCs on PDAC seen in co-culture, with no significant effect observed. However there was a significant reduction in oxidative parameters in Miapaca2, as seen in transwell co-culture, with a reduction in basal OCR of 24.8% ($p=0.02$) and OXPHOS ATP production of 42.6% ($p=0.002$). Therefore one

may conclude that the pro-glycolytic effect of PSCs is not purely attributed to secreted metabolites, and instead secondary to a more complex in vitro signaling relationship.

The effect of PDAC conditioned media on PSCs was also examined (fig 9.4); whilst transwell coculture increased oxidative parameters in Panc1, with PSC conditioned media the opposite was observed, with a significant decrease in both basal OCR of 28.9% ($p=0.0038$), maximal OCR of 21.5% ($p=0.011$) and OXPHOS ATP production of 29.6% ($p=0.019$), as well as a reduction in ECAR of 21.6% ($p=0.023$). However, much like transwell coculture, the effect of PSC-CM on Miapaca2 was variable and not significant. This again emphasizes the fact whilst secreted metabolites from either PSCs or PDAC in vitro may exert an effect on metabolic phenotype, by using the transwell coculture system one may more reliably mimic in vivo signaling, and as such this system would continue to be used throughout the project as a more clinically translational model for examining cell to cell interactions and therapeutic effects.

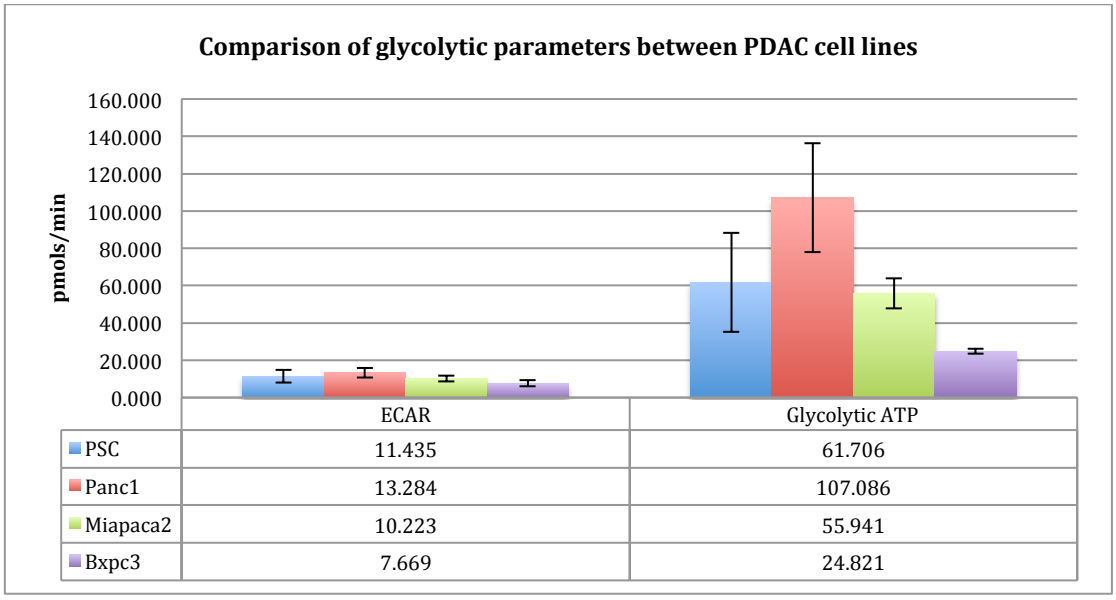
Fig 8 – Comparison of baseline metabolic parameters between PSC and PDAC cell lines;

Fig 8.1 Oxidative parameters




| | Basal OCR | Max OCR | OXPHOS ATP |
|---|---|---|---|
| PSC | 241.699 | 324.218 | 379.132 |
| Panc1 | 351.093 | 460.304 | 507.891 |
| Miapaca2 | 254.097 | 284.008 | 329.162 |
| Bxpc3 | 925.240 | 891.333 | 2180.303 |

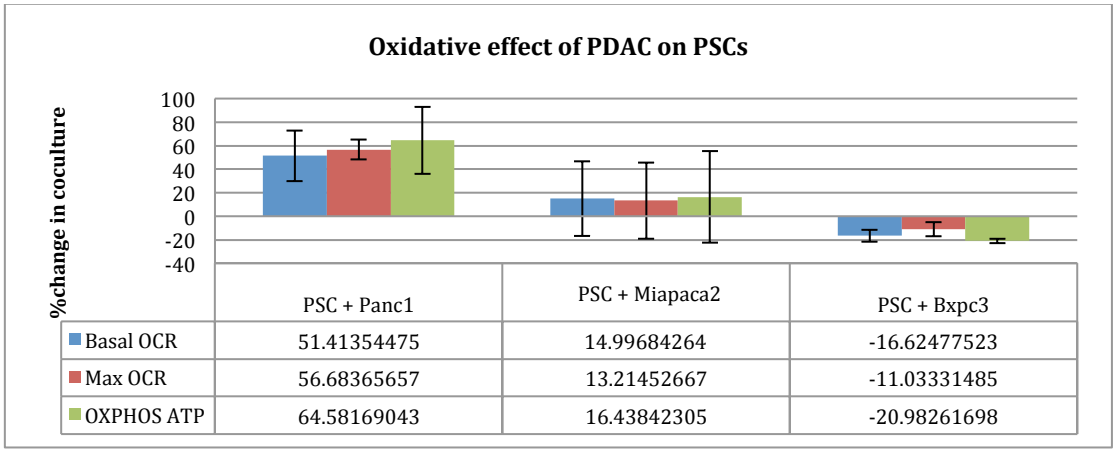
Fig 8.2 Glycolytic parameters



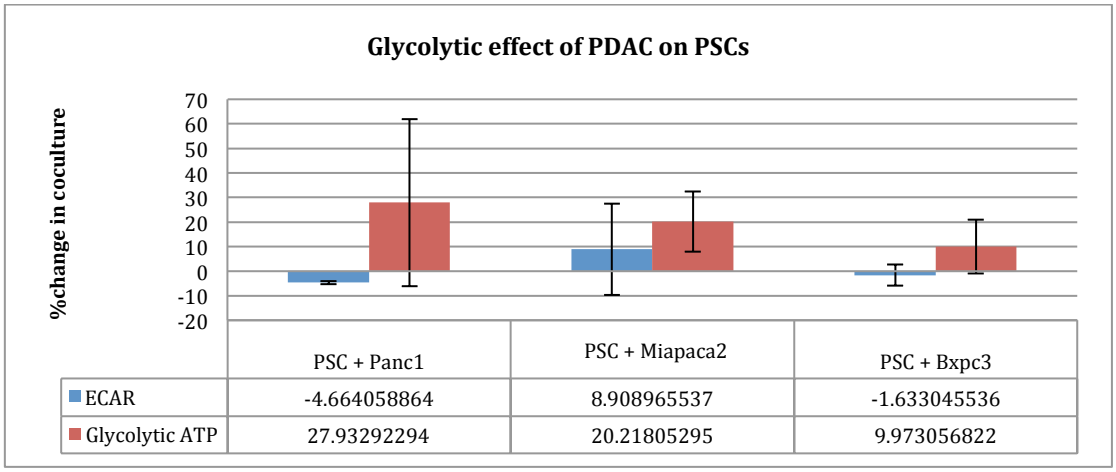

| | ECAR | Glycolytic ATP |
|---|---|---|
| PSC | 11.435 | 61.706 |
| Panc1 | 13.284 | 107.086 |
| Miapaca2 | 10.223 | 55.941 |
| Bxpc3 | 7.669 | 24.821 |

Fig 9 – The effect of PDAC-PSC co-culture/conditioned media treatment on oxidative and glycolytic metabolic parameters by Seahorse technology analysis;

Fig9.1 Effect of PDAC cell lines on PSCs

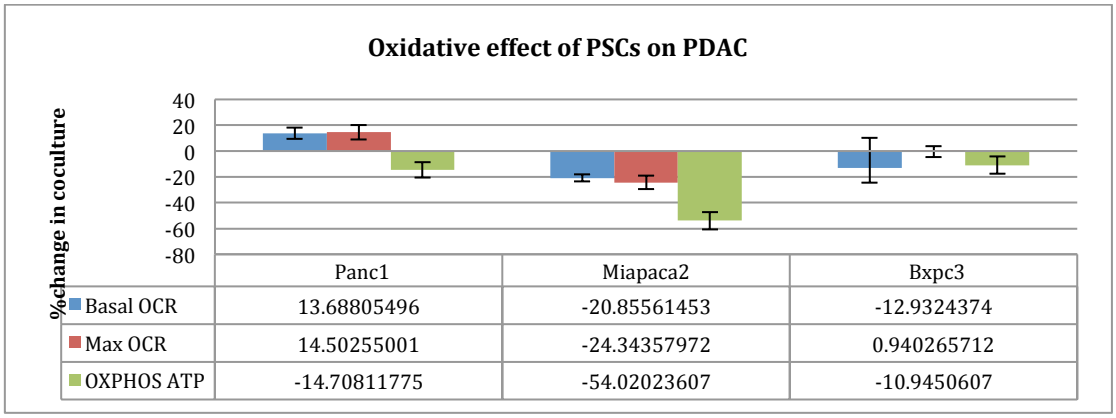



| | PSC + Panc1 | PSC + Miapaca2 | PSC + Bxpc3 |
|---|---|---|---|
| Basal OCR | 51.41354475 | 14.99684264 | -16.62477523 |
| Max OCR | 56.68365657 | 13.21452667 | -11.03331485 |
| OXPHOS ATP | 64.58169043 | 16.43842305 | -20.98261698 |




| | PSC + Panc1 | PSC + Miapaca2 | PSC + Bxpc3 |
|---|---|---|---|
| ECAR | -4.664058864 | 8.908965537 | -1.633045536 |
| Glycolytic ATP | 27.93292294 | 20.21805295 | 9.973056822 |

Fig 9.2 Effect of PSCs on PDAC cell lines




| | Panc1 | Miapaca2 | Bxpc3 |
|---|---|---|---|
| Basal OCR | 13.68805496 | -20.85561453 | -12.9324374 |
| Max OCR | 14.50255001 | -24.34357972 | 0.940265712 |
| OXPHOS ATP | -14.70811775 | -54.02023607 | -10.9450607 |

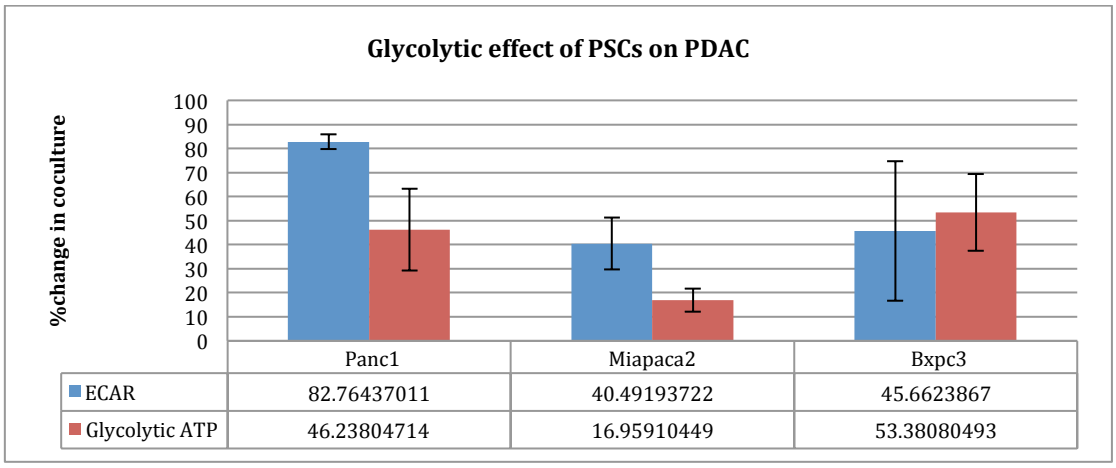



Fig 9.3 Effect of PSC conditioned media on PDAC

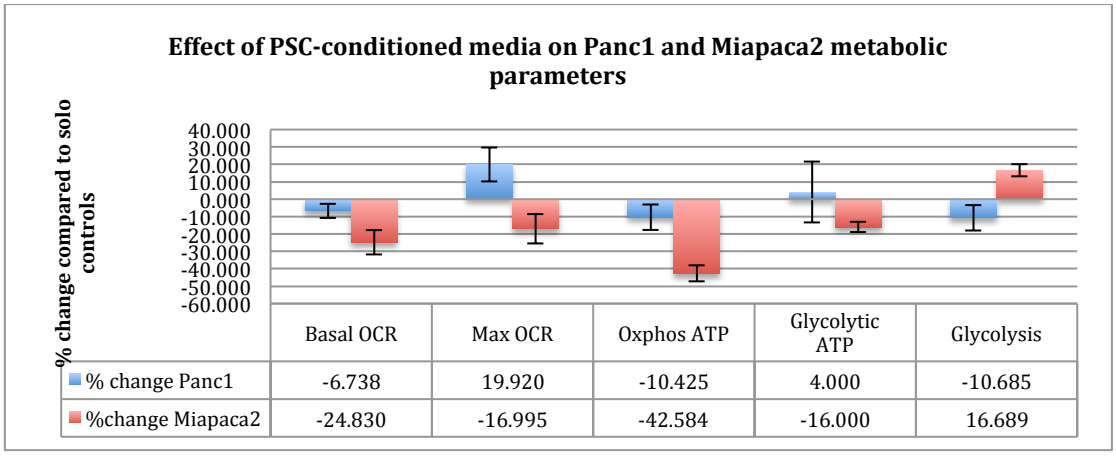
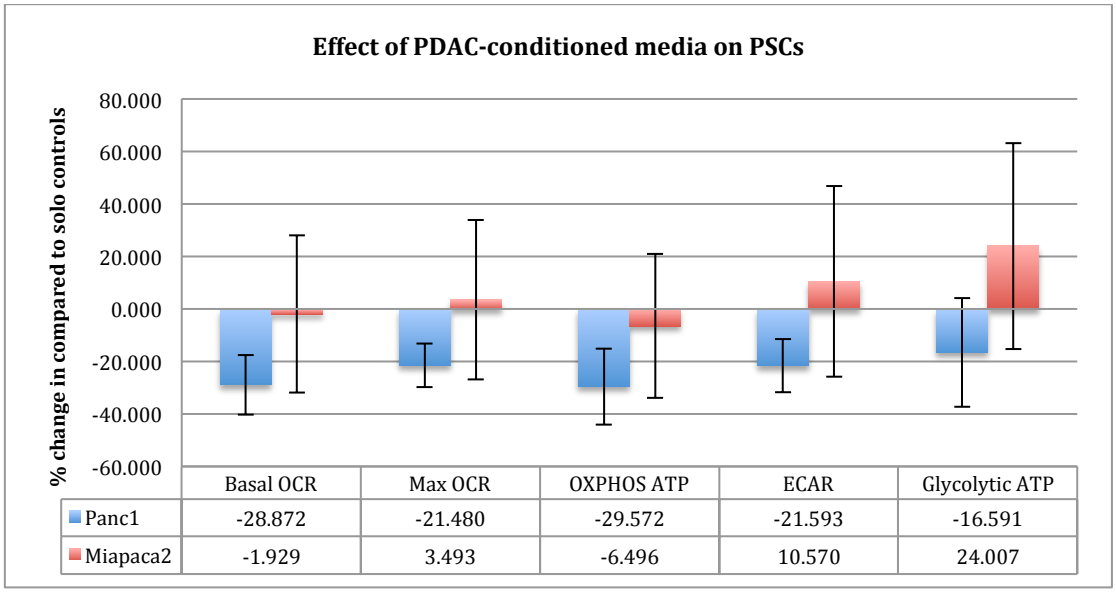



Fig 9.4 Effect of PDAC conditioned media on PSCs



### 4.2.3 Lactate shuttling

Results thus far have demonstrated how PSCs and PDAC cells impact on each other's metabolic phenotype, in particular an increase in protumourigenic acidification, whilst the proglycolytic effect of PSCs would provide a survival advantage to PDAC tumours in the hypoxic microenvironment. In view of the theorized increase in lactate production as a result of the PSC-PDAC metabolic relationship, I next aimed to investigate whether this may be attributed to interactions between the PDAC cells and PSCs that converge on a new theory of lactate shuttling with particular relevance to MCTs.

Ultimately the main stimulus for going down the experimental route in this project was the theory of the RWE. Therefore initially I simply wanted to test the author's theory that through hydrogen peroxide excretion from cancer cells, PSCs switch to glycolytic metabolism, which in theory would result in increased lactate efflux and therefore increased media lactate in vitro. In an attempt to replicate this relationship, PSCs were treated with hydrogen peroxide for 48 hours. 200uM and 500uM treatment resulted in no significant impact on media lactate concentration (Figure 10.1). Furthermore, compared to no treatment, 200uM H2O2 resulted in a significant reduction in proliferation from 0.35 O.D. to 0.019 O.D. ($p<0.0001$) (Figure 10.2). Therefore, in conjunction with seahorse results, it was becoming clear that the theory of the reverse Warburg effect did not seem applicable to PDAC.

To further investigate the simple effect of lactate on each cell type, and thus mimic the effect of in vivo lactate and ketone body accumulation each cell line was initially treated with lactate, in the form of L-lactate at a concentration of 20mM, and ketones, in the form of butyrate at a concentration of 10mM, to determine the effect on cellular activity (Fig 11).

Both lactate and ketone treatment had no significant impact on metabolic activity, therefore suggesting both PDAC and PSCs do not rely on lactate for energy production through oxidative respiration.

To further investigate the effect of PDAC-PSC interactions on lactate production/consumption, the effect of transwell co-culture on lactate concentration was investigated. This was done at both the intra-cellular level, using extracted protein for ELISA analysis, and extra-cellular level, by measuring media content. Co-culture of PSCs with Panc1 and Bxpc3 lines resulted in no significant difference in intra-cellular lactate concentration in either cell type (Fig 12). However there was a significant reduction in Miapaca2 intra-cellular lactate concentration in the presence of PSCs from 6.1mg/dl to 3.9mg/dl ($p=0.0002$), whilst PSCs again demonstrated no change in concentration.

When measuring extra-cellular lactate concentration, and given the media pools between the 2 compartments within the transwell system, the media from the base well and transwell was combined for analysis, with comparisons made with the solo PDAC controls. Furthermore alterations were examined at both 24 and 48-hour time points. A significant reduction in lactate concentration was seen in Miapaca2-PSC co-culture as compared to solo controls at both 24 (4.7 to 3.2mg/dl; $p=0.002$) and 48 hours (11.8 to 7.4mg/dl; $p<0.0001$), an intriguing result given the reduction in intra-cellular levels seen in Miapaca2. One may deduce that, in conjunction with the fact the seahorse experiments demonstrated an increase in glycolysis/acidification, PSCs may be utilizing this excessive lactate for oxidative metabolism, and hence correlating with a reduction in intra-cellular levels as this lactate would be converted to pyruvate. No significant change in extra-cellular lactate was seen in either the Panc1-PSC or Bxpc3-PSC systems.

Given this impact of co-culture on lactate concentrations, it was then theorized that expression of MCTs, which regulate lactate movement, are most likely affected by co-culture. Initially MCTs were confirmed to be expressed at a gene and protein level in both PSCs and PDAC cell lines, on RT-PCR (Fig 13.1) and Western Blot (Fig 13.2) respectively. MCT4 was more strongly expressed at the protein level in the Miapaca2 line. Unsurprisingly, given the presence of MCT in all cell lines, they also expressed CD147 at similar expression levels.

Having determined that MCTs were prevalent in PSCs and PDAC cells, and in an effort to investigate the presence of a lactate shuttling theory, and potentially the reverse Warburg effect, I then investigated whether co-culture of the two cell types affected MCT1 and MCT4 expression by qPCR and western blot (Fig 14). Following 48-hour co-culture with PSCs, a significant increase in MCT1 transcript expression was seen in Panc1 (4.7 fold; $p=0.038$), Miapaca2 (2.9 fold; $p=0.0035$) and Bxpc3 (7.3 fold; $p=0.01$). The same significant increases were seen in MCT4 expression of Panc1 and Miapaca2 by 2.1 fold ($p=0.031$) and 3.0 fold ($p=0.033$) respectively, however no effect was seen on Bxpc3. Conversely PSCs showed a significant upregulation in MCT1 in co-culture with Panc1 (2.9 fold, $p=0.011$), Miapaca2 (3.8 fold, $p=0.034$) and Bxpc3 (2.7 fold, $p=0.003$). Whilst a significant increase in MCT4 expression was seen when PSCs were co-cultured with Panc1 (2.4 fold, $p=0.022$) and Miapaca2 (5.8 fold, $p=0.013$), a significant reduction in expression was seen in the presence of the KRAS-mutant line Bxpc3 (0.5 fold reduction, $p=0.02$). The exact same findings with respect to MCT up/down-regulation were observed with regards to protein expression as demonstrated in figure 14.2 and 14.3.

The effect of transwell PDAC-PSC co-culture on glutamine metabolism was then evaluated (Fig 15), as in theory this upregulation of MCTs may induce a form of metabolic reprogramming which may increase glutamine-associated pathways of glycolysis. A significant 25.4% increase in intra-cellular glutamine content was seen in Miapaca2 when in co-culture with PSCs ($p=0.004$), however no significant effect was seen with Panc1 or Bxpc3.

Fig 10 – Effect of hydrogen peroxide treatment on PSCs

Fig 10.1 - Media lactate concentration with 200uM and 500uM treatment

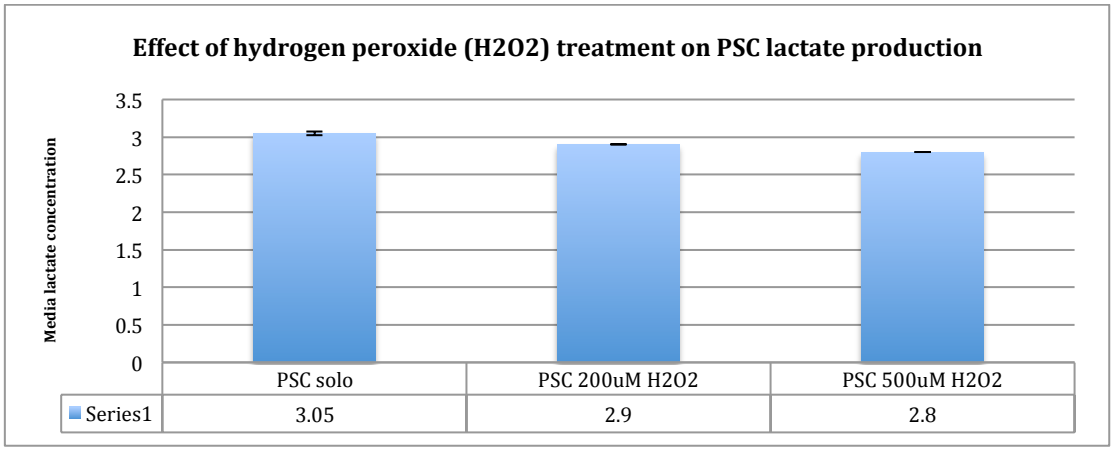



Fig 10.2 - MTT proliferation with 200uM treatment

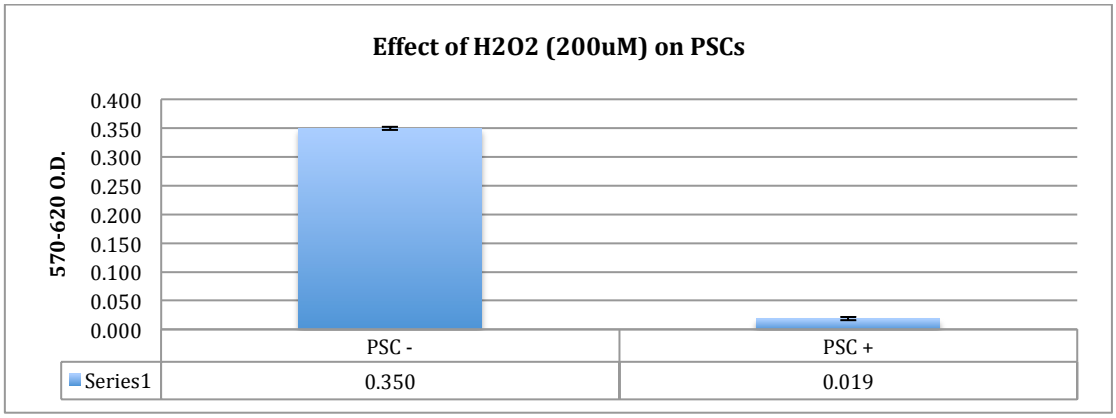



Figure 11 – Effect of lactate and ketone body treatment on PSCs and PDAC cell lines

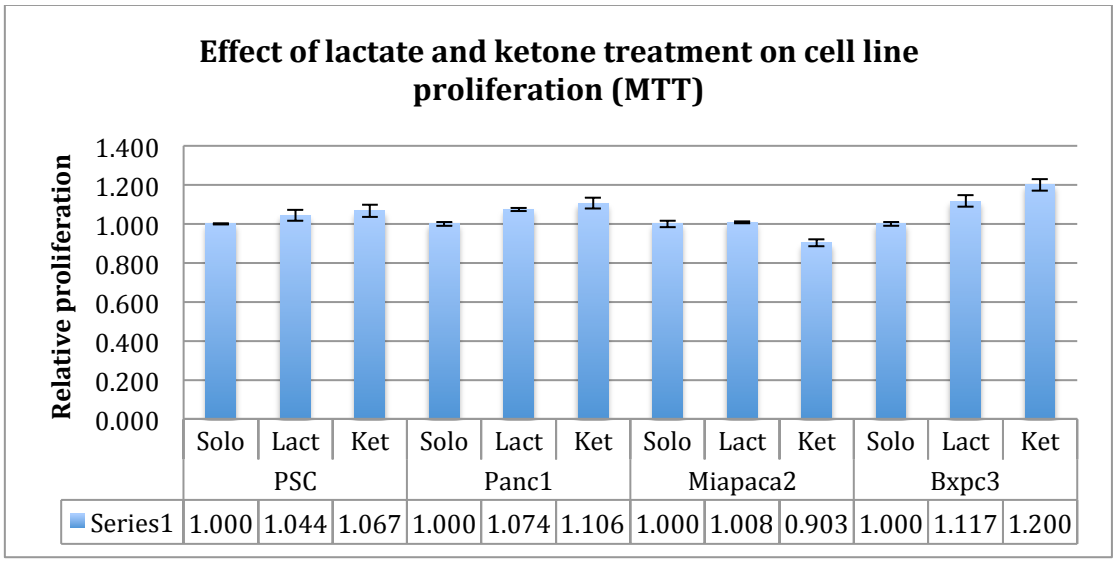
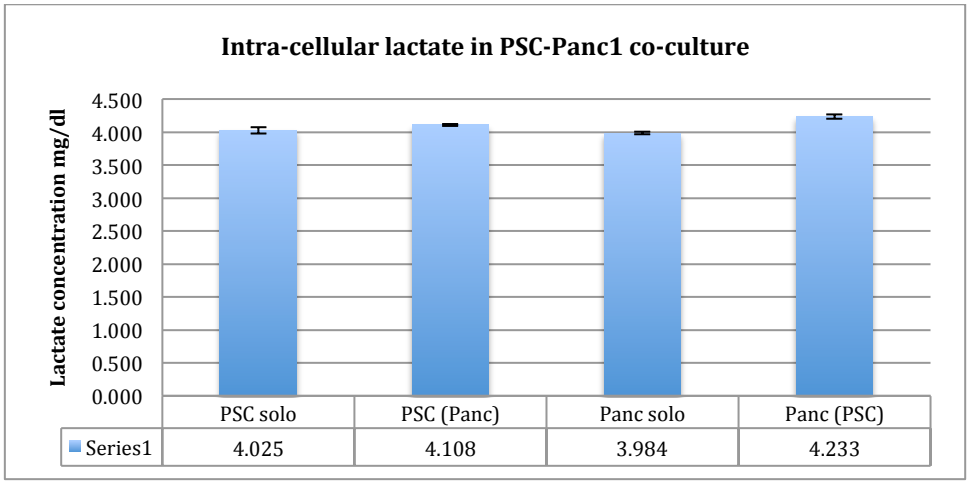
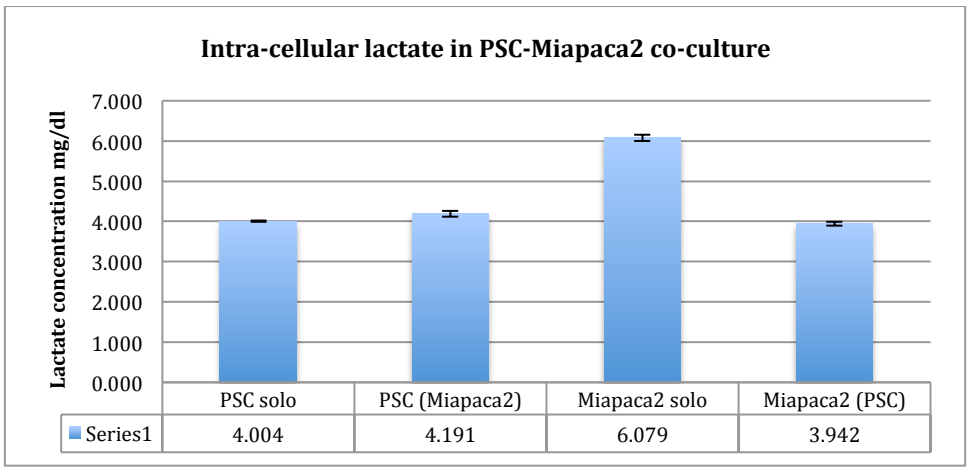


Figure 12 – Intra-cellular and extra-cellular lactate concentration in PSC-PDAC co-culture

Fig 12.1 – Impact on intra-cellular lactate



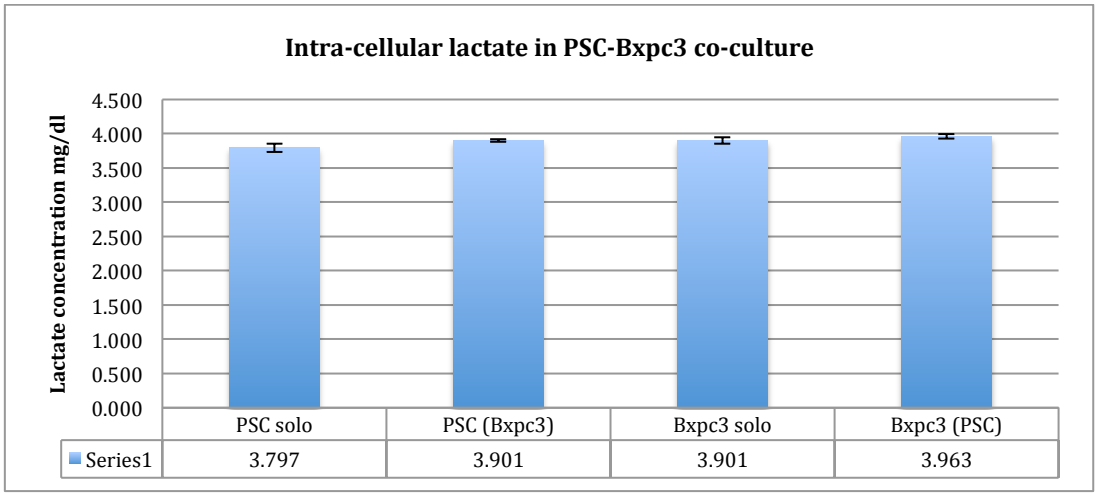
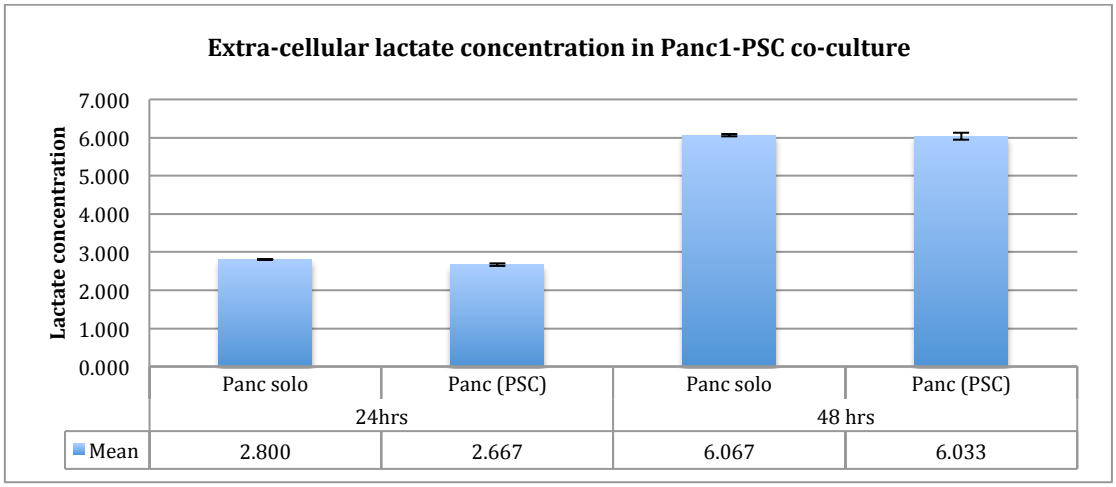
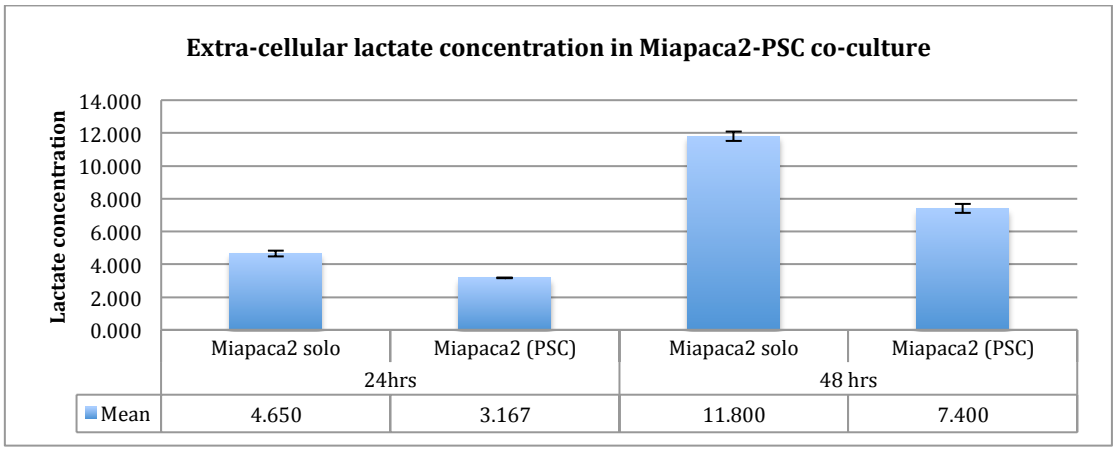



Fig 12.2 Impact on extra-cellular lactate



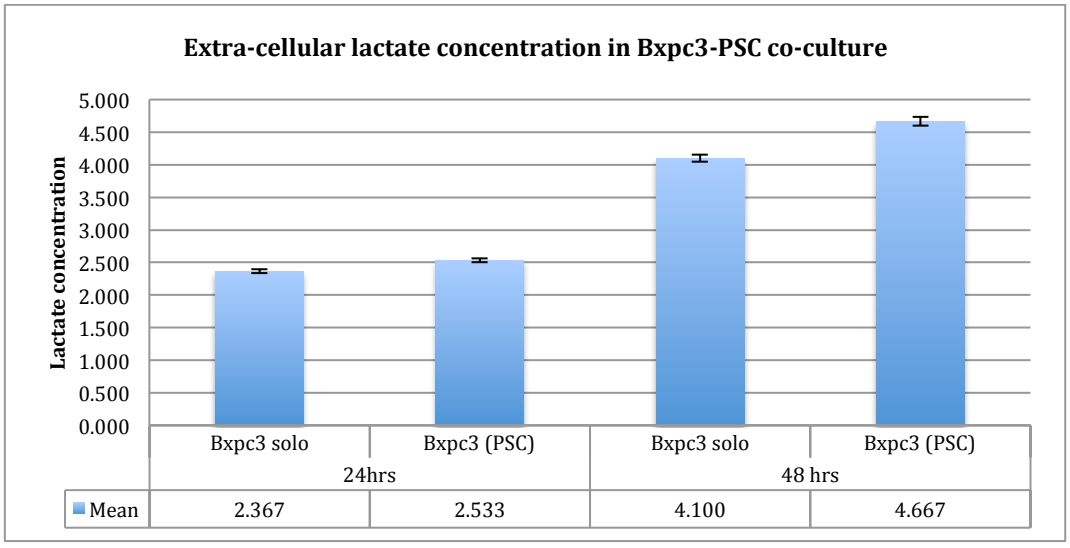



Figure 13 – Lactate transporter (MCT1, MCT4, CD147) expression analysis in PSC and PDAC cell lines:

Fig 13.1 – RNA expression by RT-PCR in triplicates

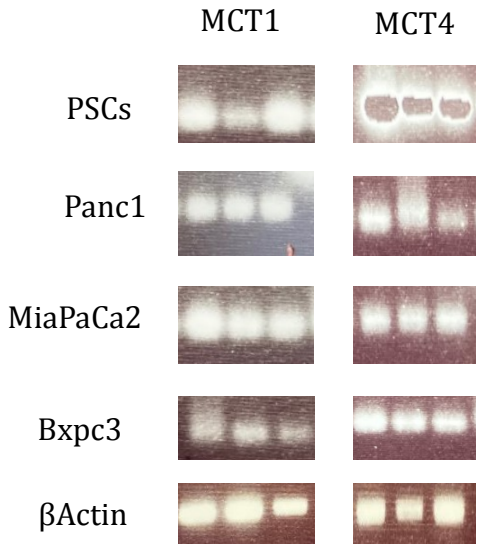



Fig 13.2 - Protein expression by western blot – note relative increased MCT4 expression in Miapaca2

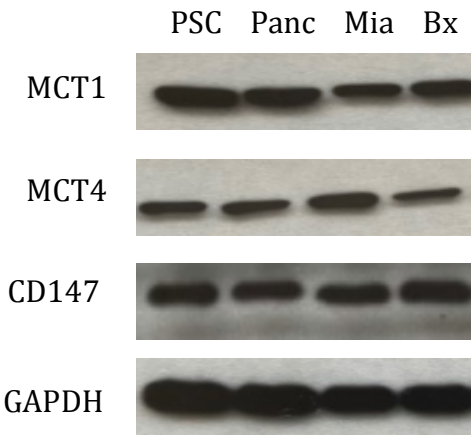
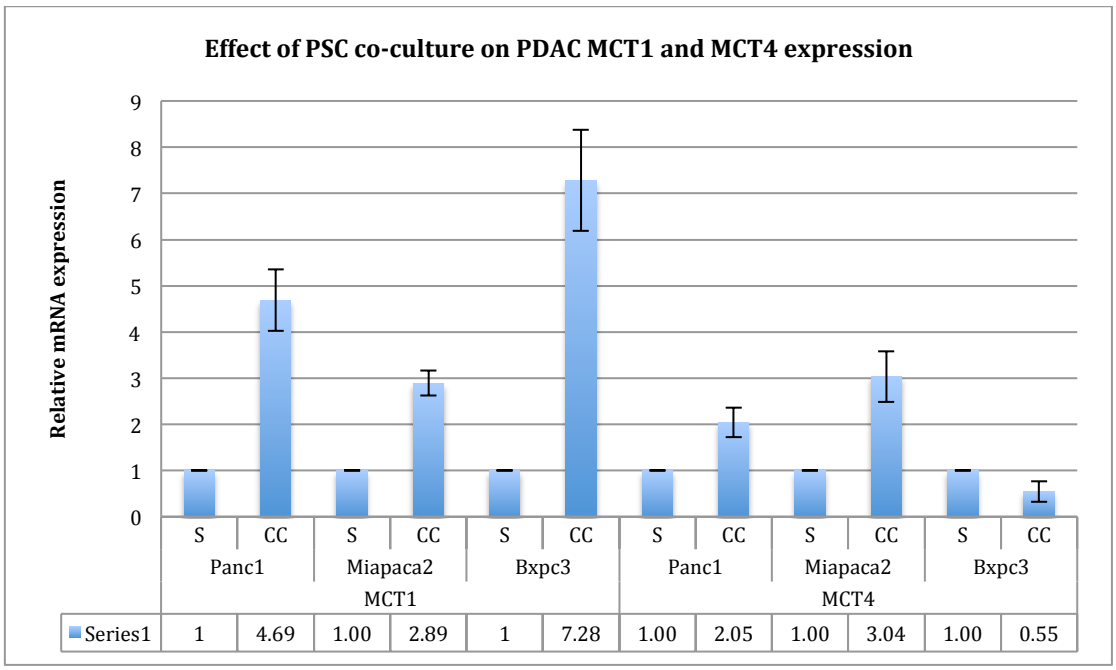


Figure 14 – Effect of PDAC-PSC co-culture on MCT1 and MCT4 gene and protein expression:
Fig 14.1 mRNA expression of MCT1 and MCT4



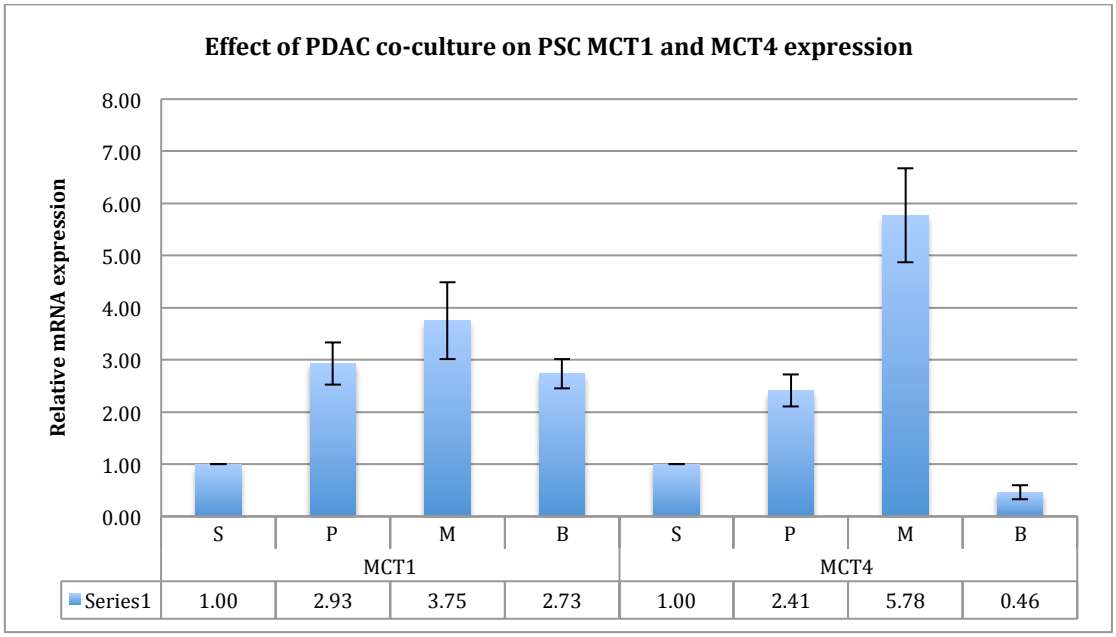



Fig 14.2 MCT1 and MCT4 protein expression of PDAC cell lines +/- PSC co-culture

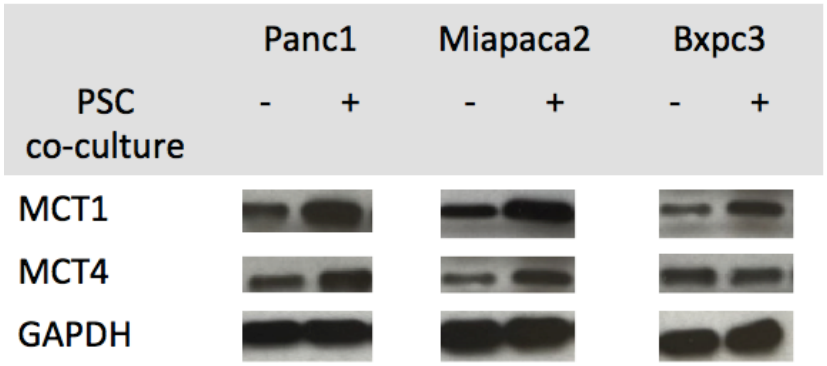



Fig 14.3 MCT1 and MCT4 protein expression of PSCs +/- PDAC co-culture

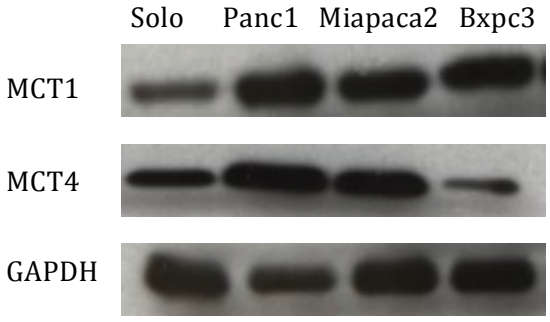
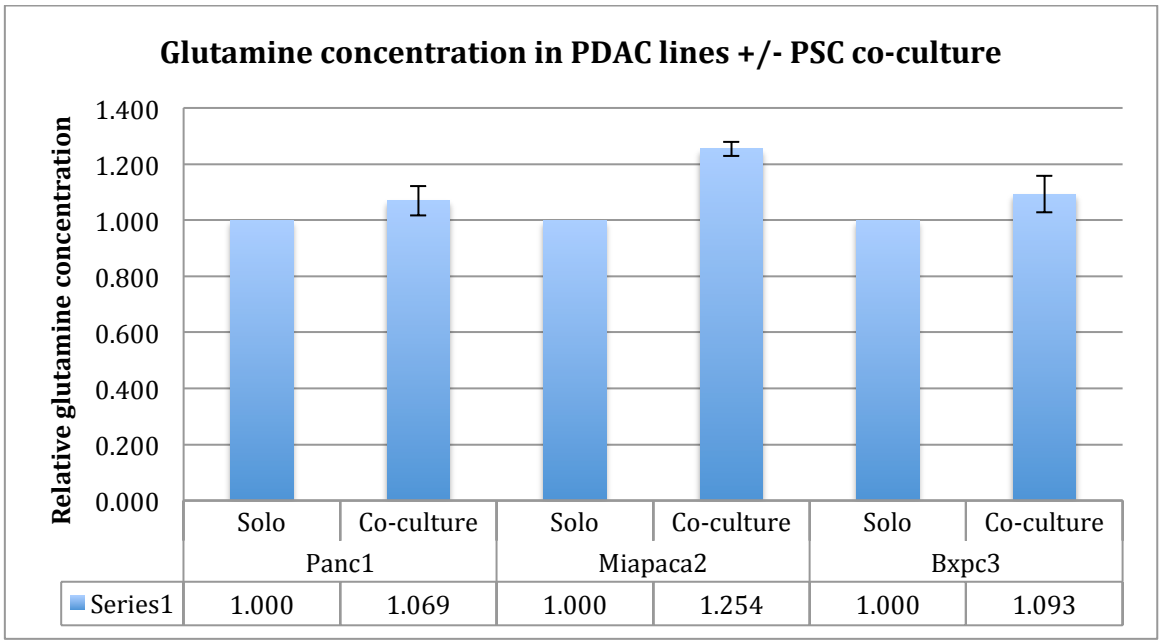



Fig 15 – The effect of PSCs on intra-cellular glutamine content of PDAC lines



## 4.3 Discussion

In summary these results have demonstrated, albeit with some inconsistency between assays, that there is some form of relationship between PDAC and PSCs which impacts on both the PDAC metabolic phenotype and lactate utilization. Baseline characteristics of metabolic phenotype were as expected, with the Panc1 and Miapaca2 lines being shown to be more glycolytic, whilst the KRAS-wild type Bxpc3 is more oxidative, which is in keeping with the fact KRAS is heavily associated with the Warburg effect glycolytic switch. Interestingly the PSCs demonstrate a similarly glycolytic phenotype to Panc1 and Miapaca2. Certainly the most significant result is the pro-glycolytic effect of PSCs, and it intriguing to see this is replicated in the Bxpc3 line as well, suggesting this mechanism of effect is not KRAS-dependent. This induction of glycolysis suggests that as well as oncogenic stimulation (by KRAS) and hypoxic conditions, PSCs may also be contributing to the Warburg phenomenon in vivo, providing tumours with a survival advantage within the harshly hypoxic environment of PDAC. Furthermore only the Miapaca2 line exhibited a converse reduction in oxidative processes, which is not the first time in this project that we start to seeing differing responses between the clinically relevant KRAS expressing Panc1 and Miapaca2 lines. As far as determining why the PDAC cell lines are behaving differently, the fact Miapaca2 more strongly expresses MCT4 at the protein level, which is heavily implicated in highly glycolytic tumours, may explain some of the opposing effects demonstrated and also makes this in intriguing cell line to use as a comparator when investigating therapeutic approaches; for example it may be able to rely on compensatory MCT4 activity when MCT1 action is inhibited. These differing observations, and associated higher MCT4 expression (in itself associated with a glycolytic phenotype) may be supported by the study by Daemen et al which stratified PDAC cell lines into distinct

metabolic categories with MIAPaCa-2 displaying a glycolytic phenotype whilst PANC-1 and BxPC-3 displayed a lipogenic phenotype with a subsequent impact on response to a variety of metabolic inhibitors [251].

Towards the completion of this project, a similar pro-glycolytic effect of CAFs was demonstrated by Zhao et al [252], and suggested the mechanism of effect is attributable to CAF-derived exosomes (CDEs) which are extracellular vesicles that can facilitate cross-talk between cancer cells and stromal cells within the tumour microenvironment by reprogramming cells resulting in regulation of proliferation and survival[253] . In vitro experiments using seahorse technology demonstrated that CDEs in both pancreatic and prostate cancer inhibited mitochondrial OXPHOS with a concurrent increase in glycolysis and glutamine-dependent reductive carboxylation in the cancer cells, supporting our theory of how CAFs support the Warburg effect. Interestingly this was not entirely in keeping with our results, whereby a concurrent reduction in oxidation was only seen with Miapaca2; nonetheless it suggests the mechanism of effect is exosome-dependent. Additionally it was shown that these CDE's contain de novo "off the shelf" metabolites (including amino acids, lipids and TCA-intermediates) which are used by the cancer cells for central carbon metabolism and thus further promote tumour growth within the nutrient deprived hypoxic environment.

The analysis of changes in MCT expression aimed to determine the overall lactate flux between the cell types, and in summary MCT1 was significantly upregulated in all PDAC cell lines and the PSCs when in co-culture, whilst a similar upregulation in MCT4 was only seen in the Panc1 and Miapaca2 lines in co-culture (as well as the PSCs), whilst a reduction in MCT4 was observed in the PSCs when in co-culture with Bxpc3. With respect to the

Panc1 and Miapaca2 lines, both of which harbor the KRAS mutation and therefore represent the most clinically relevant PDAC cell lines, we may deduce that MCT1 and MCT4 upregulation in the presence of PSCs suggests a relationship between the two cell types which relates to either the production, consumption or transfer of lactate. Conversely, with an increase in MCT1 and decrease in MCT4 in Bxpc3, we may deduce that KRAS has a key role in influencing this PDAC-PSC relationship. It is already known that KRAS is involved in the metabolic reprogramming in PDAC, with links to increased glycolysis.[182] However the fact an increase in MCT4 is only associated with the KRAS-mutant cell lines suggests some form of PDAC-PSC lactate shuttling relationship is unique to KRAS-associated tumours, and therefore MCTs may represent an effective therapeutic target in PDAC by disrupting lactate movement or usage.

It is challenging to define the exact function of each MCT with regards to whether the over-riding lactate movement is that of efflux or influx. MCT1 has been shown to bidirectionally transport lactate, with the degree of influx or efflux depending on the intracellular:extracellular substrate concentrations and the pH gradient across the cell membrane.[216] However it is generally seen that MCT1 is more prevalent in cells which oxidise lactate, whilst MCT4 is usually limited to more glycolytic cell types.[219] The only positive correlation between seahorse and MCT PCR results is the global effect of PSCs on all PDAC cell lines to cause an increase in glycolysis and increase in MCT1, thus suggesting that the over-riding function of MCT1 in this context is to export the lactate produced by excessive glycolysis, thus resulting in the increase in ECAR observed. As discussed it has most recently been considered that due to the fact MCT4 defined an aggressive glycolytic phenotype of PDAC[254], MCT4 would be a more ideal target for therapy, however this was assuming that MCT1 was chiefly involved in lactate influx. Therefore our results may

suggest that inhibiting MCT1 may have a more significant therapeutic effect in inhibiting the pro-glycolytic effect of PSCs (with resultant survival benefit in vivo) and reducing the pro-tumourigenic acidification effect of lactate pooling within the tumour microenvironment. However this would only be relevant in approximately 95% of tumours with KRAS activation. Therapeutic approaches to MCTs/glycolysis in the latter part of this project will further assist in delineating the primary role of the MCTs in lactate movement.

The experiments examining lactate concentrations, both intra-cellular and within the media (mimicking extra-cellular levels), were initially performed as a simple analysis of effect of PSCs, and I believe the seahorse experiments to be far more accurate in providing a reliable picture of how the PSCs are impacting on PDAC metabolic parameters. Nonetheless these assays imparted some useful information, firstly that the crude treatment of the cell lines with lactate resulted in no effect on proliferation, which is in effect proof in principle that an increase in lactate availability does not simply influence cellular activity through an effect on metabolism, such as an increase in oxidation with associated increases in ATP yield. Admittedly the results of the lactate assays are not entirely in keeping with the seahorse experiments, however once again it is interesting to see differing effects between the cell lines, whereby only the Miapaca2 demonstrated a significant reduction in intra-cellular lactate in the presence of PSCs, with a concomitant reduction in media/extra-cellular content, overall suggesting the underlying metabolic phenotype of Miapaca2, as opposed to the Panc1 and Bxpc3 lines, results in altered utilization of lactate. This concept is supported by our findings of preponderance for MCT4 in Miapaca2, along with the previously quoted studies proving differing metabolic phenotypes between these specific cell lines. [251, 255]

With respect to hypoxia, in view of the timing/technicalities of performing the seahorse experiments, whereby the assays needed to be exposed to the reagents over-night prior to analysis, and the fact the institutional building I was performing the seahorse experiments in did not have a hypoxic incubator, unfortunately I could not replicate these experiments in entirely hypoxic conditions. Furthermore I did not replicate the preliminary assays in hypoxic conditions, which would have added some useful information as to how each cell type is more likely to behave in vivo. Nonetheless at a later date when investigating the effect of therapeutic intervention I planned to replicate some experiments in hypoxic conditions.

Ultimately the underlying question is how do these results translate in vivo, and this is inherently an extremely difficult one to answer; nonetheless a number of theories regarding lactate shuttling have been postulated and will now be considered. In normal tissue, a cell-to-cell lactate shuttle system exists in the form of the Cori cycle, whereby lactate is taken up from the bloodstream to undergo gluconeogenesis in the liver. This concept was elaborated upon to include differing shuttling theories according to tissue type. For example, during exercise muscle cells require higher amounts of energy on the background of reduced oxygen delivery, resulting in a switch from OXPHOS to glycolysis, with the exported lactate being transported to the liver to undergo the Cori cycle.[256] However a large majority of lactate produced by myocytes (55-75%) is actually recycled by the neighbouring muscle and oxidized for energy production.[257] This presents an interesting concept in relation to cancer metabolism, whereby cells demonstrate a similar ability to utilise lactate in a heterogeneous way for more efficient energy production and survival advantage in response to a hypoxic microenvironment. Furthermore it presents a

conundrum regarding the most appropriate therapeutic approach towards MCTs, which as mentioned can bi-directionally transport lactate.

The first of these lactate shuttling concepts relates to a form of metabolic symbiosis, whereby cancer cells within the hypoxic central region of the tumour are observed to be more glycolytic, exporting lactate to cells on the periphery of the tumour which are less hypoxic and therefore can utilise this lactate for ATP production via oxidative processes. This shuttling is also thought to facilitate glucose delivery from the local vasculature to the central tumour.[219] This concept is supported by the fact MCT1 expression correlates with hypoxic regions of mouse xenografts of lung and colorectal tumours. However this study used CHC as the MCT1 inhibitor, which is not strictly specific for MCT1 and has other off target effects. However if we assume CHC is predominantly inhibiting MCT1, this theory adds support to MCT1's key role is in allowing cancer cells to take up lactate for oxidative purposes and fuel their growth. Admittedly this theory is impossible to prove in an in vitro setting in this project, and as proven thus far I suspect MCT1 inhibition may have more of an effect on disrupting the PDAC-PSC glycolytic relationship. Nonetheless, this symbiotic theory suggests the mechanism of MCT1 inhibition may be also be starving hypoxic tumours of fuel by disrupting lactate shuttling from the oxygenated periphery. IHC analysis in the following chapter will investigate this theory further as to whether there is any correlation between MCT and HIF1a expression.

A second theory relates to the interactions of tumour cells with vascular endothelial cells, where instead of acting as a metabolite, lactate functions as a signaling molecule by inducing endothelial cell migration and angiogenesis. In this concept hypoxic tumour cells export glycolytically produced lactate through MCT4, which is then imported by MCT1 on

the endothelial cells, converted to pyruvate, resulting in HIF1a stabilization and activation of NFKB/IL8 signaling.[258] However contradictory evidence exists for this concept, whereby MCT1 expression is not seen in the tumour-associated vasculature of colorectal, cervical or breast cancers [259], suggesting this form of shuttling may not be relevant in humans. Furthermore, albeit without examining endothelial cells, my results are suggesting lactate is moving bi-directionally through the MCTs. Again in vivo experimentation would be more appropriate to investigate this theory further, albeit the IHC correlation with hypoxia could provide an insight into this concept.

The final lactate shuttling theory described in the literature has been termed the "reverse Warburg effect"(RWE) and describes a metabolic coupling relationship between cancer cells and CAFs. This model proposes that by secreting hydrogen peroxide, and thereby creating a pseudo-hypoxic stromal environment, cancer cells stimulate HIF1a activation, aerobic glycolysis and MCT4 upregulation in neighbouring CAFs, thus leading to excessive lactate production which is exported out the cell by MCT4 and hijacked by adjacent cancer cells which import via MCT1 and utilise the lactate for oxidative metabolism.[248, 249] The RWE has thus far only been validated in breast cancer in vitro co-culture systems, and further supported by IHC analysis revealing CAFs have increased expression of MCT4, whilst breast cancer cells have higher expression of MCT1 and markers of mitochondrial mass.[250] Furthermore in vitro experiments revealed lactate treatment increases tumour growth and migration, whilst it was observed that lactate induced gene signatures was associated with poor prognosis in breast cancer patients. [247] Given the latter finding, it has been suggested that given the heterogeneity of transcription profiling, it may be that only selected patients would benefit from anti-metabolic therapy, and specifically

therapeutics targeting glycolysis or lactate accumulation/shuttling, in a form of personalized medicine based on metabolo-genomic profiling.

However this concept has only been rigorously investigated in breast cancer, and cannot be universally applied to other tumour types, especially when it was also observed that breast cancer cells rarely express MCT4 which defined a glycolytic phenotype. Therefore despite the CAF-rich stroma, before my experimentation I theorized that the RWE might not apply to PDAC, which can be significantly glycolytic given the hypoxic environment and high MCT4 expression observed. [224] And indeed results thus far go against this theory on an in vitro level, particularly with the pro-glycolytic effect of PSCs on PDAC, with no concurrent increase in oxidation in the PSCs. Furthermore additional crude experimentation examining the effect of hydrogen peroxide on PSCs (which the authors of the RWE theorized was the catalyst for the metabolic switch they observed) revealed no effect on lactate metabolism or proliferation, which again goes against the RWE theory in PDAC. Nonetheless, the RWE has again suggested a key role of CAFs in the metabolic reprogramming of cancer, and emphasizes the need to better understand their role in metabolism, and determine whether particular anti-metabolism therapies may incur survival benefit, either through the targeting of specific cell metabolism (cancer cells or CAFs) or the associated metabolites (e.g. lactate).

An alternative relationship between CAFs and lactate metabolism has been investigated whereby it had been shown that exposure to exogenous lactate induces fibroblast proliferation and ROS production during wound healing.[260] As discussed previously this therefore may be of particular relevance to PDAC, where repeated injury to the pancreas results in sustained ECM disorganization and damage, marshaled by PSCs, resulting in an

increased risk of cancer development. However lactate also functions on other aspects of the stroma, stimulating VEGF and promoting a pro-inflammatory environment through increased cytokine and growth factor production, thus assisting in angiogenesis and collagen synthesis that again can promote a more aggressive tumour with increased chemoresistance.[261] Crude analysis of the effect of lactate on PDAC and PSCs did not suggest any simple effect on proliferation, and as discussed this in vitro investigatory methodology may be limiting the ability to give a true picture of lactate flux, with in vivo analysis ultimately providing a more complete picture whilst accounting for other stromal elements such as endothelial cells.

Lastly I additionally examined the effect of PSCs on glutamine metabolism. As previously discussed, cancer cells are addicted to glutamine suggesting this as a viable treatment target, however its metabolism in PDAC is atypical and the importance of glutamine metabolism in the tumour microenvironment is yet to be conclusively established with relevance to interactions between cancer cells and their associated stromal cells, including fibroblasts, immune and endothelial cells. Due to differences in oxygen tension, in conjunction with altered transcriptional and post translational control mechanisms of glutamine metabolism, the expression and activity of glutamine-associated proteins is expected to differ from tumoural expression.[262] It has been shown that cancer associated fibroblasts (CAFs) have lower levels of c-Myc (which regulates Gln transporter expression) as compared to normal stroma.[263] This results in reprogramming of Gln metabolism in CAFs, which in turn increases IL-6 levels, driving the protumourigenic microenvironment through proinflammatory (increased IL-6) and pro-oxidative (increased ROS) mechanisms [264]; these findings are certainly in keeping with the validatory experiments performed on the trans-well set up used in this project. The altered Gln

metabolism in CAFs has significant implications for therapeutic approaches towards these pathways in cancer. For example, the chemotherapy agent Paclitaxel, which has shown efficacy in PDAC, has been shown to trigger endoplasmic reticulum (ER) stress, resulting in Gln transporter degradation with subsequent mTOR inactivation and apoptosis in breast cancer cells.[205] However, a similar effect of Paclitaxel on CAFs has the potential to push cancer cells towards a more aggressive phenotype.[265] Therefore, whilst Gln-targeted therapy is an attractive proposition in Gln-addicted PDAC tumours, it is essential to consider the role of stromal components such as PSCs, which is relatively under investigated in PDAC, and thus determine the most appropriate therapeutic approach towards this regulatory axis in order to disrupt glutamine associated metabolic reprogramming in PDAC.

Results thus far demonstrated how PSCs induce a significant increase in glutamine concentration in the Miapaca2 line, however not in Panc1 or Bxpc3. This result again suggests heterogeneity of response to PSC interactions depending on the PDAC phenotype, and suggests in the case of a more glycolytic tumour (i.e. MCT4-heavy such as Miapaca2) PSCs exert a more significant metabolic reprogramming effect which confers a survival advantage in vivo.

**Summary of key points**

- The transwell *in vitro* system mimics the PDAC-PSC relationship *in vivo*, and provides further information on individual cell types, and was therefore most appropriate and suitable for the ongoing methodology in this project
- PSCs induce a KRAS-independent pro-glycolytic effect on PDAC *in vitro,* with associated MCT1 and MCT4 upregulation

- PSC interactions with the PDAC lines is influenced by the underlying genetic/metabolic phenotype of each cell line, with the more glycolytic MCT4-heavy Miapaca2 distinguishing itself from Panc1, and KRAS-wild type Bxpc3 behaving inherently differently to Miapaca2 and Panc1

# Chapter 5
# Expression patterns and prognostic relevance of metabolic markers in PDAC

## 5.1 Background

Results thus far have concentrated on the in vitro metabolic relationship between PDAC and PSCs, and this has uncovered a novel pro-glycolytic influence of PSCs with associated MCT1 and MCT4 upregulation. However prior to the commencement of this project, there were no published studies examining the immunohistochemical staining patterns of MCTs in resected PDAC specimens, and whether this had any prognostic relevance. Therefore prior to performing this staining I had a number of theories as to what to expect. Certainly MCT4 was anticipated to feature heavily in the tumour with it's known tendency towards glycolytic metabolism with respect to the Warburg Effect. However the theory of the RWE suggested potentially MCT1 would be equally prevalent in the tumour, and conversely MCT4 would feature heavily within the CAF-rich stroma.

As *in vitro* results unfolded, it became clear that I should expect MCT1 to be heavily expressed in both tumour and stroma, whilst MCT4 should be equally as prevalent in the tumour given the clinically relevant KRAS-lines demonstrated upregulation in the presence of PSCs. What would be of particular interest is the correlation between tumoural and stromal MCT1:MCT4 expression, and whether this made any suggestion as to the over-riding function of each transporter, as well as giving a clue as to overall lactate flux. Ultimately however the prognostic relevance of both MCT1 and MCT4 was as yet unproven, and I was keen to investigate if either was linked to reduced OS given it's association with a

pro-tumourigenic metabolically reprogrammed environment of over-acidified lactate pooling. Additionally MCT2 would be worthwhile examining with regards to its association with MCT1, and whether it predicted survival and therefore represented a potential therapeutic target.

As previously mentioned Baek et al published their study in 2014 demonstrating how high tumoural and stromal MCT4 expression defined a glycolytic subtype and predicted poor prognosis[254]. However they did not identify any prognostic relevance of tumoural MCT1 expression, and more interestingly did not see any significant MCT1 expression in the stroma. Albeit in a larger patient cohort (n=223), I was interested to compare my results, as the lack of stromal MCT1 expression was certainly not in keeping with my in vitro results.

Admittedly the stroma contains an abundance of cell types, including inflammatory, vascular and neural components [6], and all/some of these may be expressing MCTs in different patterns of intensity. Therefore I aimed to account for PSCs by staining for the activated marker αSMA, and account for the degree of disorganized vasculature by staining for the hypoxic marker HIF1a. These could then be correlated with stromal MCT expression in an effort to determine whether any specific cell type may be expressing more/less strongly. From a clinically translational perspective, when considering trialing a drug that inhibits MCT1/MCT4, ultimately it may be irrelevant as to which stromal cell type expresses MCT more so than another. Nonetheless it could impart useful information upon the in vitro experiments thus far as to what the over-riding MCT expression pattern of PSCs is in vivo, and whether this correlates with a metabolic phenotype which influences tumour aggressiveness and therefore patient prognosis.

An alternative form of metabolic attack is through the targeting of glycolysis via glycolytic enzyme inhibition. Whilst a number of other enzymes represent viable targets as previously discussed (HK2, PFK-1, PKM2, PDK), I was particularly interested in approaches towards LDH, which as previously discussed catalyses the interconversion of pyruvate to lactate, and has strong associations with tumour initiation and progression. LDH exists in 2 subunits forms, LDHA and LDHB, with LDHA having a higher affinity for pyruvate, and therefore associated with the catabolism of pyruvate to lactate, thus being found more commonly in highly glycolytic tissues such as skeletal muscle.[201] The in vivo effect of LDHA inhibition in preventing PDAC progression[203] highlights the potential importance of LDH in the metabolic reprogramming of PDAC, and with pan-LDH inhibitors such as Galloflavin in development, defining the expression pattern of LDH in PDAC also appeared to be an attractive investigatory route.

LDHA is transcriptionally regulated by HIF1a [202]; therefore it should in theory have a profound association with PDAC, however at the commencement of this project this was as yet unproven. In contrast the role of LDHB in cancer is more complex and varied. This allows cells to use lactate as a source for oxidative metabolism through conversion to pyruvate, and whilst it is silenced in prostate cancer[266], it is over-expressed in KRAS mutated human lung adenocarcinoma.[267] Again it's expression patterns in PDAC have not been reported upon in the literature, and would be investigated in this staining analysis. Results will both determine the prognostic relevance, and may also mechanistically determine how lactate is being utilised in the tumour microenvironment of PDAC, as if lactate is more commonly oxidated in cancer cells one would expect to see more prevalent LDHB expression, and conversely stronger LDHA expression in the PSC-rich stroma.

Therefore overall this staining analysis would both aim to investigate the mechanism of lactate shuttling between the tumour and stroma compartments, and also determine the most appropriate and effective therapeutic approach to MCTs, whereby inhibition may prove beneficial through either reducing protumourigenic acidification, or removing the source of lactate as an oxidative fuel.[216, 217]

## 5.2 Methodology

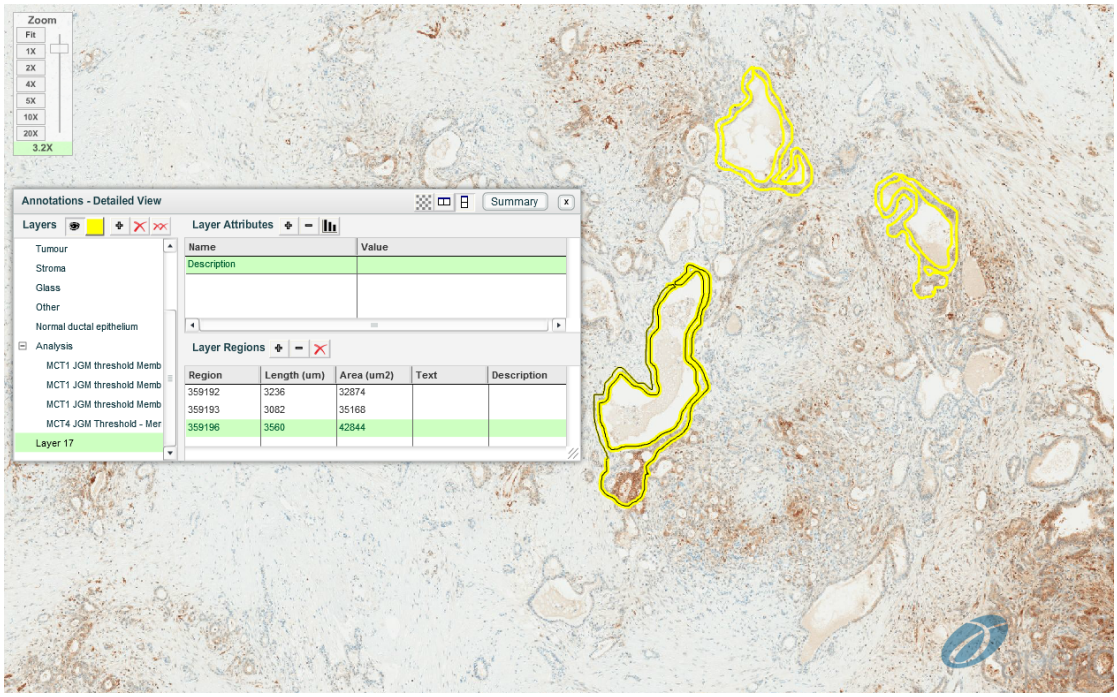
Once the relevant antibodies had been optimised as described in the relevant Materials and Methods chapter (2.8), the Ventana Autostainer (Roche, Tuscon, USA) was used to more efficiently stain multiple tumour sections with the numerous antibodies. This approach also ensured optimal staining consistency for more reliable results. In brief, this system involves staining dispensers positioned beneath a rotating plunger. The reagents are then applied to the slides within a 30-pad carousel drawer via the plunger, whilst rinsing fluids are sprayed onto the slides. The stainer also deparaffinises the slides using heat and detergents. Appropriate protocols are applied to deliver antibodies at the relevant concentrations.

The Aperio ScanScope (Aperio Technologies) was then utilised to perform IHC expression analysis. The stained slides were scanned onto the system at an absolute magnification of 400× [resolution of 0.25 μm/pixel (100,000 pix/in.)]. The background illumination levels were calibrated using a pre-scan procedure. The acquired digital images representing whole-tissue sections were viewed and analysed using The Spectrum Analysis algorithm package and ImageScope analysis software (Aperio Technologies, Inc.) were applied to quantify IHC staining which will now be described in more detail.

Training algorithms were generated for each individual stain, whereby the software was trained by myself to identify the compartments of interest (tumour and stroma). The system also identified other cell types that would then be excluded from analysis; this included duodenal epithelium, normal pancreatic epithelium, endothelial cells, acinar tissue, islets of Langerhans and any lumen. These specific areas of interest were highlighted

on the training package, as demonstrated in figure 16.1. The algorithm was then geared towards identifying the appropriate staining pattern according to the protein of interest – for example membranous staining for the membrane bound proteins (MCT1 and MCT4), and total staining (membranous and cytoplasmic) for αSMA and LDHB. The algorithm output determined a variety of parameters, including expression levels (high, medium, low and nil) and cell counts. These data were then collated and appropriate statistical analysis performed with respect to both correlations and patient survival.

Figure 16.1 – Demonstration of the Aperio Imagescope analysis software, with highlighting of tumour ductal epithelium (yellow)



## 5.3 Results

### 5.3.1 Patient data-set summary

As described in the chapter 3, ethics approval was granted to obtain pre-2006 sections of PDAC tumours from Whipple's procedure resections. A total of 59 patient tumour blocks were retrieved, with the histological diagnosis of PDAC confirmed from reports on the online trust database. Data was then collected on these patients with respect to general demographics, procedure details, morbidity, mortality, OS and prognostic indicators. Table 10 summarises key data on these patients and their tumours. The median age was 65 (range 34-78), with a male:female ratio of 33:26. Most patients were ASA grade 2 (53.8%), indicating patients with only mild systemic disease. Only 3 tumours were of a low grade T1 (5.1%), with the remainder being T2 or T3 (27.1% and 67.8% respectively). Most tumours had an associated positive lymph node, with a 92% N1 rate, and median percentage lymph node positivity of 8.3% (range 0-14.3%). 84.7% of patients were deceased at time of follow up, however this follow up period was prolonged due to the fact all procedures were performed prior to 2006. 30 day mortality was 5%. Median overall survival was 22 months (range 0.3-140 months). As previously discussed, these outcomes are generally in keeping with published literature, with accepted mortality rates of 3-5% [49] and median survival of 10-24 months depending on the T and N status.[6]

Table 10 – Summary of data points in the patient cohort with respect to baseline demographics, key prognostic indicators and outcomes

| **Patient/tumour characteristic** | **Statistic** |
|---|---|
| Age | Median 65 (range 34-78) |
| M:F | 33:26 |
| ASA grade 1:2:3:4 | 32.8%:53.8%:11.5%:1.9% |
| T 3:2:1 | T3 67.8%, T2 27.1%, T1 5.1% |
| N1 rate | 92% |
| Lymph node yield (total number) | Median 8.3 (range 0-14.3) |
| R1 rate | 57.6% |
| Pancreatic leak rate | 18.6% |
| 30 day mortality | 5% |
| OS | Median 22 mths (range 0.3-140) |

### 5.3.2 Expression patterns

As described in the relevant methodology section, the Aperio analysis system was used to stain serial sections of histologically confirmed PDAC resection specimens of the same patient using validated antibodies. The scoring algorithm scored expression as high, medium, low and nil. As well as simply looking at positive expression (regardless of intensity), in order to simplify the stratification of expression intensity, subsequent expression was classified as either high (combining high and medium intensity) or low (combining low and nil expression). These figures were then used to classify patients into high and low expression groups by splitting the cohort about the median expression percentage.

*αSMA*

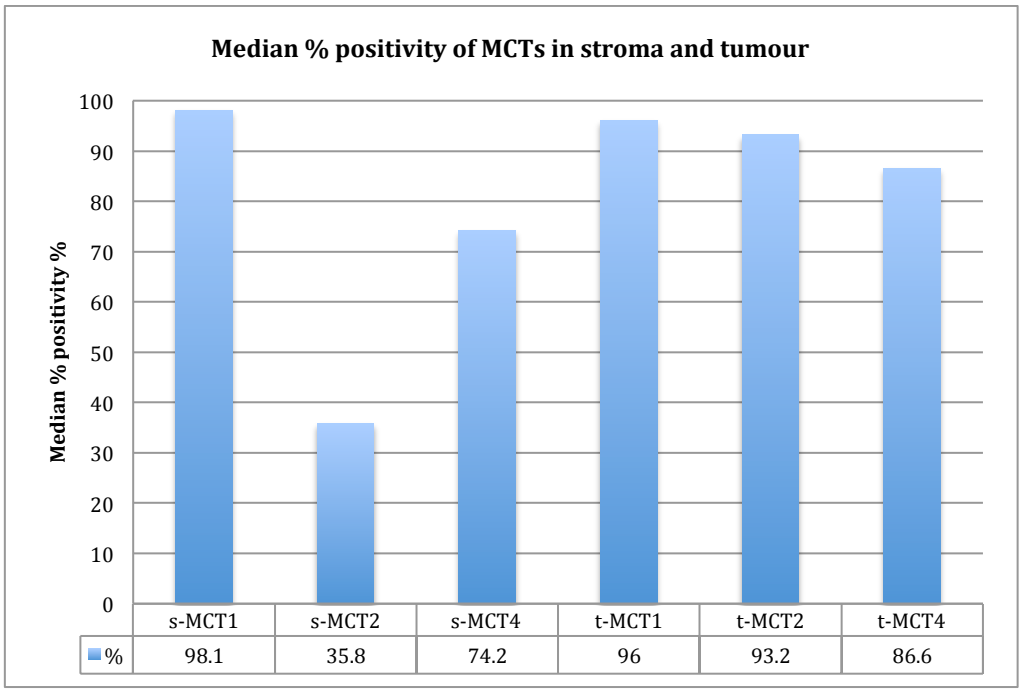
Initially it was worth demonstrating the expression pattern of αSMA in the stromal compartment. The tumour stroma contain an abundance of different cell types, and through the aperio algorithm I attempted to exclude as many of those cell types as possible to give a representative idea of fibroblast expression. Nonetheless, by collecting data on αSMA expression, one may then correlate the positivity with the extent of PSC activation within that particular tumour microenvironment, and determine whether this had any correlation with a certain pattern of MCT/LDH expression and thus potentially indicate an association of PSCs with a particular metabolic phenotype. A median area of 81.7% of stroma positively expressed αSMA (range 40.9-95.7%), whilst this was strongly expressed in 39.2% (range 8.1%-64.7%). Survival analysis indicated the expression pattern of αSMA in the stroma had no impact on patient outcomes (p=0.881).

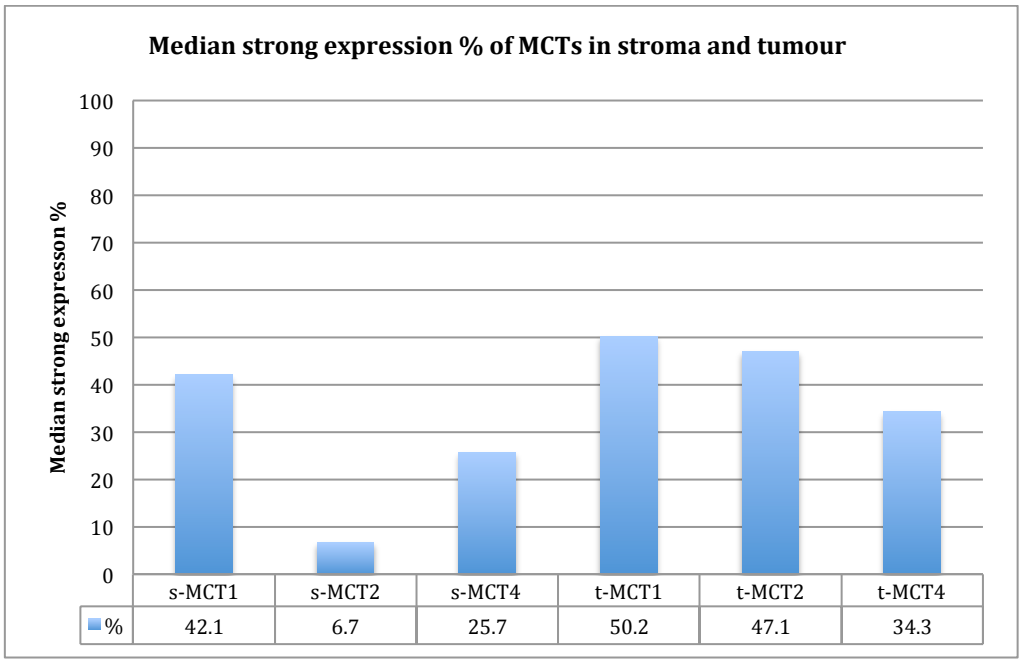
*MCTs*

The median percentage area of tumour/stroma expressing MCT1, MCT2 and MCT4 (regardless of intensity) is demonstrated in fig 16.2. MCT1 (98.1%) was expressed significantly more than both MCT2 (35.8%; $p<0.0001$) and MCT4 (74.2%; p=0.003) in the stromal compartment , whilst MCT2 was again expressed less than MCT4 (p=0.001). In the tumour all MCTs were expressed at similar levels (MCT1:MCT2:MCT4 - 96%:93.2%:86.6%; p=NS). The slices were then examined to investigate which patients expressed the MCTs at a higher intensity (defined as an expression intensity of high or medium as classified by the aperio algorithm); similarly MCT1 was more strongly expressed than MCT4 in both the stroma and tumour. And again MCT2 was expressed at a lower intensity in stroma, but similar to MCT1 intensity in tumour. One can generally see that the entire tumour

microenvironment more commonly expresses MCT1 than MCT4, whilst MCT2 is more commonly expressed in the tumour.

Figure 16.2 – Expression patterns of the different MCTs in both stroma and tumour compartments



Correlation patterns between the tumoural and stromal compartments were then investigated (table 11). This demonstrated a positive correlation of both MCT1 (p=0.002) and MCT4 (p<0.001) between tumoural and stromal compartments, however not MCT2 (p=0.673). There was a significant correlation between tumoural MCT2 and MCT4 (p<0.001), and also a correlation between tumoural MCT2 and stromal MCT4. There was also a near significant correlation (p=0.05) between MCT1 and MCT4 expression in the stroma. There was no correlation of expression of stromal αSMA or HIF1a (in tumour and stroma) with that of MCT1 or MCT4 in either stroma or tumour

Table 11 – Correlation patterns of MCTs between tumour and stroma compartments (red highlighted boxes=significant result)

| | | **Tumour** | | | **Stroma** | | |
|---|---|---|---|---|---|---|---|
| | | **MCT1** | **MCT2** | **MCT4** | **MCT1** | **MCT2** | **MCT4** |
| **Tumour** | **MCT1** | | <0.001 | p=0.953 | 0.002 | 0.871 | 0.519 |
| | **MCT2** | <0.001 | | 0.133 | 0.121 | 0.673 | 0.043 |
| | **MCT4** | 0.953 | 0.133 | | 0.180 | 0.921 | <0.001 |
| **Stroma** | **MCT1** | 0.002 | 0.121 | 0.180 | | 0.881 | 0.05 |
| | **MCT2** | 0.871 | 0.673 | 0.921 | 0.881 | | 0.759 |
| | **MCT4** | 0.519 | 0.043 | <0.001 | 0.05 | 0.759 | |

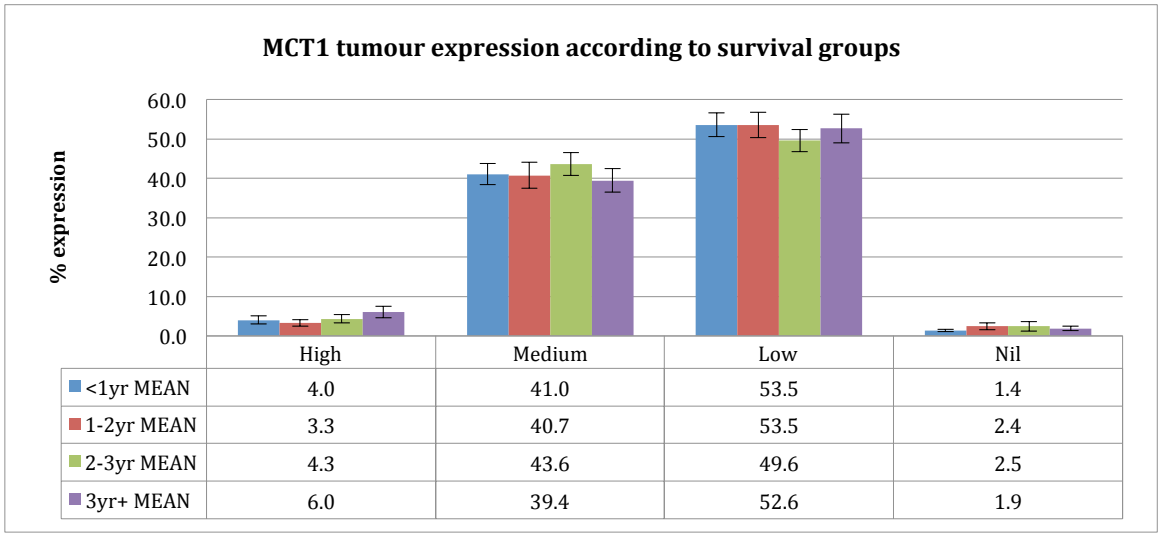
### 5.3.3 Prognostic relevance

MCTs

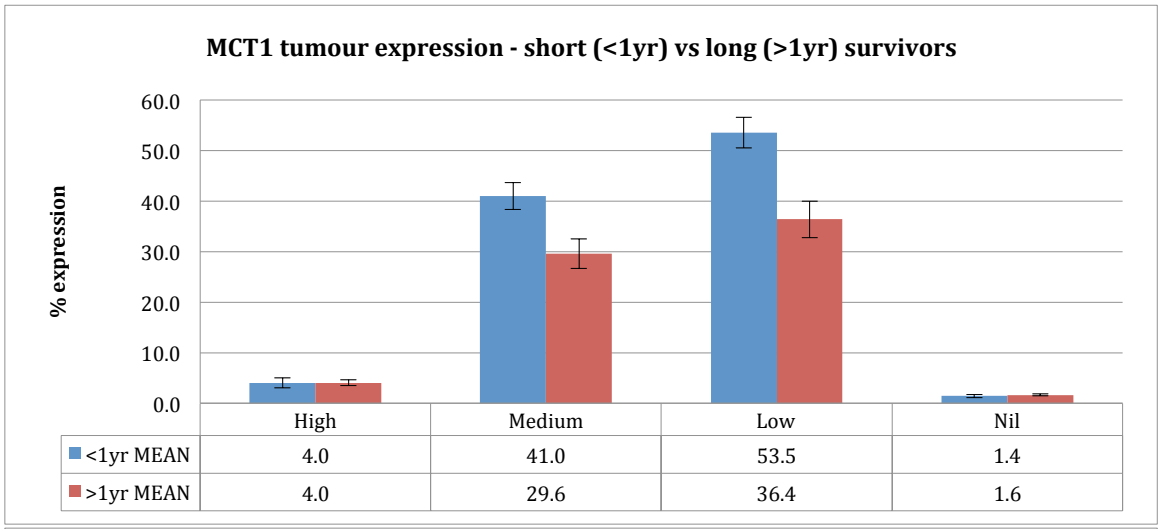
Initially I examined the impact of MCT1 and MCT4 expression on patient survival. I divided the patient cohort about the median of high expression intensity for each MCT1/4 stain in

either tumour or stroma, and performed a Kaplan Meier analysis that demonstrated no significant difference in patient survival. To interrogate further by examining different survival categories, a crude analysis was performed by splitting patients into 4 separate groups (OS <1yr, 1-2yrs, 2-3yrs and 3yrs+) and comparing the expression profile according to the intensity grading of high, medium, low and nil (fig 17); again this demonstrated no significant correlation between expression patterns and survival. As discussed previously, it has long been theorized that certain patients are destined to do better than others as they harbor a less aggressive tumour phenotype, meaning a lot of research is centred around these so-called long-term survivors (i.e. the 25% of patients who survive >2yrs). Therefore I again split the cohort into short and long term survivors (<1yr and >1yr respectively), and a similar finding was demonstrated, with no significant difference between expression profiles.

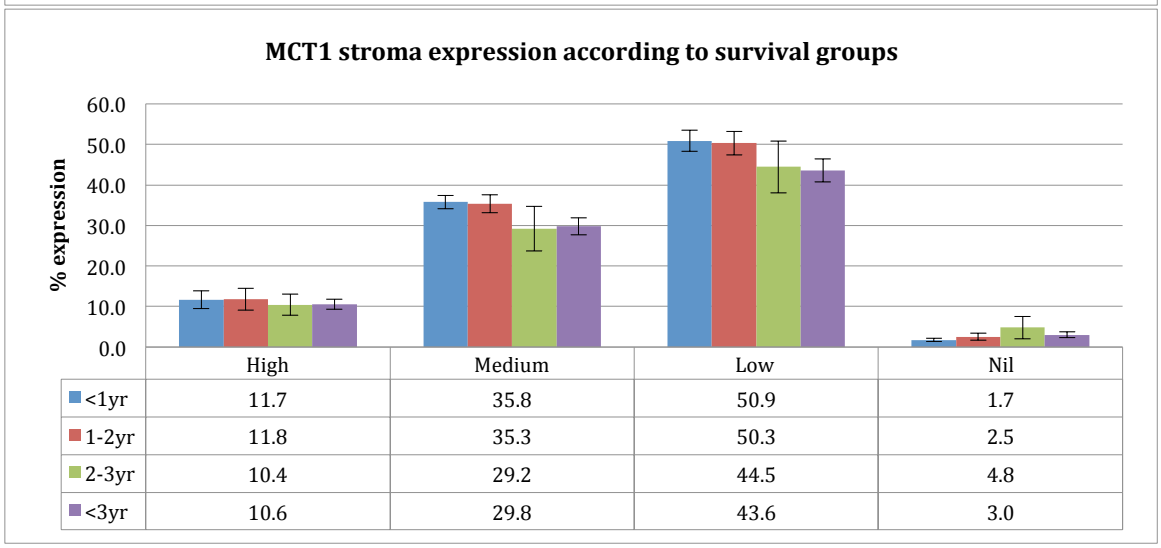
Fig 17 – MCT expression profiles according to different survival groups



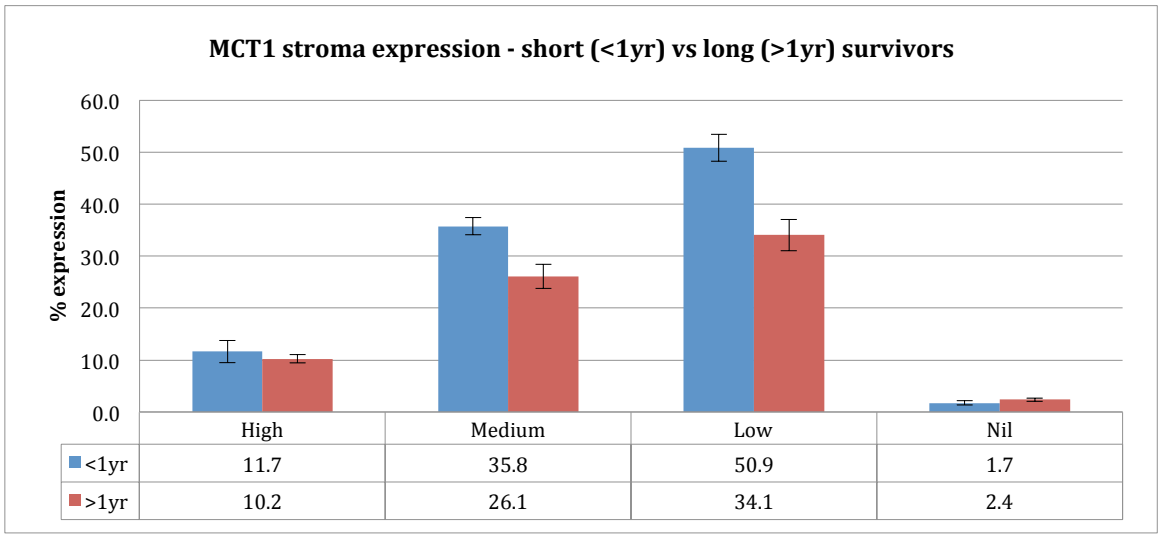

| | High | Medium | Low | Nil |
|---|---|---|---|---|
| <1yr MEAN | 4.0 | 41.0 | 53.5 | 1.4 |
| 1-2yr MEAN | 3.3 | 40.7 | 53.5 | 2.4 |
| 2-3yr MEAN | 4.3 | 43.6 | 49.6 | 2.5 |
| 3yr+ MEAN | 6.0 | 39.4 | 52.6 | 1.9 |



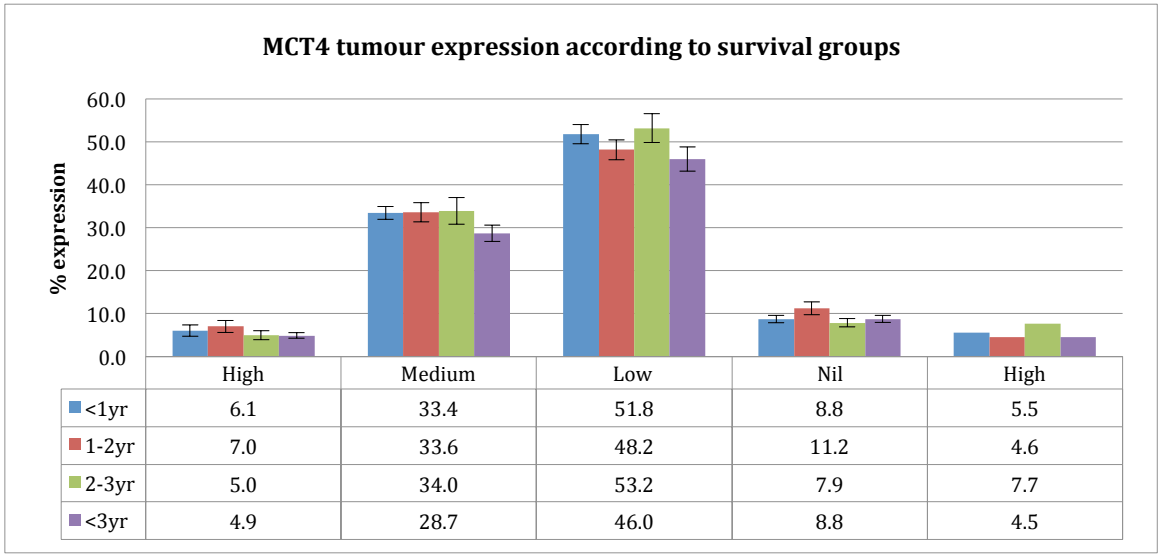

| | High | Medium | Low | Nil |
|---|---|---|---|---|
| <1yr MEAN | 4.0 | 41.0 | 53.5 | 1.4 |
| >1yr MEAN | 4.0 | 29.6 | 36.4 | 1.6 |



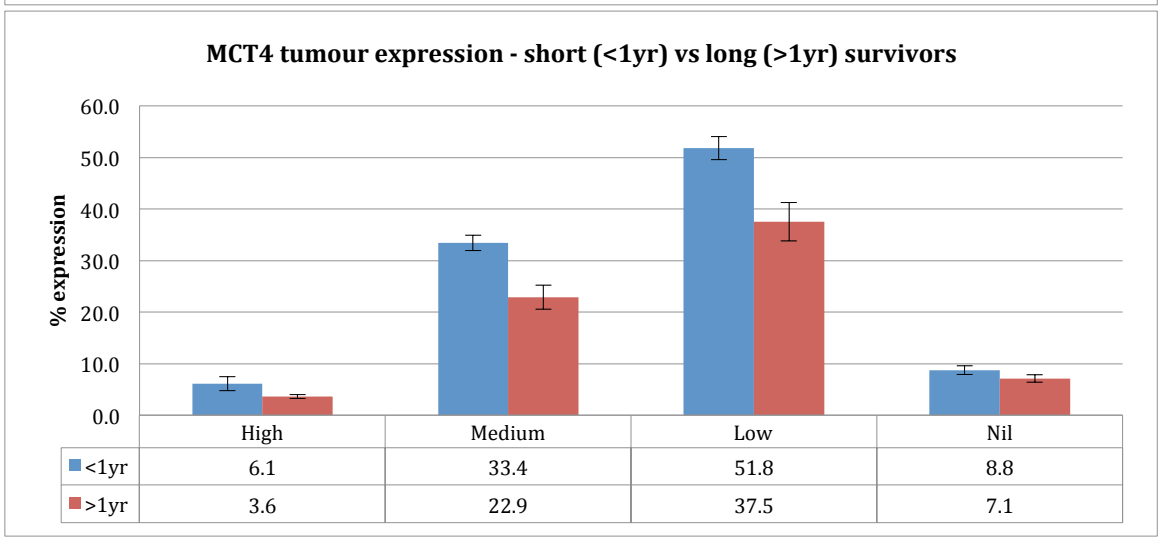

| | High | Medium | Low | Nil |
|---|---|---|---|---|
| <1yr | 11.7 | 35.8 | 50.9 | 1.7 |
| 1-2yr | 11.8 | 35.3 | 50.3 | 2.5 |
| 2-3yr | 10.4 | 29.2 | 44.5 | 4.8 |
| <3yr | 10.6 | 29.8 | 43.6 | 3.0 |



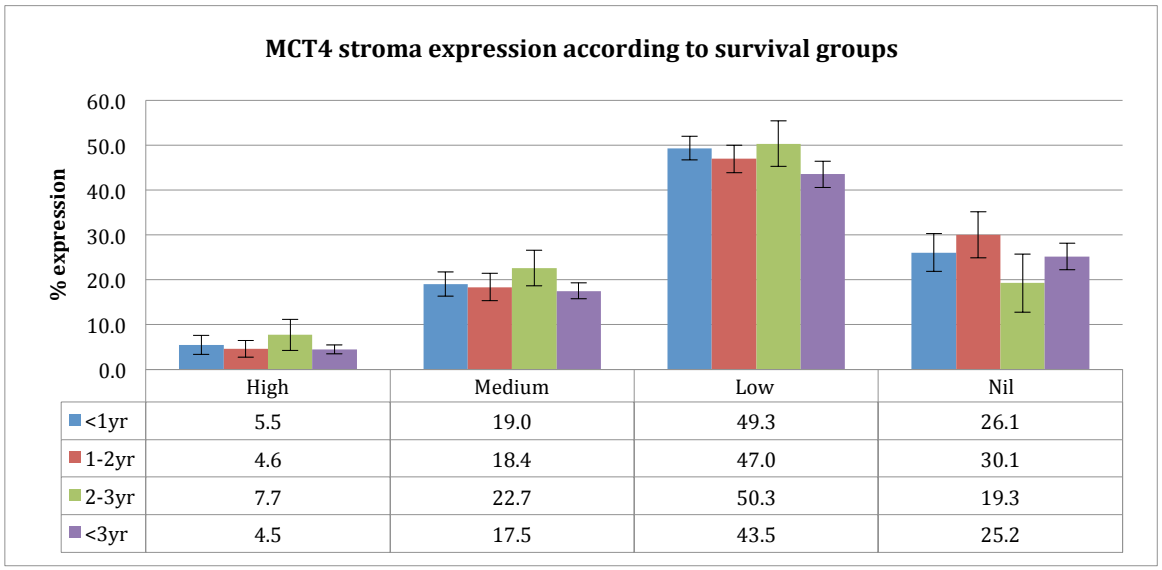

| | High | Medium | Low | Nil |
|---|---|---|---|---|
| <1yr | 11.7 | 35.8 | 50.9 | 1.7 |
| >1yr | 10.2 | 26.1 | 34.1 | 2.4 |



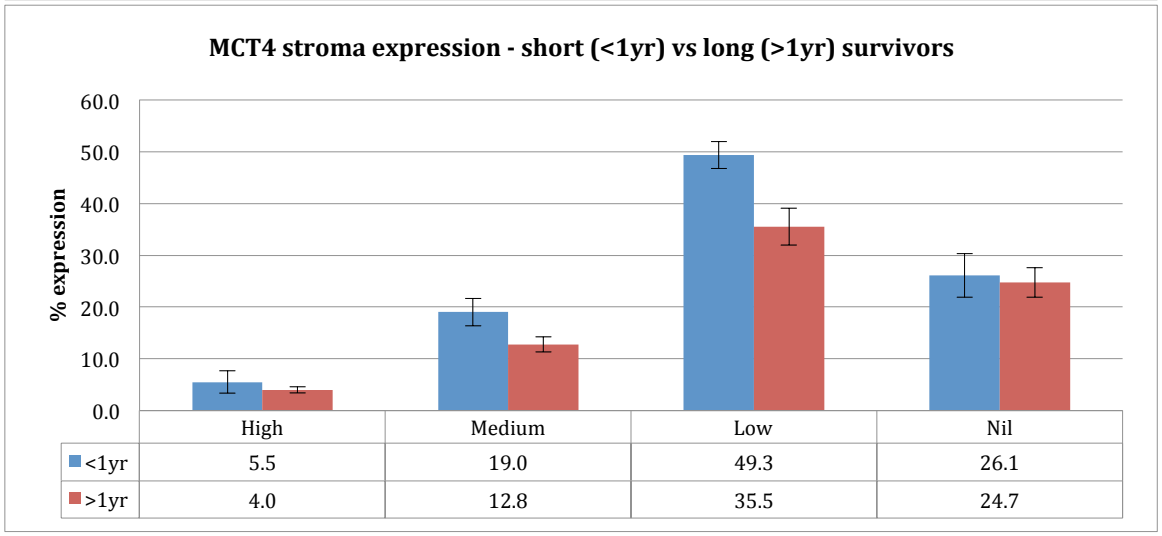

| | High | Medium | Low | Nil | High |
|---|---|---|---|---|---|
| <1yr | 6.1 | 33.4 | 51.8 | 8.8 | 5.5 |
| 1-2yr | 7.0 | 33.6 | 48.2 | 11.2 | 4.6 |
| 2-3yr | 5.0 | 34.0 | 53.2 | 7.9 | 7.7 |
| <3yr | 4.9 | 28.7 | 46.0 | 8.8 | 4.5 |




| | High | Medium | Low | Nil |
|---|---|---|---|---|
| <1yr | 6.1 | 33.4 | 51.8 | 8.8 |
| >1yr | 3.6 | 22.9 | 37.5 | 7.1 |




| | High | Medium | Low | Nil |
|---|---|---|---|---|
| <1yr | 5.5 | 19.0 | 49.3 | 26.1 |
| 1-2yr | 4.6 | 18.4 | 47.0 | 30.1 |
| 2-3yr | 7.7 | 22.7 | 50.3 | 19.3 |
| <3yr | 4.5 | 17.5 | 43.5 | 25.2 |




| | High | Medium | Low | Nil |
|---|---|---|---|---|
| <1yr | 5.5 | 19.0 | 49.3 | 26.1 |
| >1yr | 4.0 | 12.8 | 35.5 | 24.7 |

Despite these findings, it was clear from the initial expression pattern that a proportion of tumours exhibited significantly less expression of MCT1 or MCT4 as opposed to others. Therefore I divided the patient cohort into tertiles according to MCT expression, combining the upper 2 into a "high" expression group, leaving the bottom tertile as a "low" expression group, and herein a survival benefit was demonstrated (fig 18.1), whereby patients with high MCT1 expression in the stroma had a poorer prognosis as compared to the low expression group (31 months vs 55.5 months; p=0.048). This result was upheld in ANOVA analysis when accounting to key prognostic factors (fig 18.2). A similar finding was revealed with respect to tumoural MCT4, where high expression led to reduced OS as compared to low expression (27.8 months vs 57.8 months respectively; p=0.018). In this case ANOVA analysis revealed the high t-MCT4 group had a significantly higher T grading than the low expression group (mean 2.8 vs 2.25; $p<0.01$) which in turn would impart a reduced OS in that group; however given the difference in OS between T2N0 and T3N0 tumours is around 5 months (with a negligible difference between T2N1 and T3N1) [6], the 30 months difference in survival between the two groups is still a notable finding. With regards to tumoural MCT1 and stromal MCT4, as demonstrated in figure 18.1 there was a trend towards reduced OS with high expression, however this did not reach significance (p=0.133 and p=0.082 respectively). Fig 18.3 displays examples of samples with contrasting t-MCT4 and s-MCT1 expression with associated OS consistent with the survival analysis findings.

Fig 18.1 – The prognostic relevance of MCT1 and MCT4 in tumour and stroma – high stromal MCT1 and high tumoural MCT4 are associated with a significant reduction in OS

| | **MCT1** | | | | | **MCT4** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Low | | High | | P value | Low | | High | | P value |
| | n | OS | n | OS | | n | OS | n | OS | |
| **Tumour** | 19 | 47.7 | 40 | 33.5 | 0.133 | 20 | 57.8 | 39 | 27.8 | 0.018 |
| **Stroma** | 17 | 55.5 | 42 | 31 | 0.048 | 28 | 47.5 | 31 | 29.3 | 0.082 |

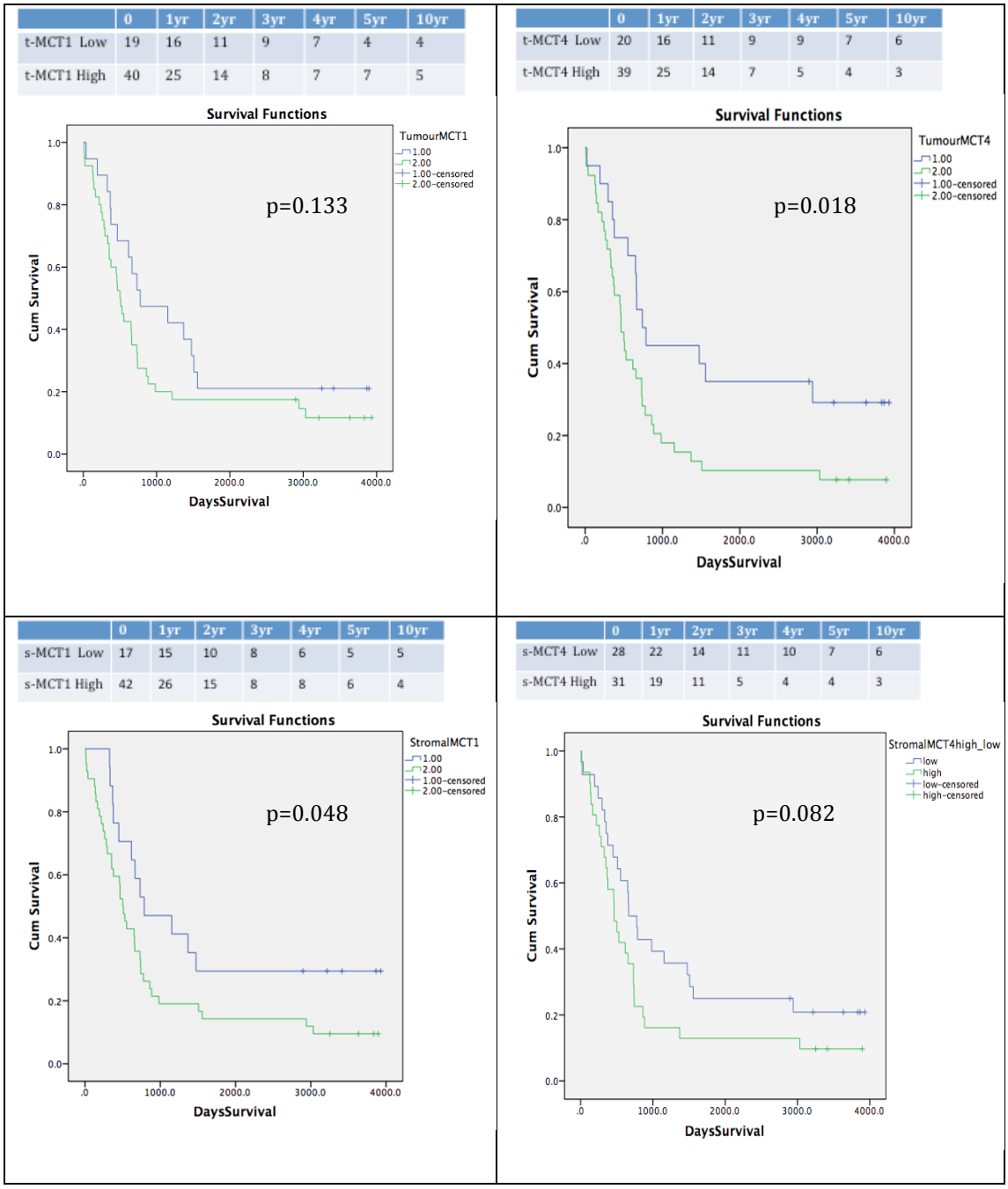
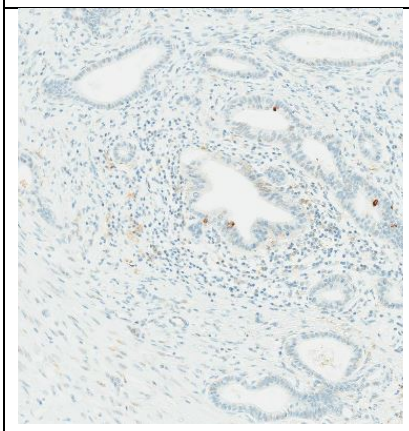
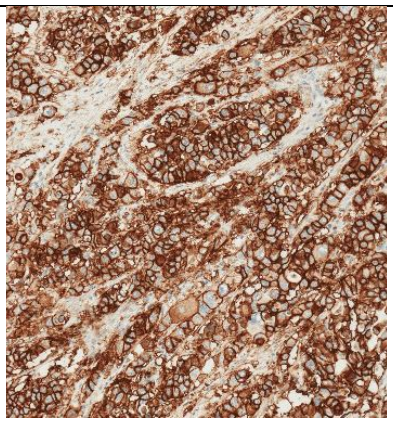
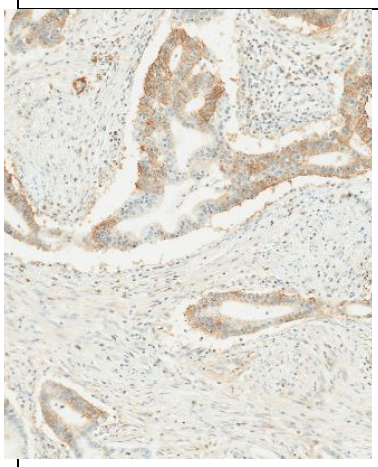
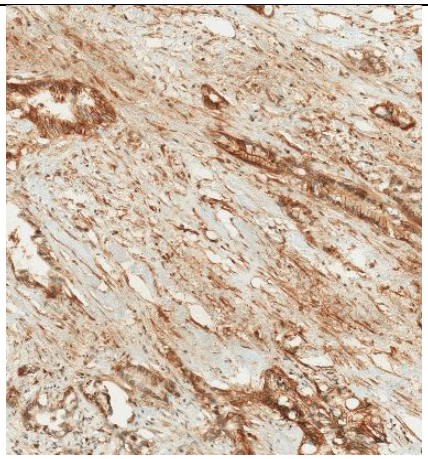


Fig 18.2 – Comparison of key prognostic indicators between the two significant survival outcome groups

| **ANOVA** | **t-MCT4** | | | **s-MCT1** | | |
|---|---|---|---|---|---|---|
| | **Low** | **High** | **p value** | **Low** | **High** | **p value** |
| **Age** | 65.2 | 63.4 | 0.598 | 63.9 | 64.5 | 0.835 |
| **ASA** | 1.89 | 1.80 | 0.697 | 1.8 | 1.8 | 0.911 |
| **T status** | 2.25 | 2.82 | <0.01 | 2.5 | 2.7 | 0.419 |
| **N status** | 0.85 | 0.95 | 0.204 | 0.82 | 0.95 | 0.111 |
| **LN% positivity** | 7.92 | 7.18 | 0.428 | 8.5 | 7.0 | 0.134 |
| **R1** | 0.55 | 0.59 | 0.775 | .47 | .62 | 0.304 |
| **Pancreatic leak** | 0.25 | 0.15 | 0.378 | .12 | .21 | 0.397 |

Fig 18.3 – IHC captions demonstrating the difference in survival according to intensity of MCT expression

| **Low t-MCT4 (2.8%); OS – 23.8mths** | **High t-MCT4 (70.2%); OS – 1mth** |
|---|---|
|  |  |
| **Low t-MCT1 (6.4%); OS – 122mths** | **High t-MCT1 (70.2%); OS – 16.3mths** |
|  |  |

LDH

Due to my initial interest in the relevance of the glycolytic enzyme LDH in PDAC, I had hoped to investigate the expression patterns of both the LDHA and LDHB isoforms. However I encountered significant issues during the work up of the LDHA staining. Despite using 3 different antibodies with a variety of optimisation techniques, I was unable to achieve specific expression without significant background staining. Nonetheless I still wished to determine the expression patterns and prognostic relevance of LDHB. Herein results demonstrated that positive staining for LDHB was detected in the tumour epithelium in 43% of cases and within the stromal compartment in 27% of cases. The relative high and low expression levels was 11.7% vs 88.3% in tumour, and 3.4% vs 96.6% in stroma, indicating when LDHB was present it tended to be a weak rather than strong stain. Analysis using the Aperio system revealed a positive correlation between tumour and stromal LDHB expression (Spearman correlation coefficient 0.593, $p<0.001$; Fig 19B). There was no significant correlation between tumour and stroma LDHB expression with MCT1, MCT4 and αSMA expression in respective compartments (Table 12).

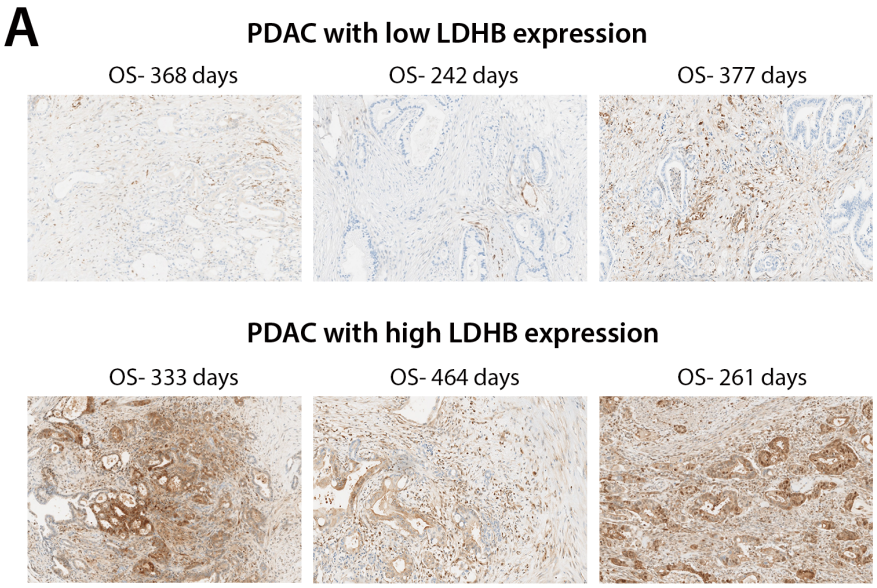
Table 12 – Correlation of MCT1, MCT4 and αSMA expression with LDHB expression in both tumour and stroma

| | **t-MCT1** | **t-MCT4** | **s-MCT1** | **s-MCT4** | **s-αSMA** |
|---|---|---|---|---|---|
| **t-LDHB** | p=0.639 | 0.655 | 0.841 | 0.655 | 0.897 |
| **s-LDHB** | 0.933 | 0.528 | 0.841 | 0.240 | 0.704 |

Neither high tumoural or stromal LDHB expression had an impact on overall survival following resection of PDAC (Tumour 590 vs. 617 days, p=0.57; Stroma 585 vs. 657 days, p=0.74; Fig 19B+C). The groups had very similar clinical characteristics although patients

with a low stromal LDHB expression were older and had a higher ASA score (Table 13). All other prognostic indicators demonstrated no significant differences, including positive resection margin (R1) rates, TNM status and lymph node ratio.

Figure 19 [290]




B

| | Tumoral LDHB expression | | | Stromal LDHB expression | | |
|---|---|---|---|---|---|---|
| | Low | High | p value | Low | High | p value |
| n | 30 | 29 | | 30 | 29 | |
| Tumoral expression % | 3.43 | 20.22 | <0.0001 | 5.82 | 17.76 | <0.0001 |
| Stromal expression % | 1.75 | 5.44 | 0.0126 | 1.3 | 5.71 | <0.0001 |
| OS (median) | 590 | 617 | 0.5713 | 585 | 657 | 0.7381 |

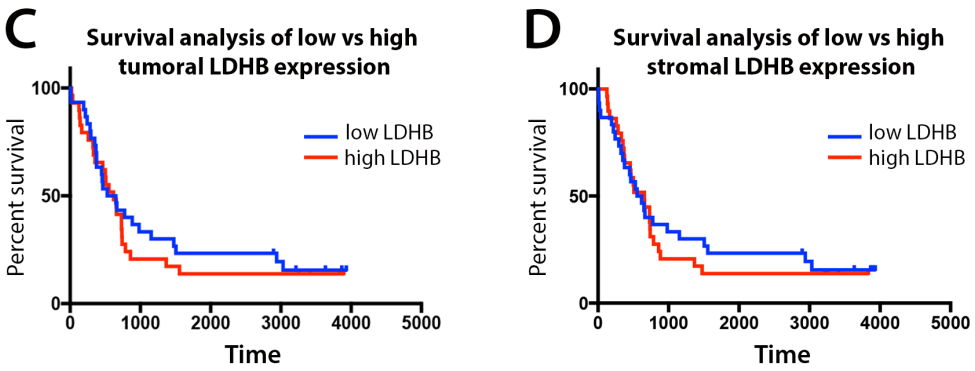


Table 13 – ANOVA analysis comparing key prognostic indictors between the low and high LDHB groups in both tumour and stroma

| | **Tumoural LDHB expression** | | | **Stromal LDHB expression** | | |
|---|---|---|---|---|---|---|
| | **Low** | **High** | **p value** | **Low** | **High** | **p value** |
| **n** | 30 | 29 | | 30 | 29 | |
| **Tumoural expression %** | 3.43 | 20.22 | <0.0001 | 5.82 | 17.76 | <0.0001 |
| **Stromal expressi %** | 1.75 | 5.44 | 0.0126 | 1.3 | 5.71 | <0.0001 |
| **OS (median)** | 590 | 617 | 0.5713 | 585 | 657 | 0.7381 |
| **Mortality %** | 83.3 | 89.7 | 0.99 | 67.5 | 63 | 0.1104 |
| **M:F** | 18:12 | 15:14 | 0.6042 | 15:15 | 18:11 | 0.4348 |
| **Age (median)** | 65 | 65 | 0.6868 | 66.3 | 62.2 | 0.0393 |
| **ASA (median)** | 2 | 2 | 0.4314 | 2.04 | 1.63 | 0.0335 |
| **R1 %** | 66.7 | 48.3 | 0.1923 | 56.7 | 58.6 | 0.99 |
| **T1:2:3** | 2:6:22 | 1:10:18 | 0.6756 | 3:6:21 | 0:10:19 | 0.8113 |
| **N1 %** | 93.3 | 89.7 | 0.848 | 90 | 90 | 0.6707 |
| **LN ratio** | 0.081 | 0.068 | 0.5775 | 0.077 | 0.072 | 0.4681 |

## 5.4 Discussion

This represents the first study to examine the expression of various metabolic markers in the tumour microenvironment of PDAC from within the same tissue specimen and separate breakdowns of tumoural and stromal expression patterns. It is clear from initial in vitro co-culture analysis that a relationship exists between PSCs and PDAC cells, whereby PSCs induce glycolysis in PDAC and influence lactate transporter expression through MCT upregulation. Whilst generally considered a bi-directional transporter of lactate, it has been reported that MCT1 is chiefly involved in lactate oxidation and therefore has a more prominent role in lactate influx. [220] In contrast MCT4 is mostly restricted to glycolytic cells, with a strong association with a hypoxic environment (HIF1a expression).[222] As such its role in PDAC is potentially profound; in 2014, during the process of performing this PhD project, Baek et al became the first group to publish data on MCT4 expression in PDAC, demonstrating that MCT4 defined a glycolytic subtype and infers a poor prognosis. [254]

If we consider the in vitro results thus far, the Panc1 and Miapaca2 cell lines (which are most representative of a PDAC genetic landscape as opposed to the KRAS-wild type Bxpc3) were seen to be more glycolytic, and as such, if this is reflected in the tumours resected in this IHC analysis, one would expect the results to reveal a higher expression of MCT4 as opposed to MCT1. However the opposite is true, whereby strong expression of MCT1 was more commonly seen in 50.2% of the tumour as opposed to 34.3% of MCT4. This may again support the theory that other elements of the stroma are impacting on the cancer cells and altering their individual metabolic profile as reflected in the *in vitro* solo cultures. The co-culture results with PSCs add weight to this theory, with upregulation in both MCT1 and MCT4; therefore it is possibly not surprising that we are seeing a high rate of positive

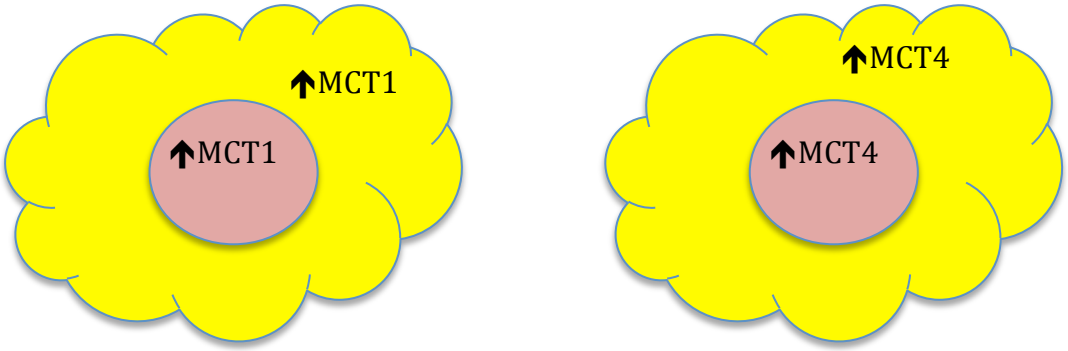
expression (regardless of intensity) of both MCT1 and MCT4 in 96 and 86.6% of resected specimens. Given one would expect the glycolytic nature of PDAC to more heavily express MCT4, one may deduce that the stromal interactions have a more significant impact on MCT1 upregulation, and one should then question whether therapeutic targeting of this as opposed to MCT4 is more worthy of further investigation. Alternatively it may be concluded that as a bidirectional transporter, MCT1 is being utilised more for lactate efflux as opposed to influx as is considered it's usual preference.

With respect to PSCs, the positive correlation of MCT expression between the two compartments is in keeping with the coculture results, whereby in the Panc1 and Miapaca2 lines upregulation of both MCT1 and MCT4 was observed in both PDAC and PSC lines. Of note Baek et al also demonstrated this positive correlation, with a non-significant 5.8% of specimens demonstrating an inverse correlation. Nonetheless this correlation is in keeping with the PSC metabolic phenotype analysis with the seahorse technology that demonstrated similar glycolytic and oxidative activity levels as compared to the Panc1 and Miapaca2 lines. However it was also shown that Panc1 induced oxidation in PSCs, whilst Miapaca2 had no impact on PSC metabolic activity. Therefore it is likely that PSCs are responding differently according to the metabolic phenotype of their associated tumour, and as such that will influence MCT1 and MCT4 expression. The results have demonstrated that, similar to the tumour, MCT1 is more strongly expressed in 42.1% of the tumour as opposed to MCT4 which is only strongly expressed in 25.7%. Meanwhile MCT1 was generally much more commonly expressed overall, with 98.1% positivity as opposed to 74.2% of MCT4. Given this preponderance for MCT1 in PSCs, we may deduce that, similar to the effect of Panc1 on PSCs in the seahorse co-culture, one may deduce that a pro-

oxidant impact on PSCs is more common *in vivo*; again one questions whether disrupting MCT1 as opposed to MCT4 is therefore of more interest.

If we now consider which therapeutic approach to MCTs might be more beneficial, the prognostic implications of MCT expression now need addressing. These results have shown a significant reduction in survival with high stromal expression of MCT1 and high tumoural expression of MCT4. The latter results are in keeping with the Beak et al publication, however they were also able to demonstrate reduced OS with high stromal MCT4 expression, as well as when stromal and tumoural compartments were combined. However our results regarding s-MCT4 (in addition to t-MCT1) were trending towards this finding, and a larger patient cohort may have allowed this to reach significance. Taking these IHC results regarding MCTs, I hypothesise two tumour microenvironment phenotypes converging on altered lactate metabolism which incur a poor prognosis (Figure 20). Based on the s-MCT1 and t-MCT4 survival analysis in combination with the positive correlations observed, it may be considered that tumour microenvironments that globally express MCT1 in both tumour and stroma, or MCT4 in both tumour and stroma, have reduced overall survival, therefore suggesting in patient's whose resection specimens exhibit his expression pattern, therapeutic targeting of either MCT1 or MCT4, with mechanism of effect against the stroma or tumour respectively, may incur survival benefit.

Fig 20 – Figure depicting the 2 proposed tumour (red) and stromal (yellow) phenotypes that incur poor prognosis, with either high MCT1 or MCT4 in both compartments




We know that in general 95% of PDAC tumours express KRAS, therefore admittedly this discussion is ignoring the potential that 5% of the patient cohort did not express KRAS (i.e. are similar to the Bxpc3 line), in whom one would expect a tendency towards a more oxidative tumour with no effect on MCT expression. However one would expect the 95% to still give a representative result, and the use of Bxpc3 will impart much more information further down the line when considering therapeutic approaches when one may consider whether to exclude patients who's tumours/biopsies do not express KRAS.

As discussed unfortunately I was unable to reliably optimise an LDHA antibody to analyse expression patterns or prognostic relevance, including investigating any potential correlation with tumoural:stromal LDHA:LDHB ratios of expression. However as so happened during my project work a paper was published revealing tumoural over-expression of the glycolytic enzyme LDHA is associated with a poorer prognosis in patients with PDAC [197]; of note I had attempted to use the same antibody utilised in this paper. It was demonstrated that the combined strong expression of pyruvate kinase type M2 (PKM2) and LDHA resulted in a significantly reduced OS from 27.9mths to 7 months

(p=0.003), and this was upheld in multivariate analysis. Whilst this was not an analysis of LDHA expression alone, and rather scored patients on either PKM2 or LDHA expression, PKM2 would be expected to go hand in hand with LDHA given it's association with glycolysis. No other studies have published on the prognostic relevance of LDHA, however these results on a reasonably sized patient cohort of 72 suggest therapeutic approaches to LDHA would be of interest.

With respect to our results concerning LDHB, it is not entirely unsurprising that in a predominantly glycolytic PDAC tumour the expression patterns were not strong as seen with the MCTs, with positive staining in tumour and stroma of only 43% and 27% respectively, and with a tendency towards weak as opposed to strong intensity. Nonetheless, in view of the seahorse analysis regarding induction of oxidative processes, combined with the theory that cells with access to oxygen on tumour peripheries are able to undergo oxidative phosphorylation and utilise lactate for ATP production in greater abundance [21] one may deduce that despite the high rate of negative LDHB expression in tumour and stroma (57% and 73% respectively), strategies towards LDHB inhibition are still worthy of pursuit in order to decrease energy production in the oxidative peripheral tumour cells. This is regardless of the lack of prognostic impact that the survival analysis demonstrated. The positive correlation between tumour and stromal LDHB expression is also noteworthy, suggesting that the metabolic change in either compartment are duplicated and therefore inhibiting LDH has the potential to target both tumoural and stromal entities.

**Summary of key points**

- The PDAC microenvironment, including both tumour and stroma, express MCT1 and MCT4 in abundance, with a tendency towards increased MCT1 expression and a positive correlation between compartments
- Patients with particularly strong expression of tumoural MCT4 and stromal MCT1 (i.e. in the top third of expression intensity) have reduced OS, whilst t-MCT1 and s-MCT4 trends in a similar fashion, suggesting inhibition of both MCT1 and MCT4 may impart prognostic benefit in PDAC by targeting the stroma and tumour respectively
- LDHB is more weakly expressed than MCTs and does not impart any prognostic value in PDAC

# Chapter 6

# Therapeutic approaches to lactate associated metabolism

## 6.1 Background

A variety of methods targeting cancer metabolism has been investigated, including inhibitory approaches to both oxidative and glycolytic processes. However these therapeutic methods have generally concentrated on targeting the metabolic reprogramming process that occurs within the cancer cells themselves, and has not accounted for the effects of key stromal components such as PSCs.

When considering drug discovery experimentation, whether that is in vitro or in vivo, a number of limitations exist, particularly in the field of anti-metabolism treatments. In vitro drug testing on PDAC cell lines is no doubt limited by the intrinsic heterogeneity of the genetic landscape of the disease. Whilst there a number of commercially available PDAC cell lines, each derived from different patients with a unique genetic signature, the immortalized nature of these lines limits the clinical applicability of data produced. However even if one isolated PDAC cells from multiple patients, one would still observe huge variations in cancer cell properties, with differences seen between primary tumour and metastases in the same patient, and even within different regions of the resected tumour.[14, 17, 268] Nonetheless, in vitro experimentation still represents the mainstay of preliminary investigation into the effects of drug treatments. However, with particular relevance to PDAC, simple treatment of cell lines on their own does not take into account

the influential tumour microenvironment previously discussed. In light of the key role of PSCs in a variety of capacities, it is essential that they be taken into account. Results in this project thus far have demonstrated an important metabolic contribution of PSCs towards PDAC cells, by both inducing a proglycolytic effect and inducing MCT upregulation. This is not only an important mechanistic discovery into how metabolic reprogramming affects the microenvironment as well as tumour, but also emphasizes the need to account for stromal components such as PSCs in drug discovery experimentation, such as utilizing transwell coculture in this project.

Furthermore in vivo experimentation with PDAC animal models is again limited but numerous factors in drug discovery, which makes converting successful pre-clinical studies into patient treatments a non-exact science. A number of animal models exist for pancreatic cancer, such as various genetically engineered mouse models (GEMMs - the KPC model being the most prevalent), cell line or patient tumour-derived xenografts and syngeneic mouse xenografts. Limitations in clinical translational capacity are in part due to the genetic heterogeneity previously discussed. In addition there are important differences between humans and mice; whilst 76% of mouse genes have direct human orthologs, the function and structure of these can vary significantly, meaning PDAC tumour in mice may exhibit key differences in pathogenesis, mechanisms of metastasis and response to therapeutics.[269] Lastly, and with most relevance to this project, many mouse models do not accurately mimic the tumour stroma which is so influential in all aspects of PDAC development. Cell-line xenografts either do not form stroma, or do so to a limited extent and being of mouse origin.[270] Patient-derived xenografts initially possess the stromal elements of their parent tumour, however this is replaced with mouse stroma over time.[271] GEMMs do exhibit a fibrotic stroma, however again this being of mouse origin is

a limitation. Organoid models have more recently been developed, and whilst they accurately mimic tumourigenesis, as well as a desmoplastic reaction with αSMA positive cell aggregation, these may still be replaced with mouse stroma over time.[272] Therefore, when delineating effects of metabolism targeting treatments towards both PDAC and its associated stromal elements, in vitro testing represents the ideal starting point to both elucidate the inter-stromal relationships, and then determine whether certain drugs may incur a beneficial and translational effect by shutting down key interactions within the tumour microenvironment, and work out whether any compensatory mechanisms occur within the microenvironment which may negate any beneficial anti-tumour impact.

Data in this project so far has shown a pro-glycolytic effect of PSCs towards PDAC, and a potential relationship of lactate shuttling which may be associated with MCT activity. During the infancy of this project a variety of therapeutic approaches was investigated, the results of which will be detailed in this chapter. This included treatment directed at oxidative processes, such as with the OXPHOS inhibitor Sodium Azide (NaN3), and ROS scavenger N-Acetylcysteine (NAC). However subsequent results as detailed in the preceding chapter directed the project more towards anti-glycolytic therapies; nonetheless these results are presented initially, as I subsequently examined any inhibitory approaches towards both oxidation and glycolysis which may exert a synergistic effect.

Given the prognostic impact of stromal MCT1 expression, which may be attributed to PSC presence, in combination with the upregulation seen in vitro, MCT1 was identified as a promising avenue of investigation. This was further influenced by the fact MCT1 is known to be common in lactate consuming cells, which may well be relevant given the increase in lactate utilization demonstrated in lactate assays. Furthermore a recent study

demonstrated MCT1 inhibition also targets Ras-transformed fibroblasts by reducing tumourigenicity and growth[273], adding support to the fact this targeted approach may disrupt the PSC-PDAC metabolic relationship.

A number of agents have been utilised as MCT1 inhibitory strategies, such as a-cyano-4-hydroxycinnamate (CHC), stilbene disulfonates (DIDS/DBDS), p-chlorormercuribenzene sulfonate (pCMBS) and phloretin, however these drugs lack the specificity towards MCT1, with off-target effects making it difficult to determine the true translational significance of the treatment. [216, 217, 223] A turning point came when Astrazeneca recently fabricated AZD3965, a highly specific MCT1 inhibitor, with Ki values in the nM region. The Freeman Hospital (Newcastle) and Northern Institute of Cancer Research (NICR), in conjunction with the Royal Marsden Hospital (London), are currently recruiting patients for phase 1 trial of AZD3965 in the treatment of patients with advanced cancer, aiming to determine the recommended safe and biologically active dose of the drug (NCT01791595). I therefore came into the privileged position of having access to this drug with the assistance of my co-supervisor Prof Steve Wedge, who was also involved in the trial.

An alternative therapeutic approach I wished to examine was inhibition of glycolytic enzymes such as LDH. With the potential reliance of PDAC tumours on glycolysis with the oxygen starved microenvironment, it appears logical that shutting down the key final step of pyruvate and lactate interconversion by targeting LDH could prove to be particularly efficacious. This has been supported by preclinical studies of the LDH-A inhibitor FX-11, whereby treatment has reduced PDAC tumour progression in vivo.[203] However this has limited potential to become a clinically applicable drug. In addition LDHA has been shown to relate to poor prognosis in PDAC, therefore despite my IHC analysis revealing no

prognostic relevance of tumoural or stromal LDH-B in PDAC, a pan-LDH inhibitory strategy still represents potential to further impact on protumourigenic acidification, and as such the pan-LDH inhibitor galloflavin, which had never been investigated in PDAC, represents an intriguing avenue of investigation, as this may also impart information on the mechanisms of the lactate shuttling that occurs between PDAC and PSCs.

As well as examining the overall effect on cancer cells/PSCs by inhibiting either MCT1 or LDH, i.e. anti-proliferative/apoptotic effects, this approach would also assist into shedding light on the metabolic relationship between PDAC and PSCs. Results in chapter 3 did not conclusively reveal how MCTs were behaving towards overall lactate flux. By inhibiting MCT1 specifically, rather than using the non-specific compounds already mentioned with their associated off-target effects on metabolism, one may examine the overall effect on lactate concentrations (intra/extra-cellular), MCT expression and metabolic phenotype (seahorse technology) in an attempt to explain preceding results. Admittedly the same observations/conclusions may be made, wherein PDAC cell lines behave differently according to their underlying metabolic phenotype/MCT expression profile. However it may be then possible to determine which kind of tumours may be more susceptible to MCT inhibitory strategies, thus guiding onward in vivo protocols, and potentially suggesting a patient/tumour-tailored approach is warranted from a clinical translation perspective.

Unlike previous chapters, given the plethora of data included, results will be discussed as they are presented to allow improved understanding of my hypotheses regarding mechanism of effect and reasoning for onward investigatory experimentation.

Of note, statistical analysis was performed comparing the affect of treatment to either the corresponding solo or co-culture condition, i.e. the effect of treatment on Panc1 in co-culture with PSCs was compared to the expression level of Panc1 in co-culture without treatment (not solo conditions). Furthermore, with respect to the seahorse experiments, it is worth noting that each run on the machine could only examine 4 different cell conditions, making it logistically difficult to perform experiments accounting for all conditions (i.e. affects on both PSCs and corresponding PDAC lines +/- treatment). Therefore I elected to perform the solo and co-culture treatments separately, given I had already demonstrated the effect of co-culture alone, and this could then be accounted for in any subsequent observations.

## 6.2 Results

### 6.2.1 Targeting oxidation

N-Acetylcysteine (NAC), at a concentration of 20mM, was used to examine the effect on metabolic activity (MTT) of blocking oxidative processes through the inhibition of ROS (Fig 21.1). In contrast the pro-oxidant buthionine sulfoxamine (BSO), which inhibits the synthesis of anti-oxidant glutathione, was used to examine any opposing effect of encouraging oxidative respiration. NAC was observed to significantly increase Miapaca2 proliferation when in co-culture with PSCs, from 0.71nM to 1.15nM ($p=0.004$), however no converse effect with BSO. No significant effect was seen in on Panc1 with either NAC or BSO treatment. With regards to effect on PSCs, BSO significantly reduced PSC proliferation when in co-culture with Panc1 ($p=0.049$), with no effect in the presence of Miapaca2, whilst no effect was seen with NAC.

Before interpreting these results, the effect of NAC on extracellular (media) lactate concentration needed to be considered, and this revealed a significant reduction in lactate concentration in both solo and co-culture conditions with both all PDAC cell lines +/- PSCs (figure 21.2; $p<0.0001$ in all conditions). It is difficult to interpret these results of NAC in isolation, however what is clear is that the simple attempt at reducing oxidative processes does not consistently impart a survival benefit to the PDAC cells through the neutralization effect of a reduction in lactate. Furthermore it is not the case that NAC imparts a pseudo-Warburg effect by encouraging a switch to glycolytic processes, as one would expect an increase in lactate in this instance. Nonetheless it is intriguing to see that proliferation increased in Miapaca2 but only when in co-culture with PSCs; the key point to elucidate

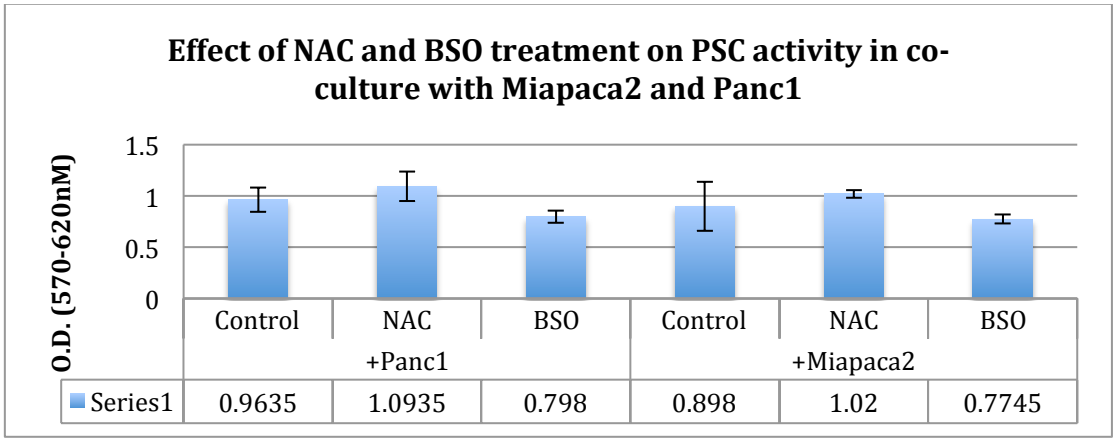
here is the fact the presence of PSCs somehow altered the mechanism of effect of NAC, in this instance for the worse.

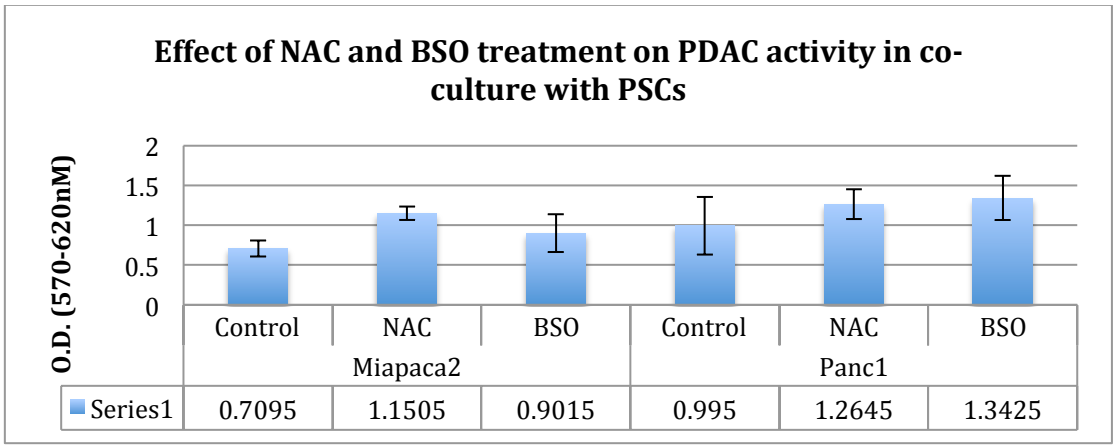
Another compound examined for potential effect was sodium azide (NaN3), which inhibits OXPHOS through blockade of mitochondrial respiratory complex IV. Cells were treated for 24 hours in solo conditions at 100uM concentration (Fig 21.3). This only demonstrated a significant reduction in proliferation (MTT) in the Miapaca2 cell line ($p=0.001$), with no effect on the other PDAC lines and PSCs.

The effect of NAC treatment on lactate imparted intriguing information on how reducing ROS impacts on lactate metabolism, and whilst admittedly these experiments would have had added value if performed in hypoxic conditions, soon after these experiments the project began to go down a different route following the findings regarding MCT upregulation in PDAC-PSC co-culture with a pro-glycolytic relationship as described in chapter 2. Furthermore due to the lack of potential for clinically translational benefit of NaN3 due to its toxicity, it was decided not to pursue further investigation of this compound. The project direction was then concentrated upon methods of targeting lactate metabolism/glycolysis, which will now be described in more detail with relevance to inhibition of MCT1 (AZD3965) and LDH (Galloflavin). Nonetheless I aimed to use combinatorial treatments with NAC to help elucidate mechanisms of effect in subsequent experiments.

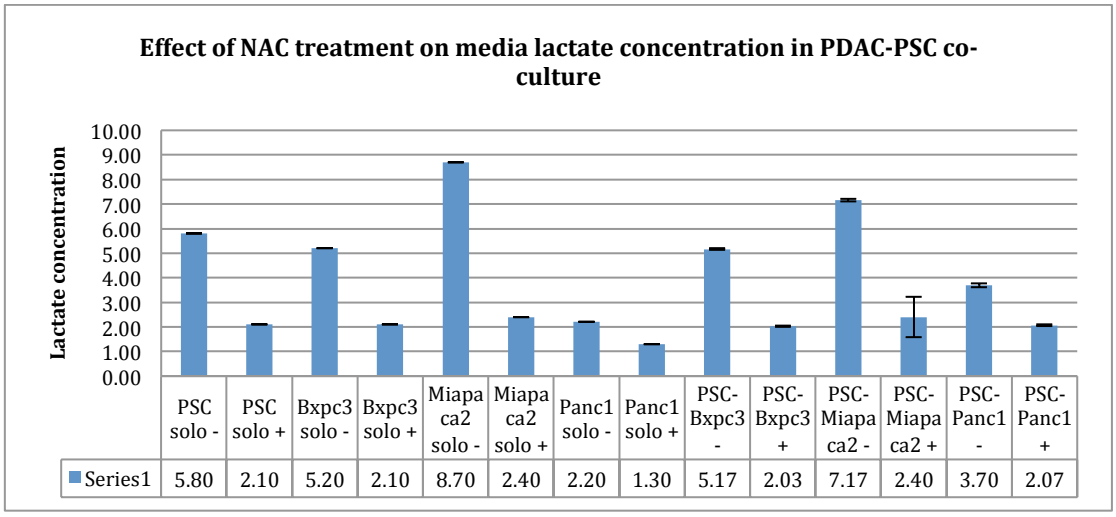
Figure 21 – Effect of therapeutic approaches to oxidative processes.

21.1 - MTT assay demonstrating effect of anti-oxidant (N-Acetylcysteine 20mM) and pro-oxidant (BSO, 1mM) treatment on co-culture of PSCs with Panc1 and Miapaca2

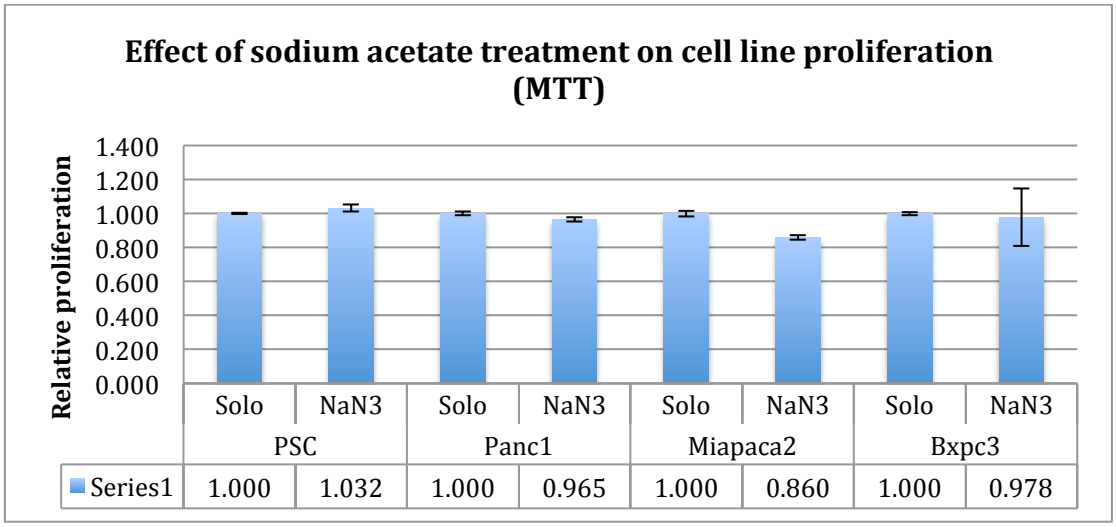



21.2 – Extra-cellular lactate concentration +/- NAC (20mM) in PDAC-PSC co-culture.



21.3 MTT assay demonstrating effect of NaN3 (100uM, 24 hrs) on PDAC-PSC co-culture

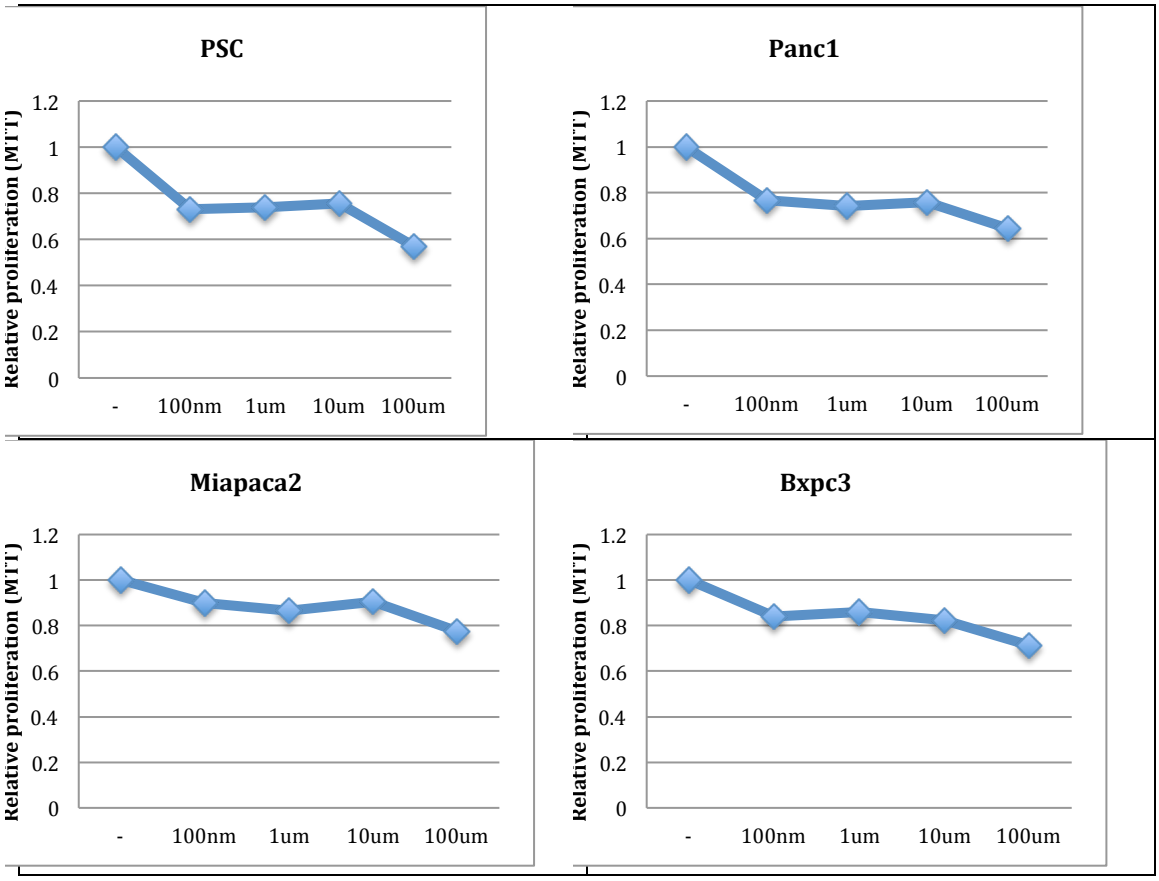


### 6.2.2 MCT1 inhibition

#### 6.2.2.1 Cell line specific efficacy

The MCT1 inhibitor AZD3965 was obtained from AstraZeneca as previously discussed. Dose response was assessed at a variety of concentrations from 100nM to 100uM (figure 22). The response was not significantly different between all concentrations, and given 100nM has been deemed the most clinically applicable dose as determined in AZ's drug discovery, and re-affirmed by further studies at the NICR (Newcastle) on lymphoma cell lines (unpublished work at time of writing), this dose was selected as the most appropriate for subsequent in vitro experimentation.

Figure 22 – Dose response curve to AZD3965 treatment of all cell lines at 0, 100nM, 1uM, 10uM and 100uM



Initial studies investigated effect on metabolic activity (MTT) and proliferation (BrDu). Fig 23.1 demonstrates significantly reduced metabolic activity with solo treatment of all cell lines (PSC p=0.017, Panc1 p=0.027, Miapaca2 p=0.009, Bxpc3 p=0.018), whilst this reduction was not replicated in co-culture between either Panc1/Bxpc3 and PSCs. However in co-culture the Miapaca2 line still exhibited reduced activity with treatment (p=0.001), intriguingly with a converse significant increase in PSC activity (p=0.0015). In contrast to the MTT results, AZD3965 only exerted a significant anti-proliferative effect (Brdu assay) on PSCs with solo treatment (p=0.005) however not in co-culture, and no effect was seen on all PDAC lines (fig 23.2). In combination, this suggests any beneficial effect that MCT1 disruption may exert (as demonstrated by the MTT assays) could be reversed by the metabolic relationship between PDAC and PSCs, resulting in no effect on overall proliferation. Furthermore, the conflicting results with the Miapaca2 line, which as previously described is more MCT4 dependent, implies each cell line is likely to behave differently given their differing MCT expression profiles, which in fact is an accurate reflection of the heterogeneity observed in the IHC staining of resected specimens.

Further into the project, it became clear that performing these *in vitro* experiments in hypoxic conditions could add further translational understanding regarding the mechanism of action of treatment and any in vivo translational impact. Unfortunately I had already performed the majority of the experiments thus far, and due to time constraints could not repeat all of these. Nonetheless I deemed it important to at least examine the effect on cellular activity that AZD3965 imparts on the different cell types within hypoxic as opposed to normoxic conditions; these results are demonstrated in fig 24. Treatment of PSCs alone in hypoxia had the same effect on reduced metabolic activity (p=0.0009), however whilst an increase in activity was seen following normoxic treatment of PSC-

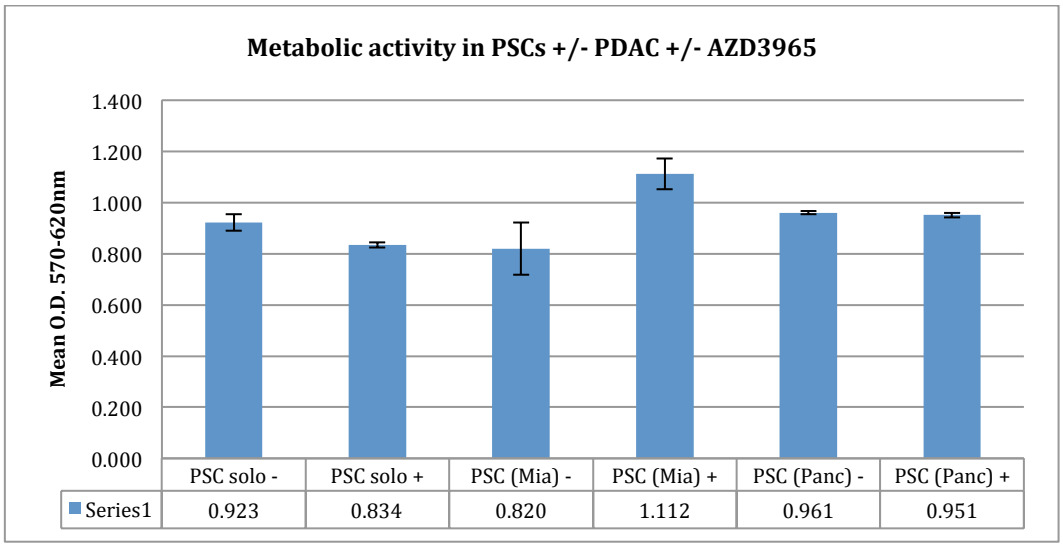
Miapaca2 co-culture, in hypoxia there was a significant reduction in metabolic activity (p=0.025). The Miapaca2 line demonstrated the same results as in normoxia, with a significant reduction in both treatment alone (p=0.0114) and in co-culture with PSCs (p=0.0245). The Panc1 line had only demonstrated a reduction in activity in solo conditions in normoxia, however treatment in hypoxia resulted in a significant reduction in metabolic activity in solo (p=0.0352) and co-culture (p=0.0243) conditions. Lastly treatment of the Bxpc3 line in hypoxia had no effect in solo and co-culture conditions, which is in contrast to the reduction in metabolic activity seen in solo treatment in normoxia. Overall these results suggest hypoxic conditions are influencing the mechanism of effect of MCT1 inhibition with a tendency towards increased chances of an anti-proliferative effect on both PSCs and PDAC cell lines, which has promise from an translational perspective given the hypoxic in vivo tumour microenvironment.

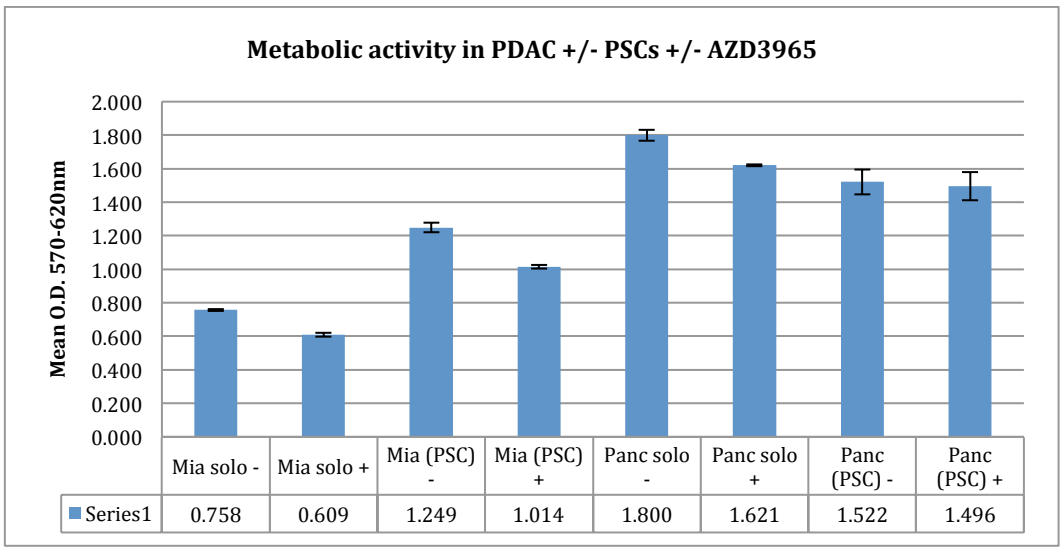
Returning to the experiments in normoxia, the apoptotic effect of AZD3965 was then examined by caspase-3 assay (Fig 23.3), where it was demonstrated that whilst solo treatment of PSCs had no effect on apoptosis, when in the presence of either Panc1 or Miapaca2 there was a significant increase in PSC apoptosis of 7.3 fold (p=0.045) and 4.8 fold (p=0.048) respectively. However this was not replicated with the Bxpc3 line, suggesting this apoptosis effect on PSCs is dependent on a KRAS-regulated metabolic relationship between PDAC and PSCs. There was no significant apoptotic effect on the Panc1 or Miapaca2 lines, however the Bxpc3 line behaved differently with treatment, whereby in solo and co-culture treatment there was a significant reduction in apoptosis of 2.1 fold (p=0.034) and 1.8 fold (p=0.041) respectively, again implying the KRAS mutation is necessary to facilitate the apoptotic action of AZD3965. Therefore, by inducing cell death in PSCs, albeit with no observed over-riding anti-proliferative effect on the aforementioned

assays, MCT1 inhibition may therefore herald great promise in vivo by specifically targeting PSCs.

Fig 23 – Effect of AZD3965 (100nM, 48hrs) on metabolic activity, proliferation and apoptosis of individual cell types in PDAC-PSC co-culture

Fig 23.1 – Effect of AZD3965 on metabolic activity (MTT)



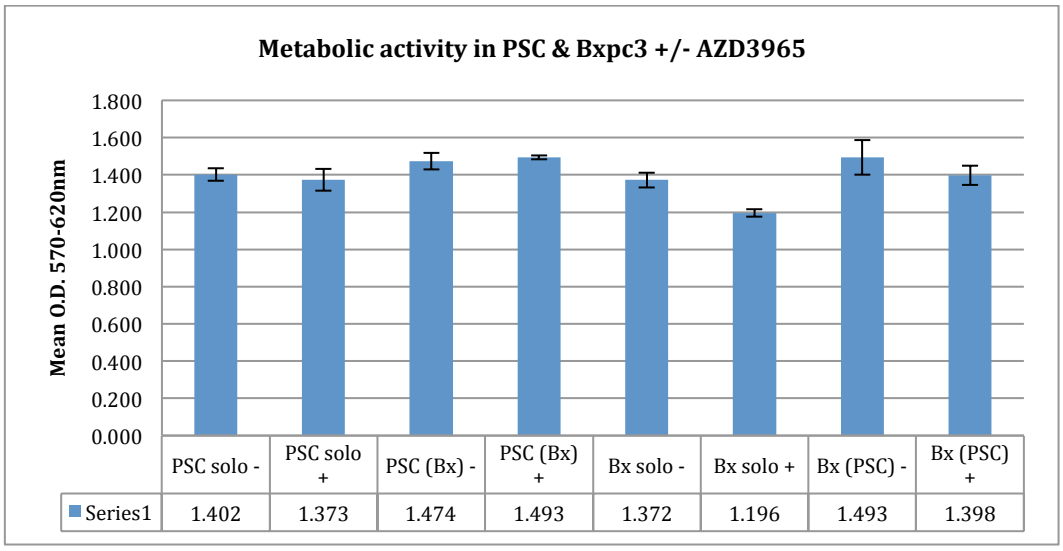
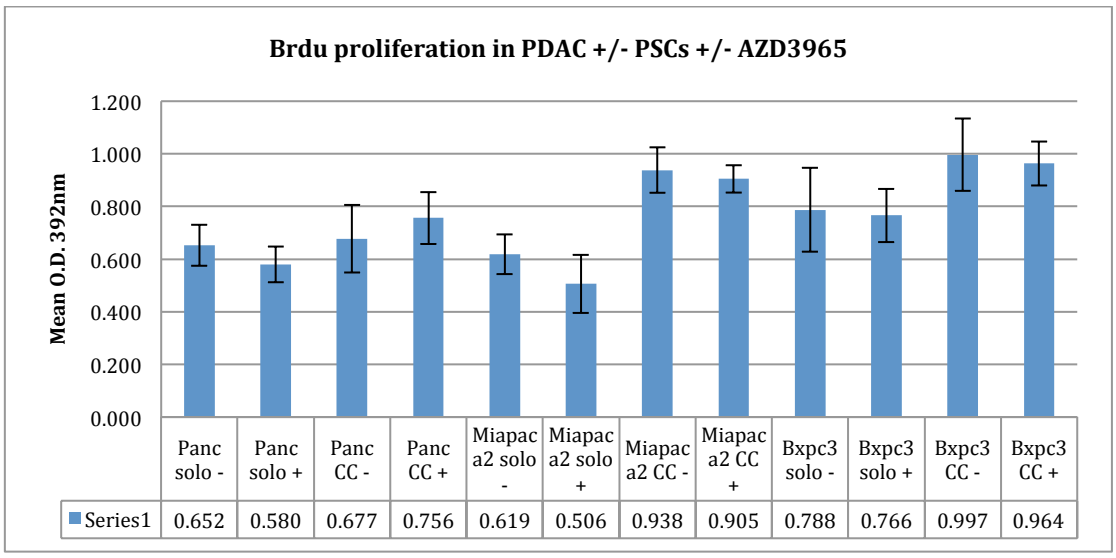



Fig 23.2 – Effect of AZD3965 on proliferation (Brdu)



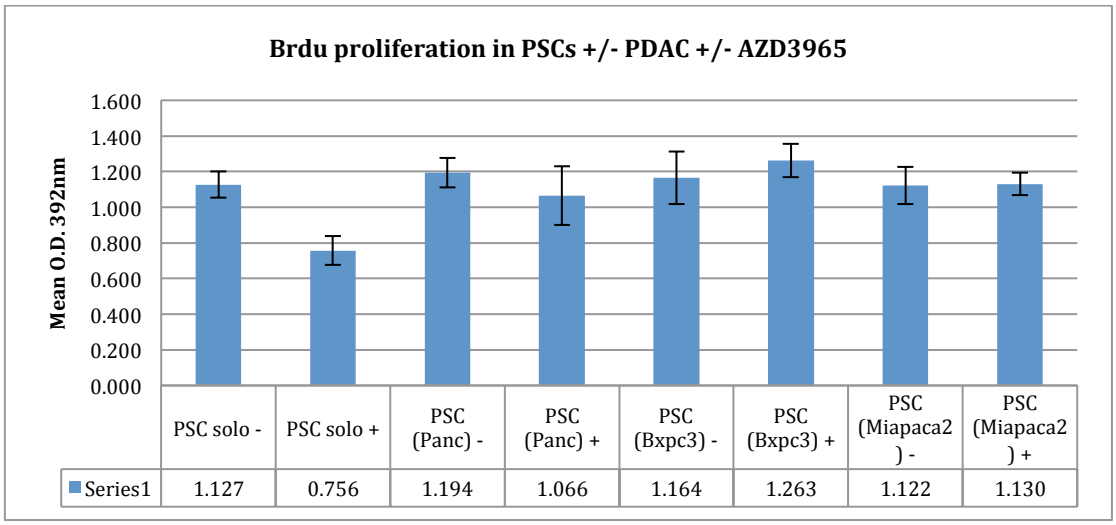
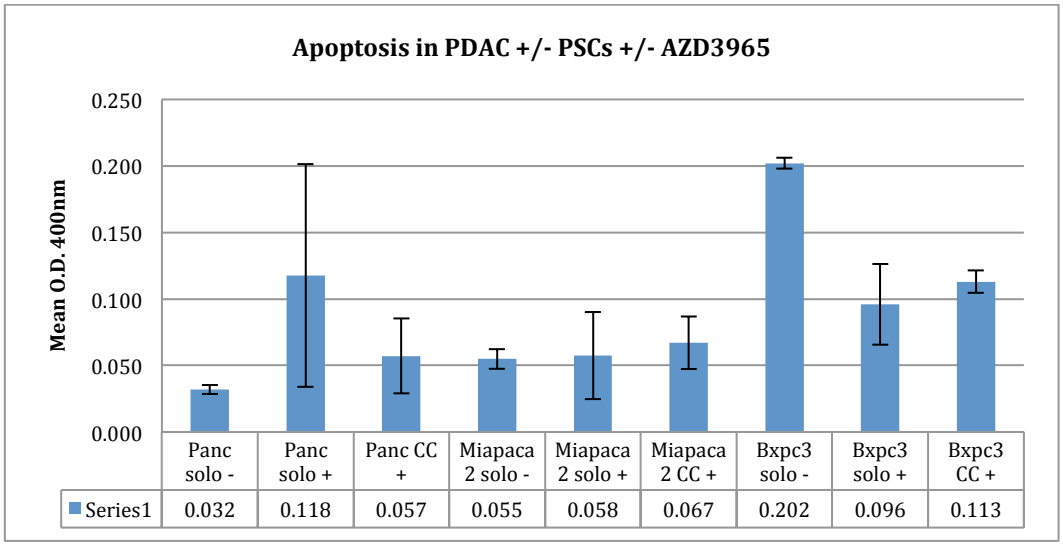



Fig 23.3 – Effect of AZD3965 on apoptosis (Caspase-3)



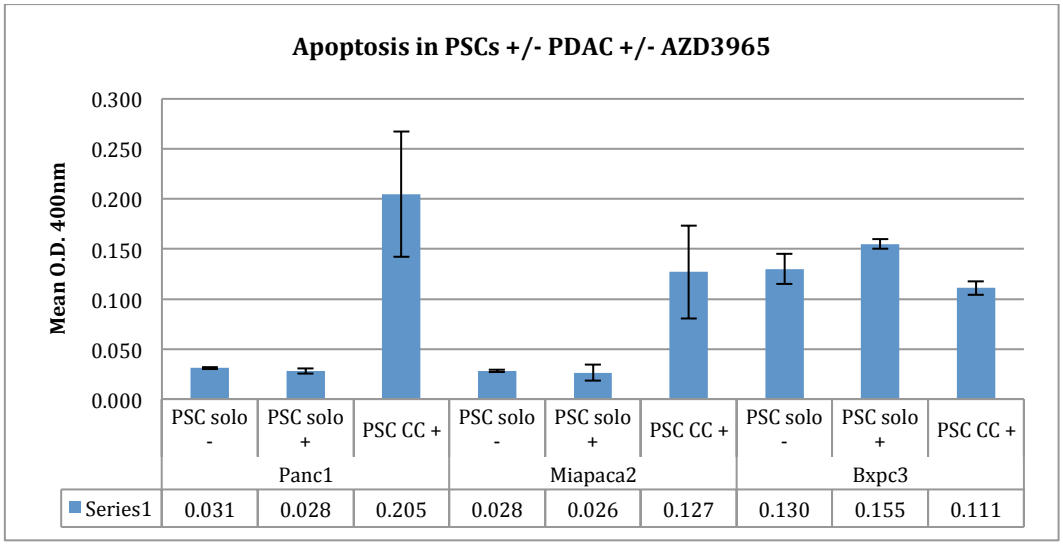
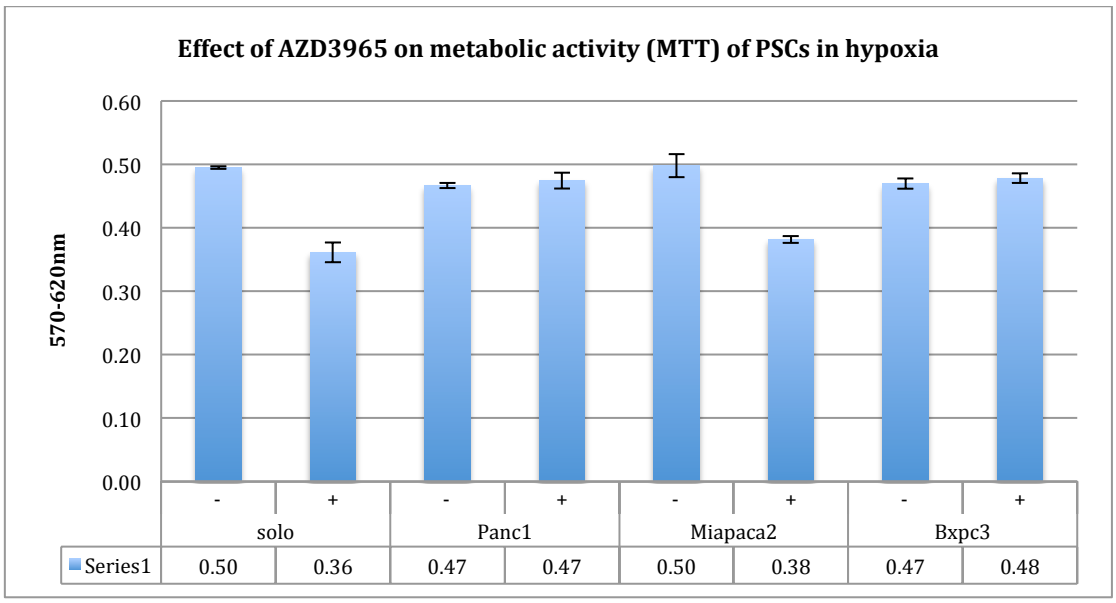
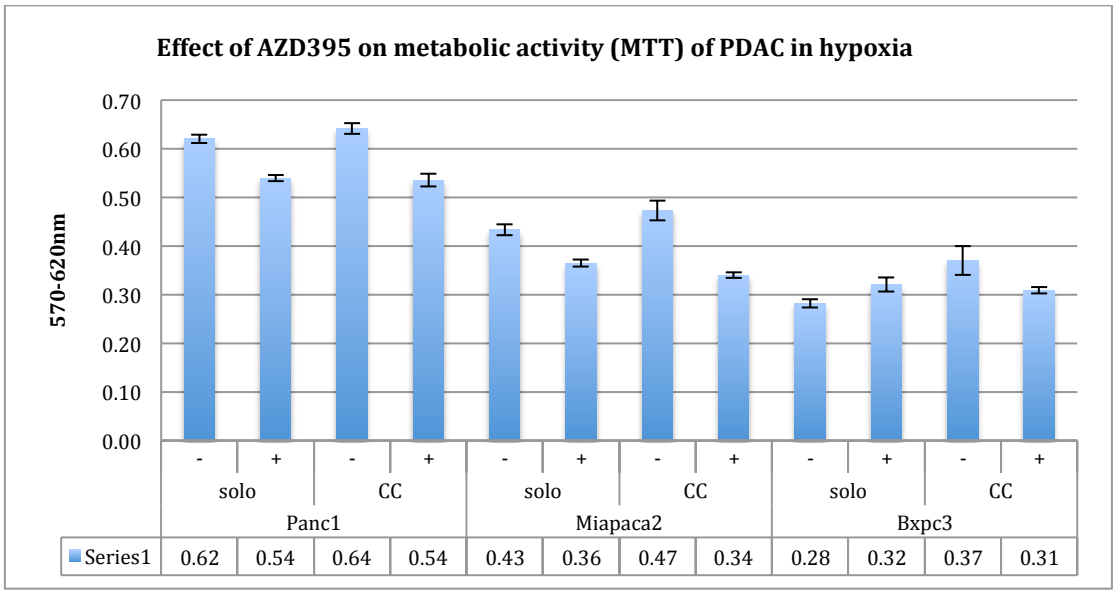



Fig 24 Effect of AZD3965 on metabolic activity of PDAC and PSCs in hypoxic conditions

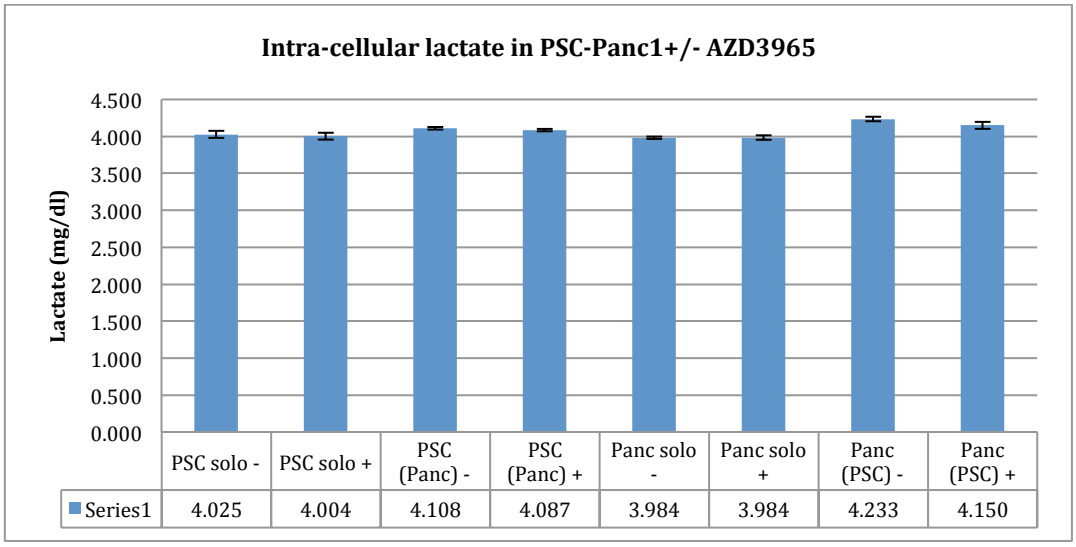


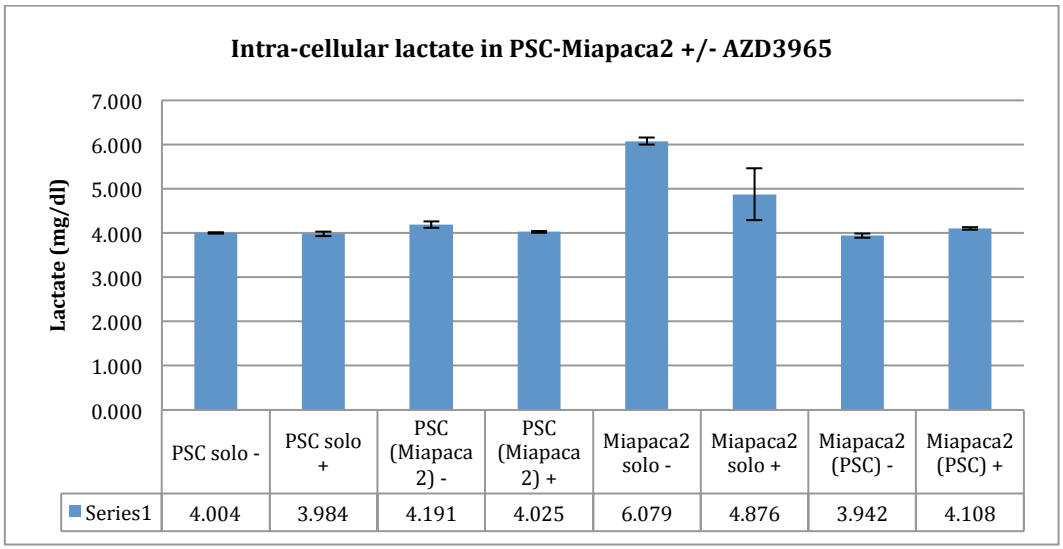



The effect of AZD3965 on lactate levels, at both the intra- and extra-cellular level, was then examined, with 24 and 48-hour time points in the latter analysis (fig 25). The only significant result observed regarding intra-cellular lactate was a reduction with solo treatment of Miapaca2 (from 6.1mg/dl to 4.9mg/dl; p=0.045), however this effect was not replicated in co-culture (fig 25.1). In correlation with the extra-cellular lactate findings, solo treatment of Miapaca2 resulted in a significant reduction of 63.6% (p<0.0001) at 48 hours; in combination this suggested that somehow MCT1 inhibition was reducing lactate usage and consumption across the board. However in co-culture with PSCs no effect on lactate (both intra and extra-cellular was observed), suggesting in the case of a more MCT4-expressed tumour, PSCs are influencing the mechanism of effect of MCT1 inhibition. With respect to Panc1 and Bxpc3, there was a global reduction in extra-cellular lactate in both solo and co-culture conditions, with a reduction at 48hrs in co-culture with PSCs of 46.4% (p<0.001) and 42.4% (p<0.001) respectively (fig 25.2).

Fig 25 – Effect of AZD3965 (100nM, 48hrs) on lactate concentrations in PDAC-PSC co-culture

Fig 25.1 – Effect of AZD3965 on intra-cellular lactate





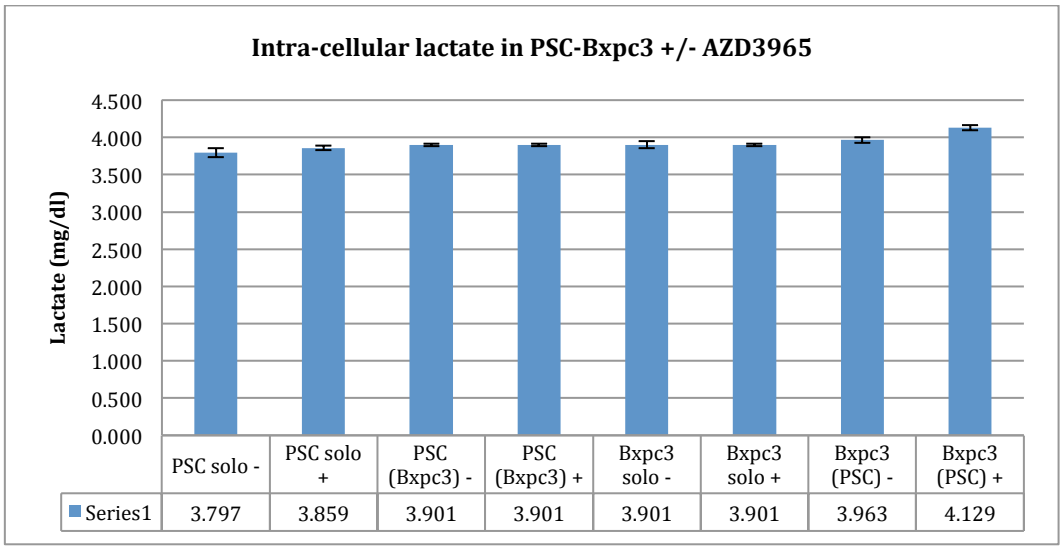
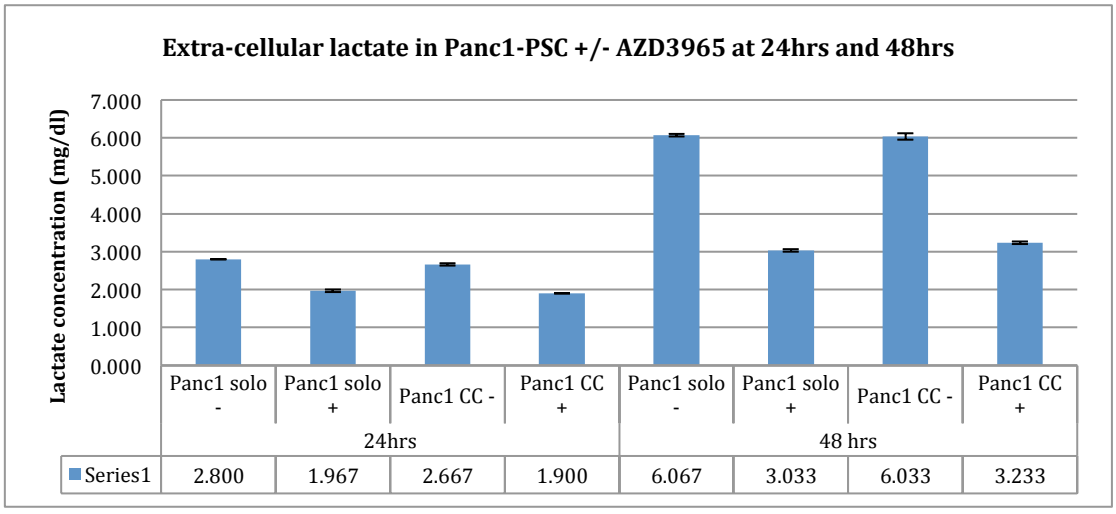
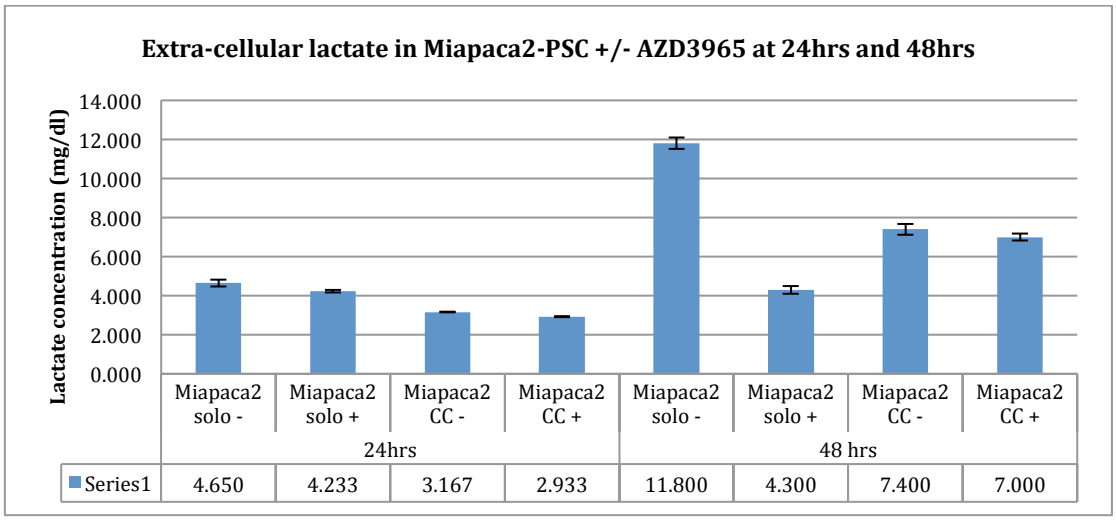
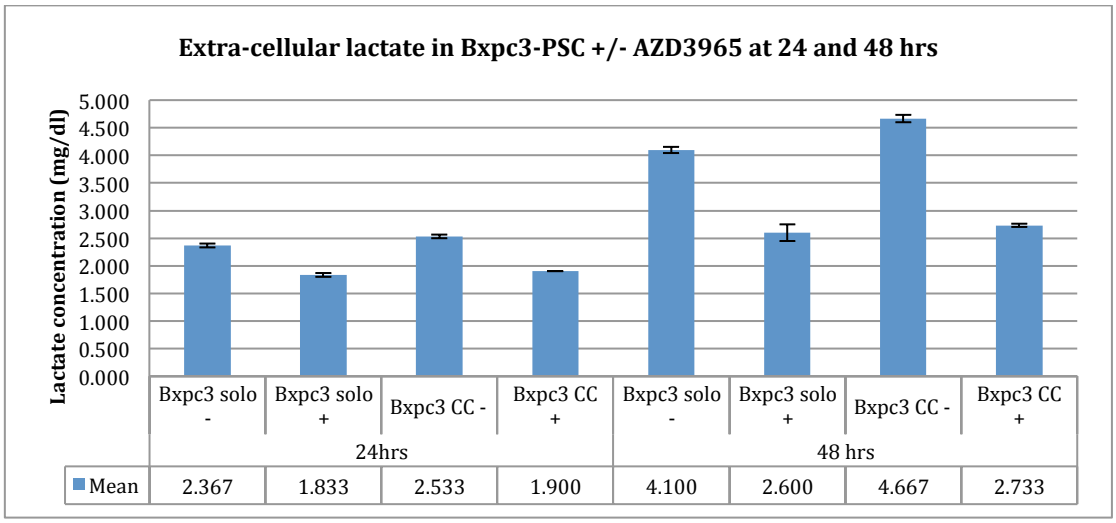




Fig 25.2 – Effect of AZD3965 on extra-cellular lactate




Extra-cellular lactate in Miapaca2-PSC +/- AZD3965 at 24hrs and 48hrs
Lactate concentration (mg/dl)
14.000
12.000
10.000
8.000
6.000
4.000
2.000
0.000
Miapaca2 solo -
Miapaca2 solo +
Miapaca2 CC -
Miapaca2 CC +
Miapaca2 solo -
Miapaca2 solo +
Miapaca2 CC -
Miapaca2 CC +
24hrs
48 hrs
Series1
4.650
4.233
3.167
2.933
11.800
4.300
7.400
7.000


Extra-cellular lactate in Bxpc3-PSC +/- AZD3965 at 24 and 48 hrs
Lactate concentration (mg/dl)
5.000
4.500
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
Bxpc3 solo -
Bxpc3 solo +
Bxpc3 CC -
Bxpc3 CC +
Bxpc3 solo -
Bxpc3 solo +
Bxpc3 CC -
Bxpc3 CC +
24hrs
48 hrs
Mean
2.367
1.833
2.533
1.900
4.100
2.600
4.667
2.733

Lastly the effect of treatment on PDAC intra-cellular glutamine concentration was examined (fig 26), where it was revealed the only significant effect of treatment was on the Miapaca2 line in solo conditions (increase in concentration of 28%; p=0.031) however this was not replicated in co-culture. Once again, an overall contrasting effect on the Miapaca2 line was observed with the addition of PSCs negating any treatment effect.

Fig 26 – Effect of AZD3965 on cancer cell line glutamine concentration in solo and co-culture conditions

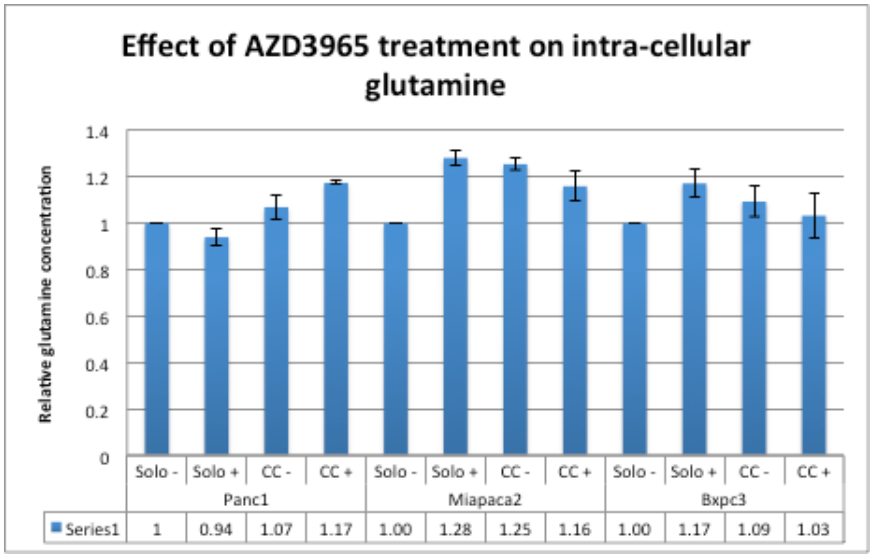



Table 14 summarises the AZD3965 results thus far (barring combinatorial approaches) in tabular form. Given the heterogeneity of PDAC, at this stage of the project when considering therapeutic intervention I deemed it reasonable to be considering each of these cell lines as individual patients, with differing genetic/metabolic phenotypes that may behave differently to anti-metabolic treatment, and as such I will now attempt to delineate any treatment effects on these cell lines individually whilst discussing the concomitant effect on MCT expression (qPCR) and metabolic phenotype (seahorse) observed; this is including the concomitant effects on PSCs.

Table 14 - Table summarizing effect of AZD3965 treatment of PSC and PDAC cell lines in solo and co-culture conditions on metabolic activity (MTT), proliferation (Brdu), apoptosis (Caspase-3), intra-cellular lactate and extra-cellular lactate (at 48hrs)

| | PSC | | | | Miapaca2 | | Panc1 | | Bxpc3 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | S | CC-M | CC-P | CC-B | S | CC | S | CC | S | CC |
| **Metabolic activity:** | | | | | | | | | | |
| **Normoxia** | ↓ | ↑ | - | - | ↓ | ↓ | ↓ | - | ↓ | - |
| **Hypoxia** | ↓ | ↓ | - | - | ↓ | ↓ | ↓ | ↓ | - | - |
| **Proliferation** | ↓ | - | - | - | - | - | - | - | - | - |
| **Apoptosis** | - | ↑ | ↑ | - | - | - | - | - | ↓ | ↓ |
| **i-c lactate** | - | - | - | - | ↓ | - | - | - | - | - |
| **e-c lactate** | - | - | ↓ | ↓ | ↓ | - | ↓ | ↓ | ↓ | ↓ |
| **Glutamine** | | | | | ↑ | - | - | - | - | - |

AZD3965 vs Miapaca2

The effect of MCT1 inhibition on Miapaca2 in solo conditions is to induce a neutralizing effect on the media and associated reduction in metabolic activity (MTT), suggesting a reduction in export and/or increase in import of lactate, and overall potentially an increase in lactate utilization as an oxidative fuel. The lack of effect on proliferation, with an anti-metabolic effect, in part supports this theory as oxidation is picking up the slack and maintaining proliferation, therefore in solo conditions MCT1 inhibition is pro-oxidative towards Miapaca2. However seahorse analysis did not fully support this theory, with no effect on OCR or OXHPOS-ATP observed, and in fact an increase in glycolytic parameters was seen, with a significant increase in ECAR of 98.5% ($p<0.0001$) and glycolytic ATP of 88.3% ($p<0.0001$) (fig 27), and as we already know this effect would impart survival benefit in vivo, much like the pro-glycolytic effect of PSC previously demonstrated. However, when considering co-culture experiments with PSCs, this effect is negated, with no effect on glycolysis seen when treated in co-culture, and furthermore no concomitant

increase in oxidative processes, therefore suggesting in the least that AZD3965 has no over-riding impact on methods of metabolism/ATP production.

As discussed, regardless of the effect in solo conditions, what is more pertinent is the effect whilst in co-culture with PSCs, better mimicking the effect in vivo, and we do in fact see differing results, whereby in the presence of PSCs this neutralizing effect seen in solo treatment is not observed. We may consider this result to be expected given we now know that PSCs exert a pro-glycolytic effect, therefore despite inhibition of MCT1, PSCs are stimulating the Miapaca2 cells to pump out excessive lactate thus continuing to acidify the media and negate the effect of AZD3965 seen in solo conditions. This theory is supported by the fact PSC metabolic activity (MTT) is increased whilst in co-culture with Miapaca2. However the seahorse analysis demonstrated conflicting results to this hypothesis, whereby treatment in co-culture resulted in no significant effect on either oxidative or glycolytic processes (fig 27).

However alterations in MCT expression may suggest the underlying mechanism for these observations of treatment effect. Whilst AZD3965 had no impact on MCT1 expression, though no doubt an impact on it's functionality given the aforementioned findings, most intriguingly a concomitant increase in MCT4 expression was observed in Miapaca2 in both solo ($p<0.0001$) and co-culture ($p<0.0001$) conditions, suggesting the blockade of MCT1 results in a compensatory increase in MCT4 functionality (fig 28). Of note this is in a cell line which we already known is more MCT4 dependent, and suggests that is the way this cell line is compensating for the lack of MCT1 functionality, and thus blocking any over-riding anti-tumoural effect the drug is attempting to impart. At the same time, an impact on

the PSCs was also observed with a significant reduction in MCT4 expression by 54% (p=0.004), with no change in MCT1.

In summary, whilst we may be reassured of the anti-metabolic effect exhibited in both normoxia and hypoxia, these results suggest that with respect to Miapaca2, and in particular due to the lack of influence on metabolic phenotype and extra-cellular acidification, MCT1 inhibitory strategies in tumours of a similar phenotype may not be effectively exerting a clinically beneficial effect against the cancer cells themselves. Furthermore the increase in PSC proliferation may not translate into clinical benefit, albeit this may be a result of a pro-oxidative switch and therefore not translate in the hypoxic in vivo setting. Nonetheless, these results suggest an effect towards PSCs, and any additional effects on their inflammatory profile or activity will be discussed later as, regardless of the lack of in vitro anti-tumourigenic effect towards the cancer cells, this may still translate to in vivo benefit.

AZD3965 vs Panc1

The effect of MCT1 inhibition towards the Panc1 line will now be examined, which as discussed/demonstrated is similar to Miapaca2 in some ways (KRAS, similar PSC interactivity), however not in others (less MCT4 expression, more oxidative, different genetic phenotype). When considering in vitro results thus far, the key difference with respect to Miapaca2 is the fact treatment of Panc1 results in a reduction in extra-cellular lactate acidification in both solo and PSC co-culture conditions, however in co-culture a reduction in metabolic activity is not observed (albeit this was seen in hypoxia), and there is no concomitant alterations in PSC activity.

The key differences with respect to Miapaca2 are the converse effects of AZD3965 on MCT expression. Whilst again no effect on MCT1 expression was seen in solo or coculture conditions, with Panc1 there was no concomitant increase in MCT4 expression, suggesting MCT4 transporters are continuing to function at a normal level in the cancer cells. An important observation is also seen with respect to the PSCs, whereby treatment results in a reduction in both MCT1 ($p=0.009$) and MCT4 ($p=0.0148$), suggesting a global shutdown in the lactate shuttling potential of the PSCs, which we have already revealed to be key with respect to the pro-glycolytic relationship observed in the first seahorse experiments (fig 28). These findings certainly support the apoptotic effect towards PSCs in co-culture treatment with AZD3965.

The seahorse results help to decipher these results further. MCT1 inhibition with AZD3965 against Panc1 cells alone resulted in a significant increase in glycolytic parameters, with an increase in ECAR and glycolytic ATP of 46.1% ($p=0.0014$) and 165.4% ($p=0.015$) respectively, with no impact on oxidation, which in essence is an overall effect that may be detrimental in an in vivo setting (fig 27). However in the presence of PSCs, the opposite is observed with a significant reduction in ECAR and glycolytic ATP of 79% ($p=0.0005$) and 72.5% ($p=0.0002$) respectively in the Panc1 cells when treated with AZD3965. Whilst one would expect a concomitant switch to oxidative processes, in fact the opposite is observed whereby treatment resulted in a significant inhibitory effect towards oxidation, with a reduction in both basal and maximal OCR of 39.6% ($p=0.0165$) and 42.8% ($p=0.0104$) respectively, with a trend towards reduced ATP production. Certainly these findings support the observations regarding MCT downregulation in PSCs, suggesting the mechanism of effect of AZD3965 hinges upon the effective disruption of the PDAC-PSC relationship. One may then conclude that in the case of Panc1, a global shut down of

metabolism, with a significant influence on the PSCs and lactate shuttling, could herald immense promise in neutralizing the tumour microenvironment and thus translating to effective anti-tumourigenic activity in vivo.

AZD3965 vs Bxpc3

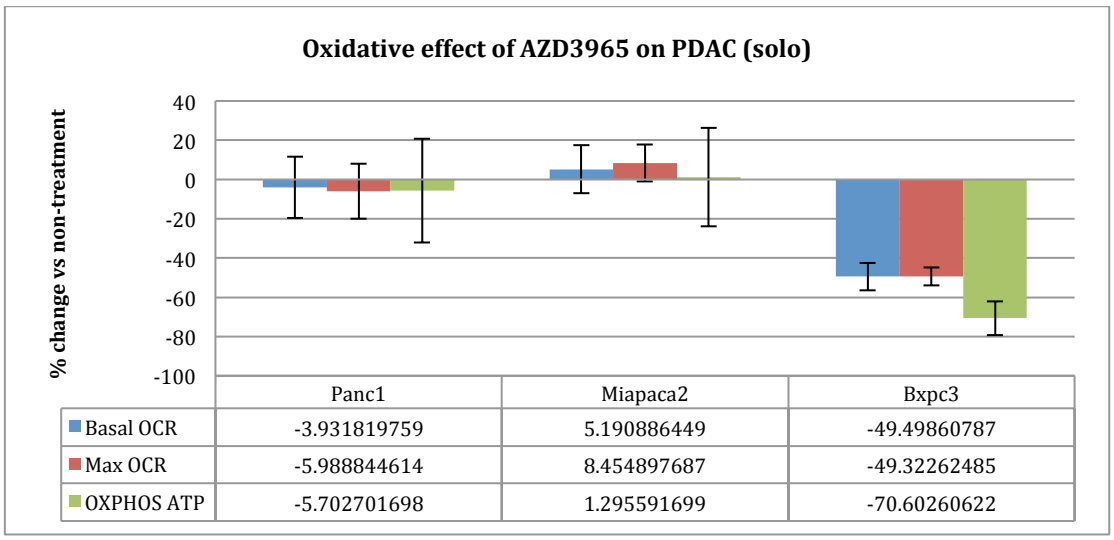
Lastly, we consider the effect on the KRAS-wild type line Bxpc3, which has previously demonstrated contrasting differences to both Miapaca2 and Panc1 with a different metabolic phenotype (more oxidative). However it has also demonstrated a similar increase in glycolysis with PSCs, and has been shown to have a similar genetic phenotype to Panc1.

Thus far MCT1 inhibition has revealed a similar trend to Panc1, with reduction in extra-cellular lactate in both solo and co-culture conditions. However the key finding was an apoptotic effect of inhibition regardless of PSC presence. Needless to say this is a promising result, albeit it's translational relevance is less significant with respect to the KRAS-mutant nature of Bxpc3.

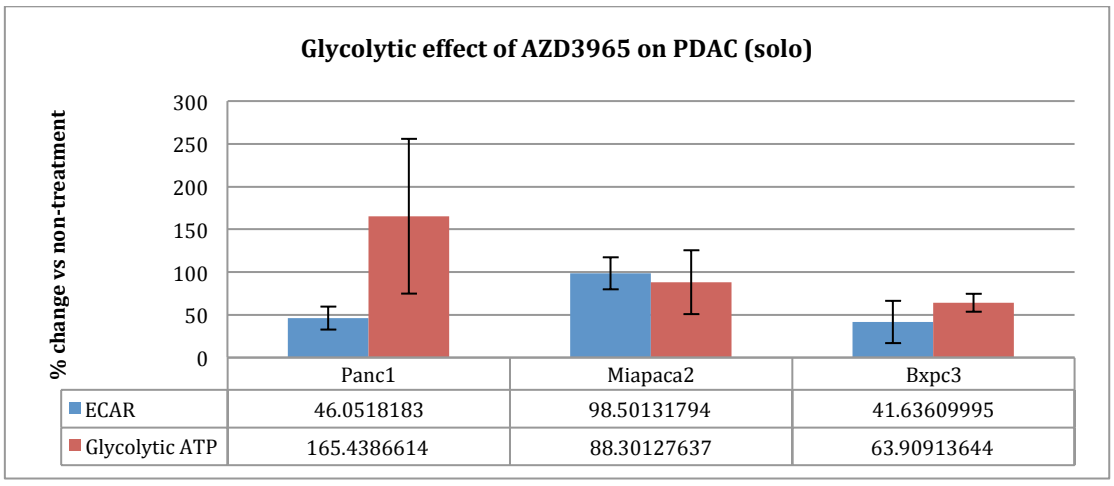
Deciphering mechanism of effect needs to now consider the effects on MCT expression and metabolic phenotype. Herein we see no significant change in MCT1 expression in the cancer cells in either solo or co-culture treatment, however a decrease in MCT1 by 37% ($p=0.003$). With regards to MCT4 however a significant 10-fold increase ($p=0.0008$) in MCT4 was observed only in solo treatment however not in co-culture, with no concomitant effect on PSCs (fig 28).

These results are fairly similar to what was observed with Panc1, however the effect on metabolic processes is markedly different, whereby solo treatment resulted in a significant reduction in all oxidative processes in the Bxpc3 line, with a reduction in basal OCR, maximal OCR and OXPHOS ATP of 49.5% (p=0.0013), 49.3% (p=0.004) and 70.6% (p=0.001). Correspondingly there was a 63.9% increase in ECAR (p=0.0037), albeit no change in the amount of ATP produced by glycolysis (fig 27). In co-culture with PSCs, the same picture was seen with respect to inhibition of oxidation, with a decrease in basal OCR by 68.2% (p=0.0003), maximal OCR by 70.3% (p=0.0001) and OXPHOX ATP production by 82.7% (p=0.0001), and with a concomitant increase in glycolytic ATP production by 59.8% (p=0.0018). Therefore, again albeit without any affect on proliferation, we can see that MCT1 inhibition is switching the Bxpc3 line towards glycolysis; this in theory would impart survival benefit in vivo. As far as using these results to explain a mechanism of effect of AZD3965, we may deduce that the presence of KRAS is highly influential, which is unsurprising given its influence on metabolic pathways.

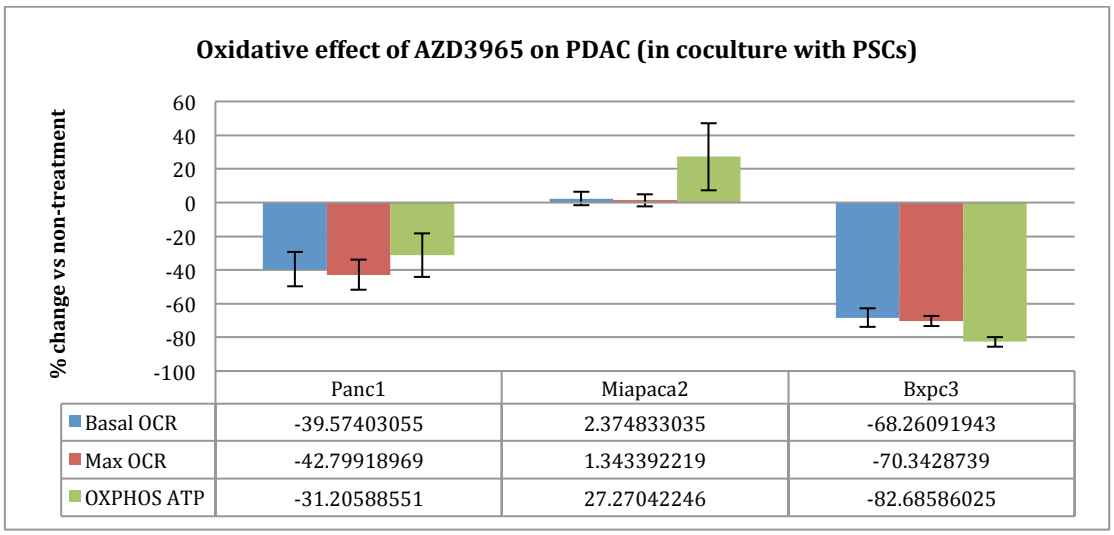
Fig 27 Effect of AZD3965 on PDAC metabolic parameters in seahorse analysis




| | Panc1 | Miapaca2 | Bxpc3 |
|---|---|---|---|
| Basal OCR | -3.931819759 | 5.190886449 | -49.49860787 |
| Max OCR | -5.988844614 | 8.454897687 | -49.32262485 |
| OXPHOS ATP | -5.702701698 | 1.295591699 | -70.60260622 |




| | Panc1 | Miapaca2 | Bxpc3 |
|---|---|---|---|
| ECAR | 46.0518183 | 98.50131794 | 41.63609995 |
| Glycolytic ATP | 165.4386614 | 88.30127637 | 63.90913644 |




| | Panc1 | Miapaca2 | Bxpc3 |
|---|---|---|---|
| Basal OCR | -39.57403055 | 2.374833035 | -68.26091943 |
| Max OCR | -42.79918969 | 1.343392219 | -70.3428739 |
| OXPHOS ATP | -31.20588551 | 27.27042246 | -82.68586025 |

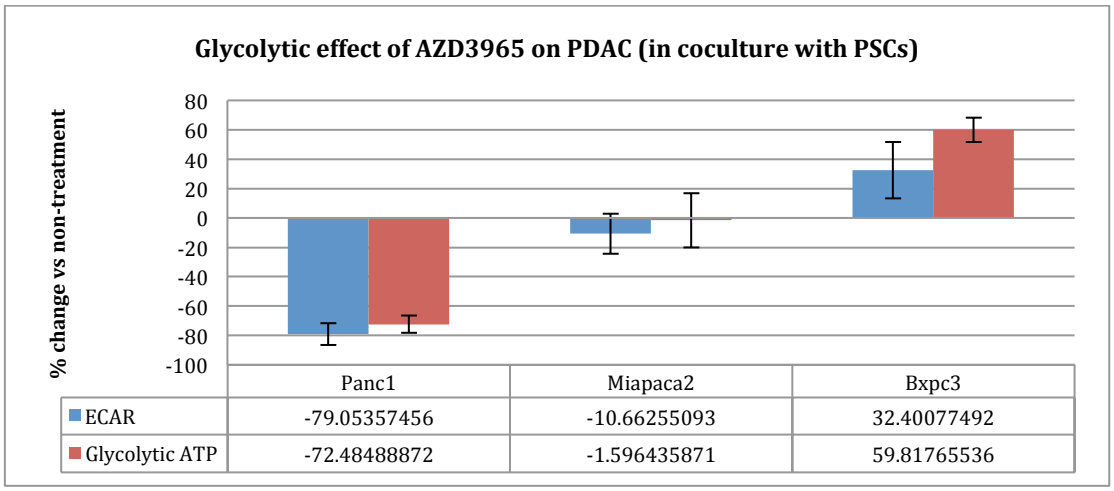
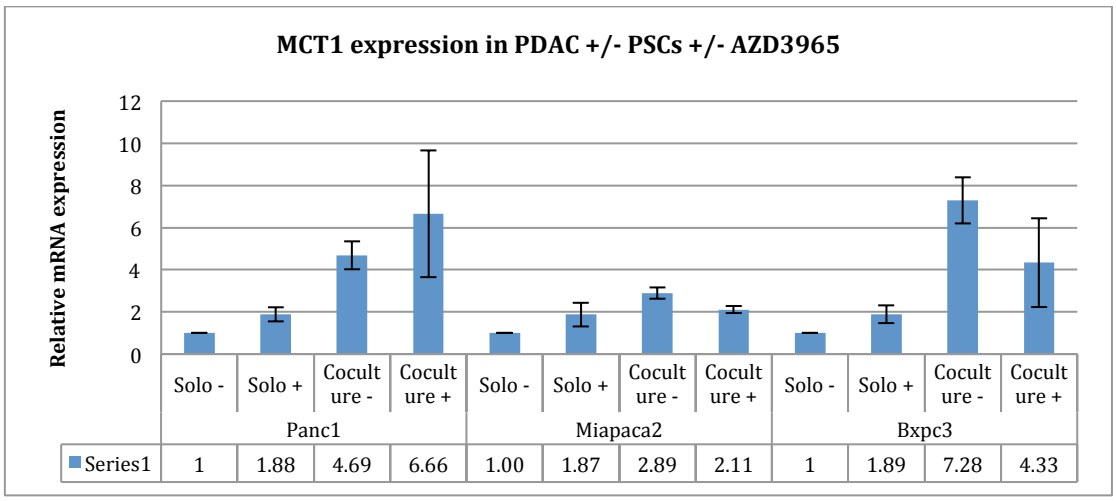
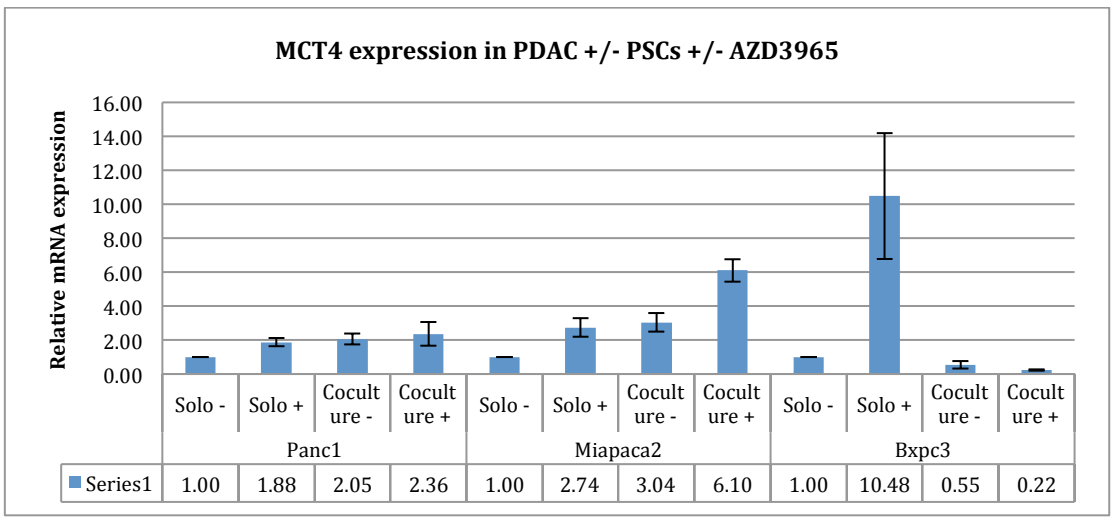
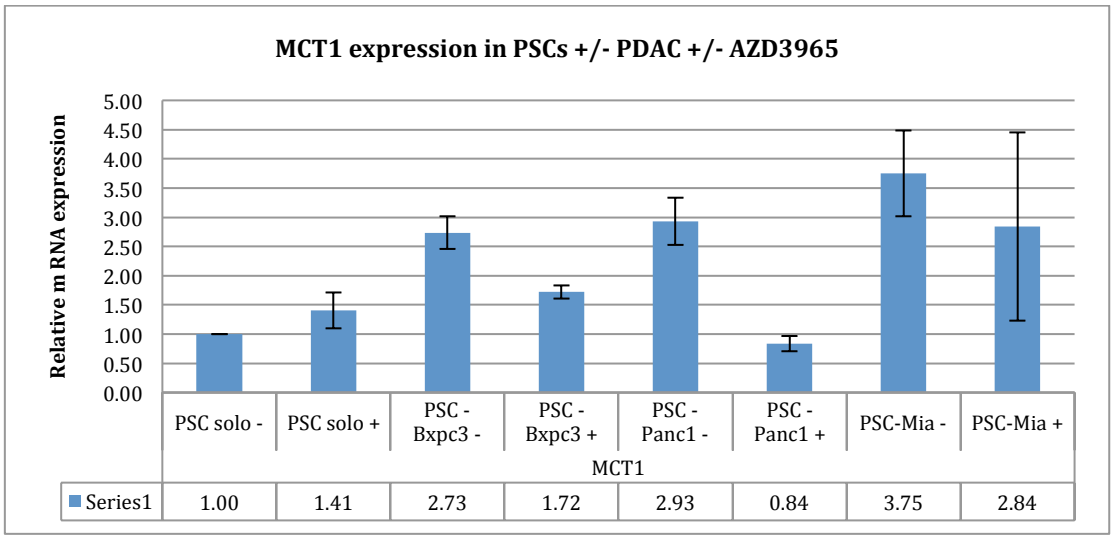
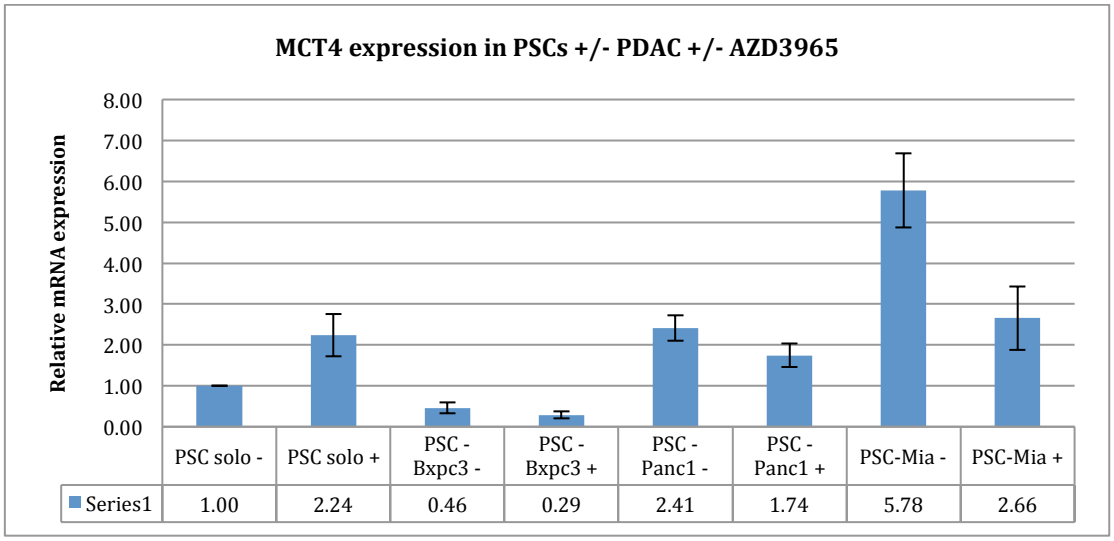



Fig 28 Effect of AZD3965 on MCT1 and MCT4 expression in both PDAC and PSC cell lines during solo and co-culture conditions



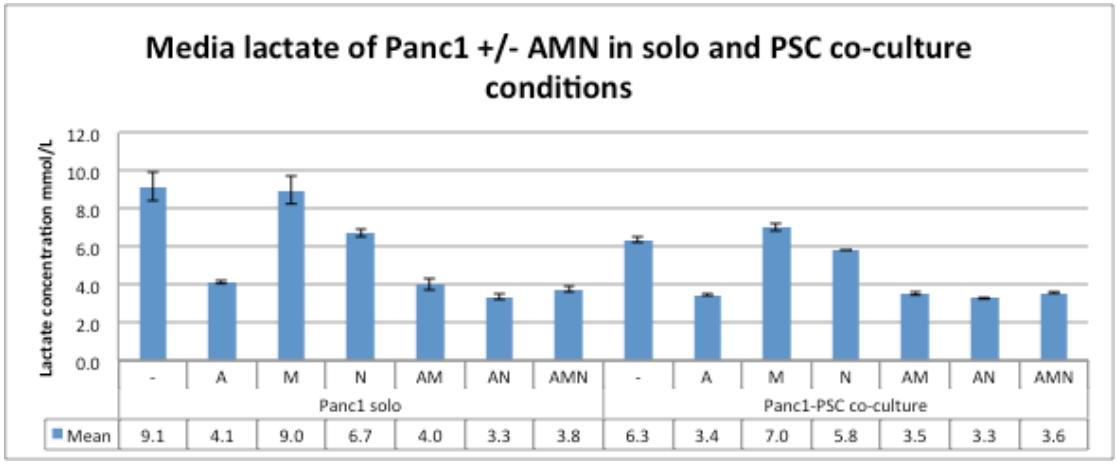



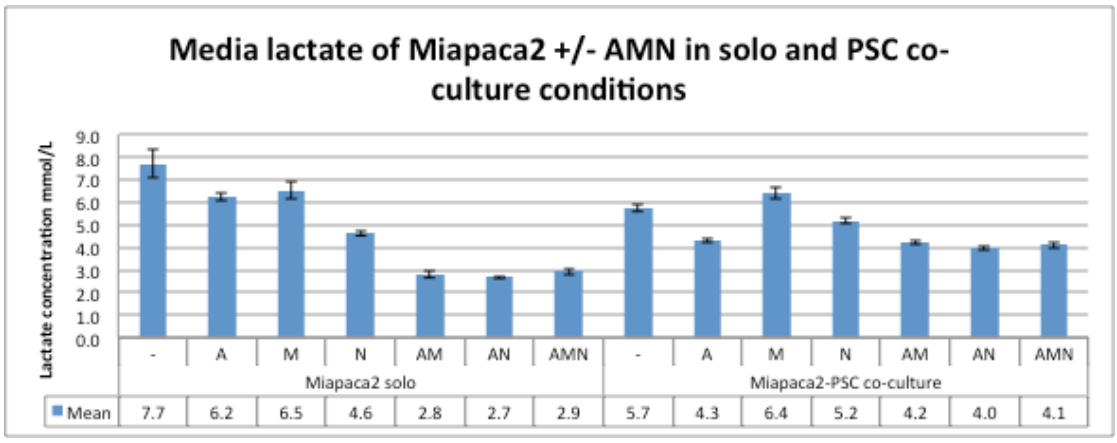

MCT1 expression in PSCs +/- PDAC +/- AZD3965
Relative m RNA expression
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
PSC solo -
PSC solo +
PSC - Bxpc3 -
PSC - Bxpc3 +
PSC - Panc1 -
PSC - Panc1 +
PSC-Mia -
PSC-Mia +
MCT1
Series1
1.00
1.41
2.73
1.72
2.93
0.84
3.75
2.84

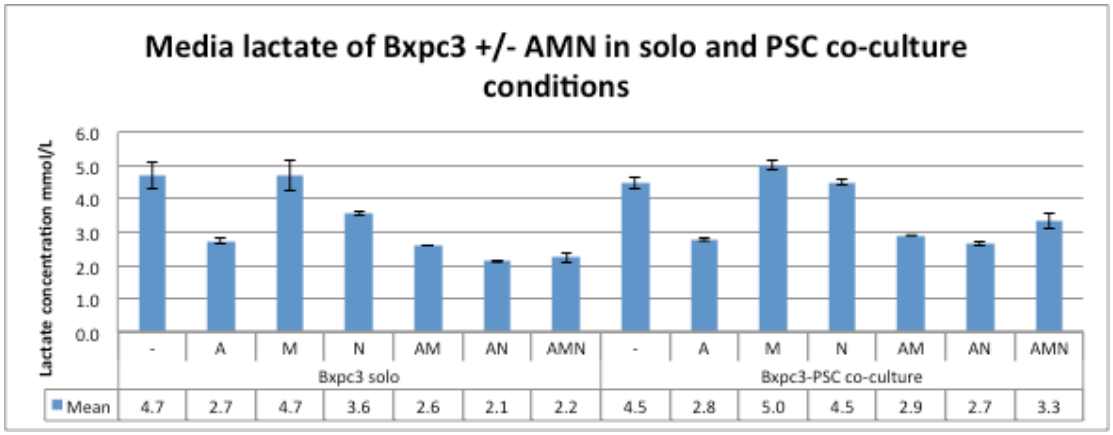

MCT4 expression in PSCs +/- PDAC +/- AZD3965
Relative mRNA expression
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
PSC solo -
PSC solo +
PSC - Bxpc3 -
PSC - Bxpc3 +
PSC - Panc1 -
PSC - Panc1 +
PSC-Mia -
PSC-Mia +
Series1
1.00
2.24
0.46
0.29
2.41
1.74
5.78
2.66

Thus far it has been shown that both targeting both oxidation (with NAC) and lactate transportation (with AZD3965) results in a reduction in media lactate levels, suggesting this would lead to a reduction in acidification in the tumour microenvironment in vivo, and in theory impart an overall suppression on tumour activity (invasion and dissemination). In an effort to examine any potential synergistic effect, I performed a preliminary analysis examining how combination treatment of both AZD3965 and NAC may impact on media lactate (fig 29). During these experiments I also briefly investigated the use of metformin (1mM) as an alternative mode of anti-oxidative attack, with it's mode of action consisting of interacting with the ND3 subunit of the mitochondrial respiratory chain complex, hence inhibiting the ETC and impacting ATP synthesis. This approach towards both oxidation and lactate shuttling may also impart mechanistic information as to how this lactate is being utilised in the context of inhibition of oxidation with NAC/metformin treatment. As previously demonstrated both AZD3965 and NAC significantly reduced media lactate levels in both solo and PSC-co-culture conditions ($p<0.05$ in all conditions). Meanwhile combination treatment of AZD3965 and NAC (AN) only demonstrated a synergistic effect with solo treatment of Miapaca2 (reduction of 19% with AZD3965, 40% with NAC, and 65% with both treatments; $p<0.001$), whilst this was not replicated in co-culture. With respect to metformin, treatment did not impart any effect on lactate concentration in all cell line conditions, and as such unsurprisingly no synergistic effect was seen in combination with AZD3965 +/- NAC (AMN). In view of these findings and due to time constraints I did not pursue any further combinatorial treatment strategies.

Fig 29 Effect of combination treatments with AZD3965 (A), metformin (M) and NAC (N) on extra-cellular lactate



#### 6.2.2.2 Influence on PSC inflammatory profile

As evidenced thus far, it appears the underlying metabolic phenotype differs between each PDAC cell line, and therefore affecting their resultant response to anti-metabolic therapy. I have theorized that the difference may be contributed to by the difference in MCT expression profile and underlying phenotype. However experiments have also shown how PSCs tend to affect both the PDAC metabolism (induction of glycolysis) and response to treatment. In view of this, it is important to consider how the PSCs are being affected by treatment, both with respect to their own metabolism, as well as any additional effects on their activity that may translate to an influential impact *in vivo*. Furthermore the apoptotic effect of MCT1 inhibition towards PSCs in the presence of Panc1 and Miapaca2 suggests therapy may be influencing PSCs more so than the cancer cells, and therefore represents a promising method of shutting down PSC activity and their associated pro-tumourigenic interactions within the microenvironment.

Initially I examined how AZD3965 treatment affected αSMA expression, which has already been demonstrated to be upregulated in the presence of PSCs in the Panc1 and Miapaca2 but not Bxpc3 lines. Herein it was revealed that in co-culture with Panc1 and Miapaca2 treatment resulted in a significant and dramatic reduction in αSMA expression in the PSCs by 97% ($p=0.0004$) and 74% ($p=0.0034$) respectively as compared to expression levels in co-culture without treatment (fig 30). However no effect was seen following treatment in co-culture with Bxpc3. This is a particularly noteworthy effect, suggesting MCT1 inhibition is somehow deactivating PSCs that could have a significant effect in vivo.

I then went on to correlate this with any potential effect on the inflammatory profile of PSCs by looking at IL6/IL8 expression (fig 31); again in co-culture with Panc1 and

Miapaca2 a significant reduction in IL6 expression in the PSCs was observed by 84% (p=0.0004) and 42.2% (p=0.0016) respectively as compared to co-culture without treatment, whilst there was a significant increase in IL6 expression in co-culture treatment with Bxpc3 by 64.2% (p=0.0008) , whilst no effect was seen on IL8 expression.

At the same time, I deemed it worthy to also examine the effect on PDAC interleukin expression. Herein solo treatment resulted in a significant increase of IL6 in Panc1 (3-fold; p=0.022) and Miapaca2 (2.8-fold; p=0.0005) and Bxpc3 (3.3-fold; p=0.0002) expression of IL6, however this was not replicated in co-culture in any of those cell lines (fig 31).

Therefore one may deduce that AZD3965 treatment is exerting a “deactivating” (reduced αSMA) and anti-inflammatory (reduced IL6) on PSCs, in combination with an apoptotic effect. Certainly this may herald great promise in an in vivo setting.

Fig 30 – Effect of AZD3965 on αSMA expression in PSCs in solo and PDAC co-culture conditions

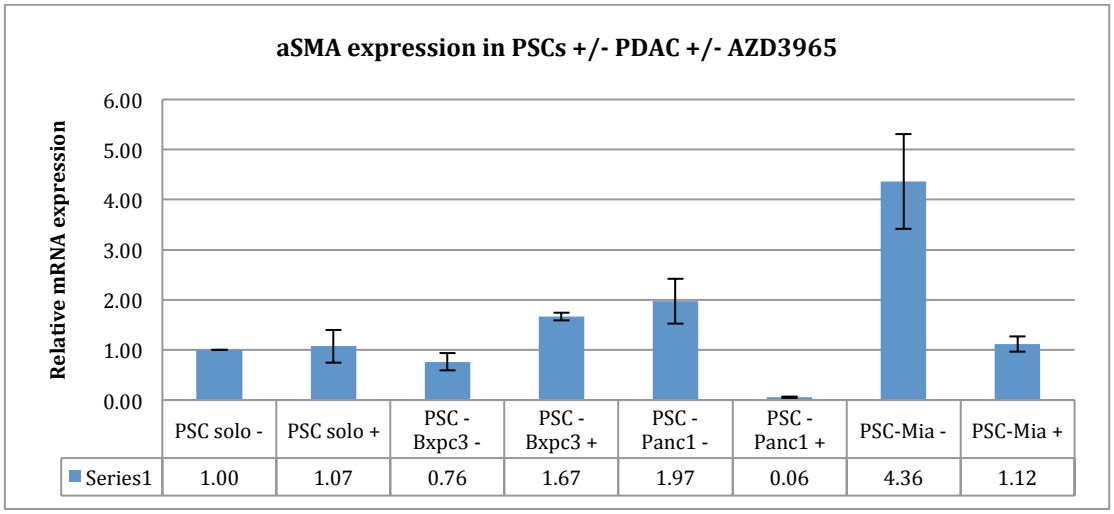
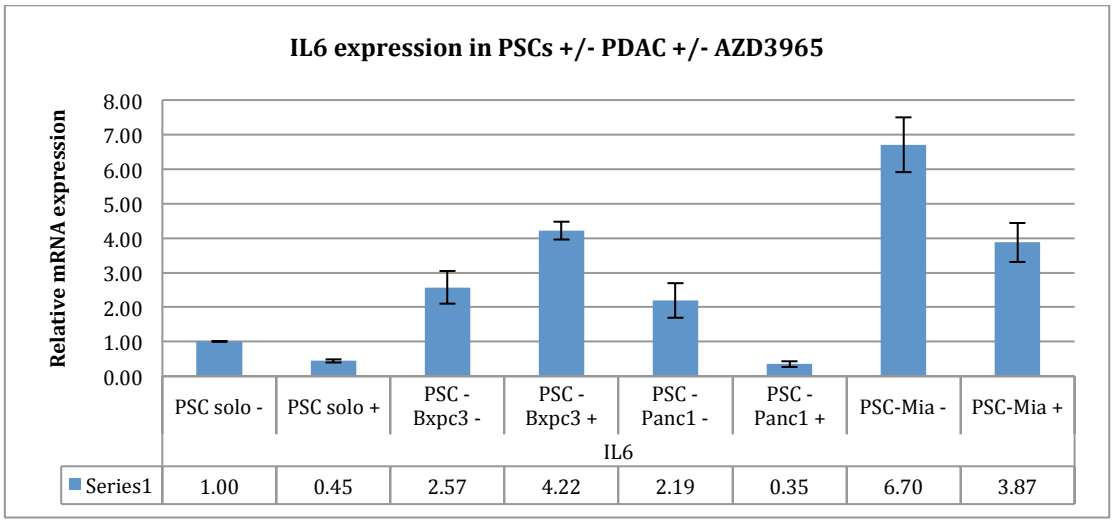
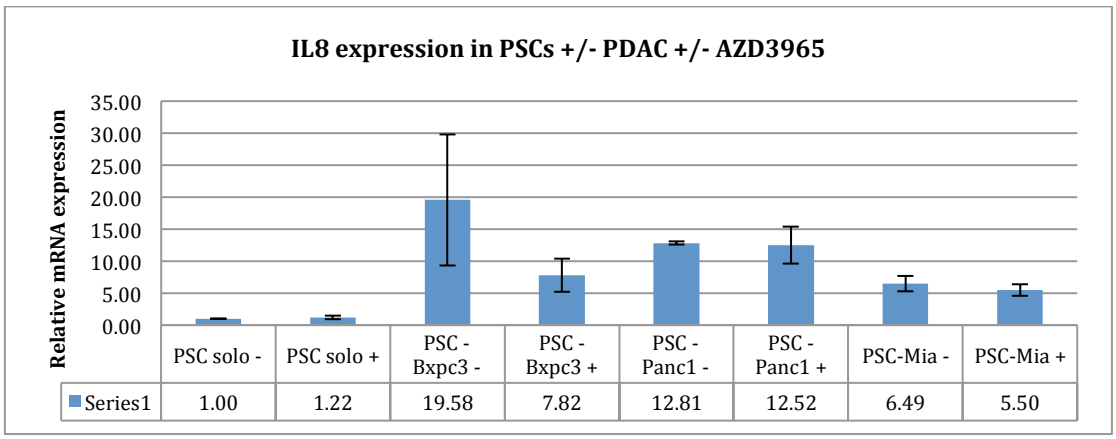
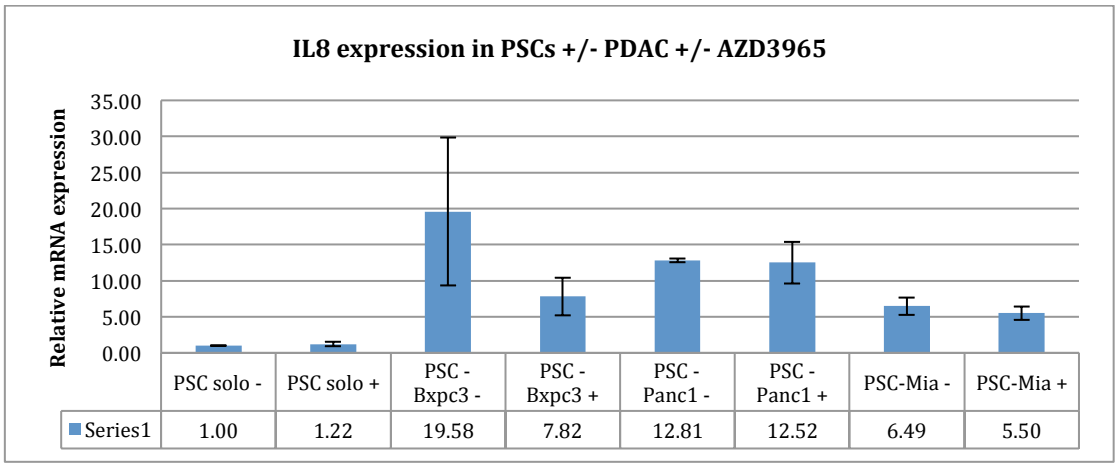
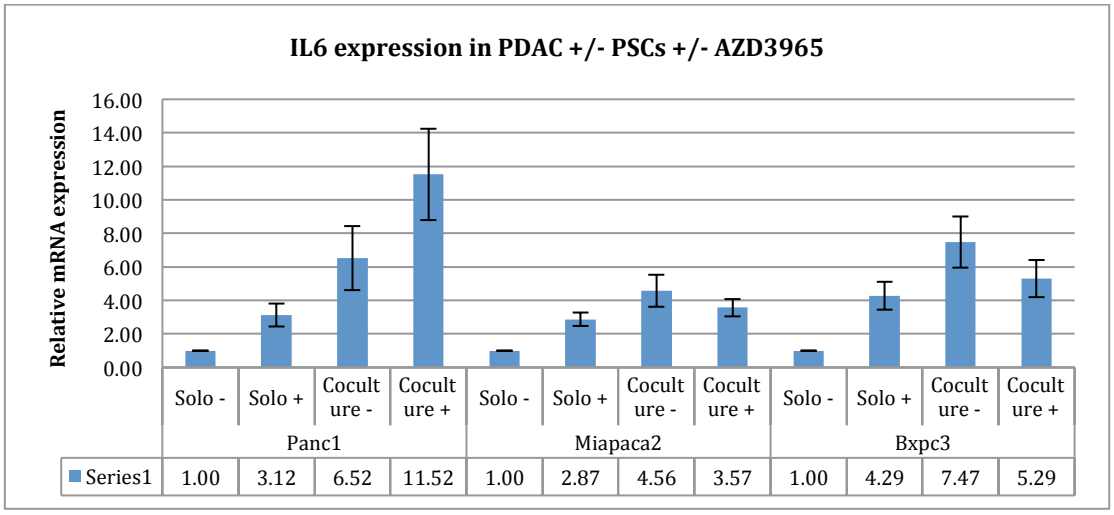


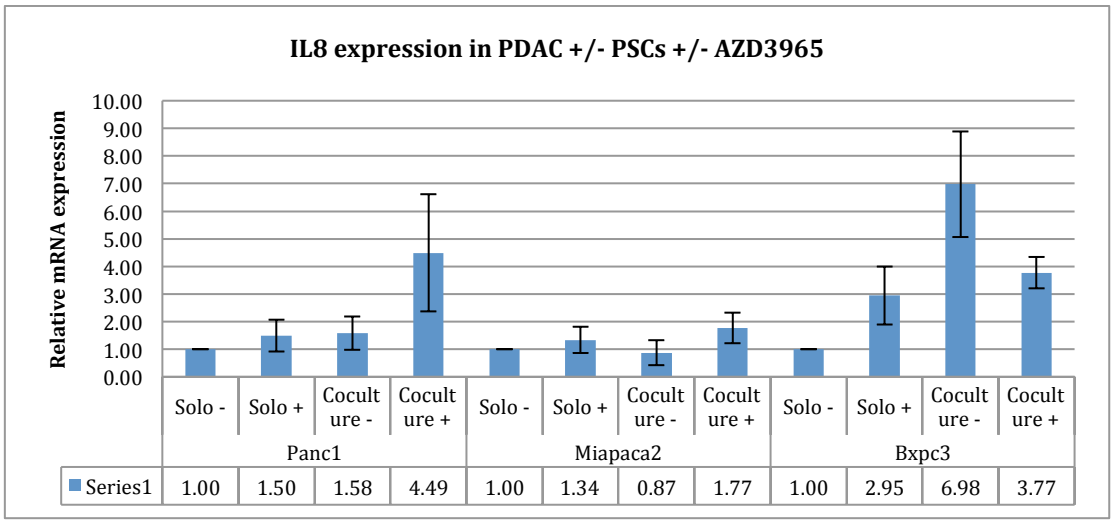

Fig 31 Effect of AZD3965 on interleukin (IL6 and IL8) expression in PDAC and PSCs in solo and co-culture conditions




IL8 expression in PSCs +/- PDAC +/- AZD3965
Relative mRNA expression
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
PSC solo -
PSC solo +
PSC - Bxpc3 -
PSC - Bxpc3 +
PSC - Panc1 -
PSC - Panc1 +
PSC-Mia -
PSC-Mia +
Series1
1.00
1.22
19.58
7.82
12.81
12.52
6.49
5.50


IL6 expression in PDAC +/- PSCs +/- AZD3965
Relative mRNA expression
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Solo -
Solo +
Cocult ure -
Cocult ure +
Panc1
Miapaca2
Bxpc3
Series1
1.00
3.12
6.52
11.52
1.00
2.87
4.56
3.57
1.00
4.29
7.47
5.29


IL8 expression in PDAC +/- PSCs +/- AZD3965
Relative mRNA expression
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Solo -
Solo +
Cocult ure -
Cocult ure +
Panc1
Miapaca2
Bxpc3
Series1
1.00
1.50
1.58
4.49
1.00
1.34
0.87
1.77
1.00
2.95
6.98
3.77

### 6.2.3 LDH inhibition

Given the increased evidence as a poor prognostic predictor, LDHA inhibition has received most attention in drug discovery. FX-11 is a gossypol derivative demonstrated selectivity of LDHA over LDHB through preferential inhibition of LDH5, with enhanced efficacy in cells with a tendency towards the glycolytic phenotype.[274] In vitro treatment demonstrates efficacy against lymphoma, renal and breast cancer cell lines, with induction of oxidative stress, reduced ATP and apoptosis, whilst in vivo treatment of PDAC inhibits tumour progression. [203]

As discussed, LDHB allows cells to use lactate as a source for oxidative metabolism through conversion to pyruvate, and its association with cancer is more complex and varied. For example it is silenced by promoter methylation in some cancers such as prostate[266], however over-expressed in others such as KRAS mutated human lung adenocarcinoma.[267] Therefore the most appropriate method of LDH inhibition (LDHA/LDHB/both) is unclear and will vary depending on the tumour type, associated genetic landscape and metabolic phenotype. Nonetheless, theoretically in PDAC, if one can reduce protumourigenic acidification, or remove lactate as a metabolite source within the reprogrammed microenvironment, LDH inhibition represents an ideal therapeutic approach.

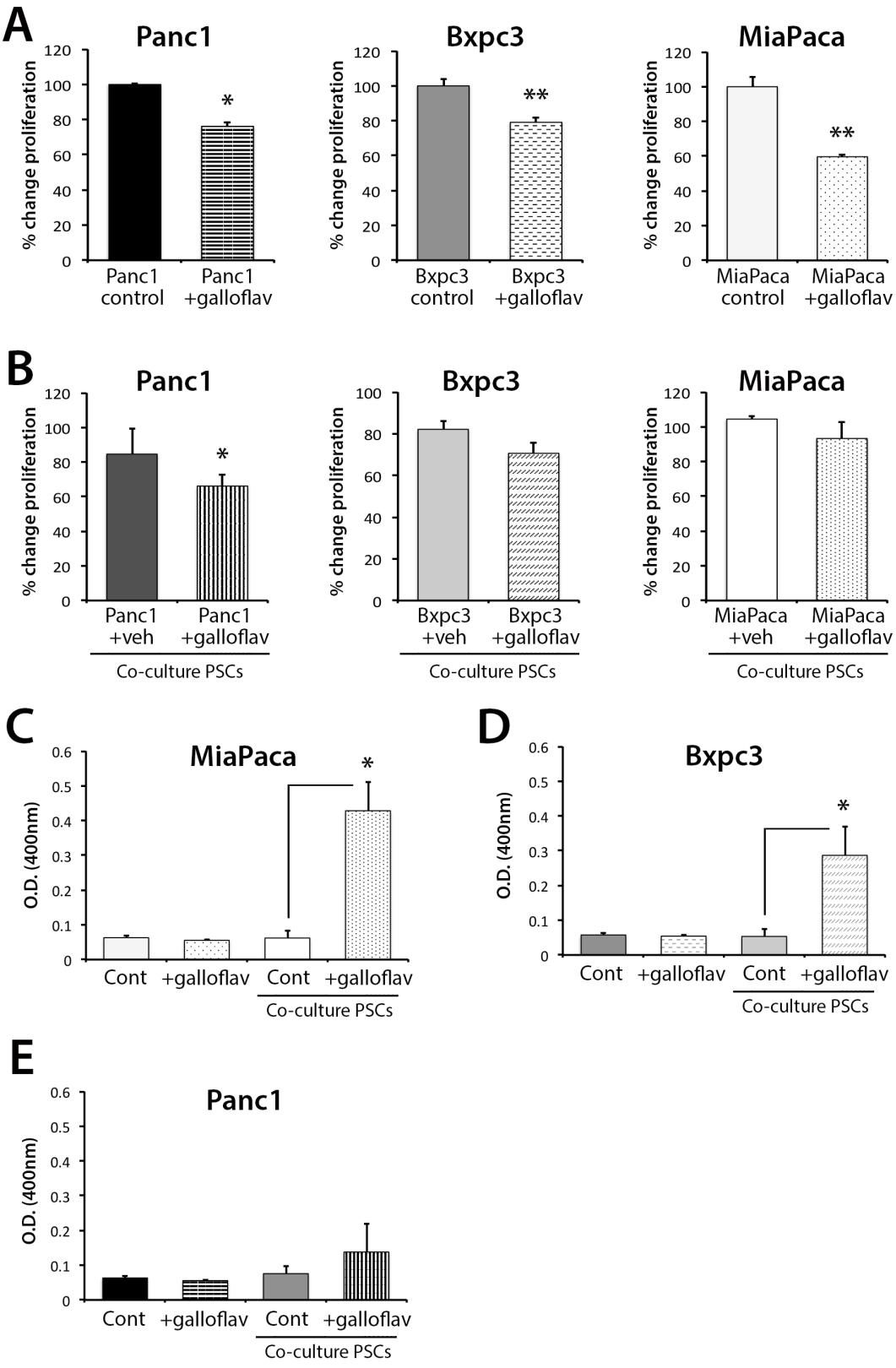
More recently much work has been geared towards fabricating novel LDH inhibitors which may be translated into clinic. Through this drug discovery galloflavin has been identified as a novel LDH inhibitor which targets both isoforms, with demonstrated efficacy against hepatocellular and breast carcinoma cells resulting in reduced lactate and ATP production.[275-277] The drug was well tolerated in preliminary murine studies,

suggesting promise for being translated into the clinic. However efficacy against PDAC is yet to be established and I aimed to investigate this.

Treatment of all three PDAC cell lines in monoculture with the LDH inhibitor Galloflavin (100uM) led to a reduction in proliferation (Fig 32A; $p<0.01$). In contrast when these cells were co-cultured with PSC's both the MIAPaCa-2 and BxPC-3 cell lines were protected from the anti-proliferative effect of Galloflavin (Fig 32B) although there was still a 34% reduction in proliferation in the PANC-1 cell line ($p=0.0012$).

Galloflavin had no impact on the apoptotic rate of any of the cell lines in monoculture experiments (Fig 32 C-E). In contrast there was a significant increase in the proportion of apoptotic cells when the PDAC cell lines MIAPaCa-2 (6.9 fold; $p=0.03$) and BxPC-3 (5.4 fold; $p=0.04$) were co-cultured with PSC's in the presence of Galloflavin. Whilst there was a trend towards increased apoptosis in the PANC-1 cell line this did not reach statistical significance.

Figure 32 – Effect of galloflavin on proliferation (MTT) in solo conditions (32A) and co-culture (32B); effect on apoptosis in both solo and co-culture (32C-E) [290]



To determine the impact of LDH inhibition on extracellular lactate production the PDAC cell lines were cultured either alone or in the presence of PSCs and treated with Galloflavin. In monoculture experiments there was no impact of LDH inhibition on the concentration of extracellular lactate (Fig33A) whereas in the presence of PSCs there was a significant reduction in the concentration of extracellular lactate in all 3 PDAC cell lines; PANC-1 (6.47 to 5.27 mmol/L; p=0.006), MIAPaCa-2 (5.47 to 4.1 mmol/L; p=0.008) and BxPC-3 (5.1 to 4.5 mmol/L; p=0.007) (Fig 33B).

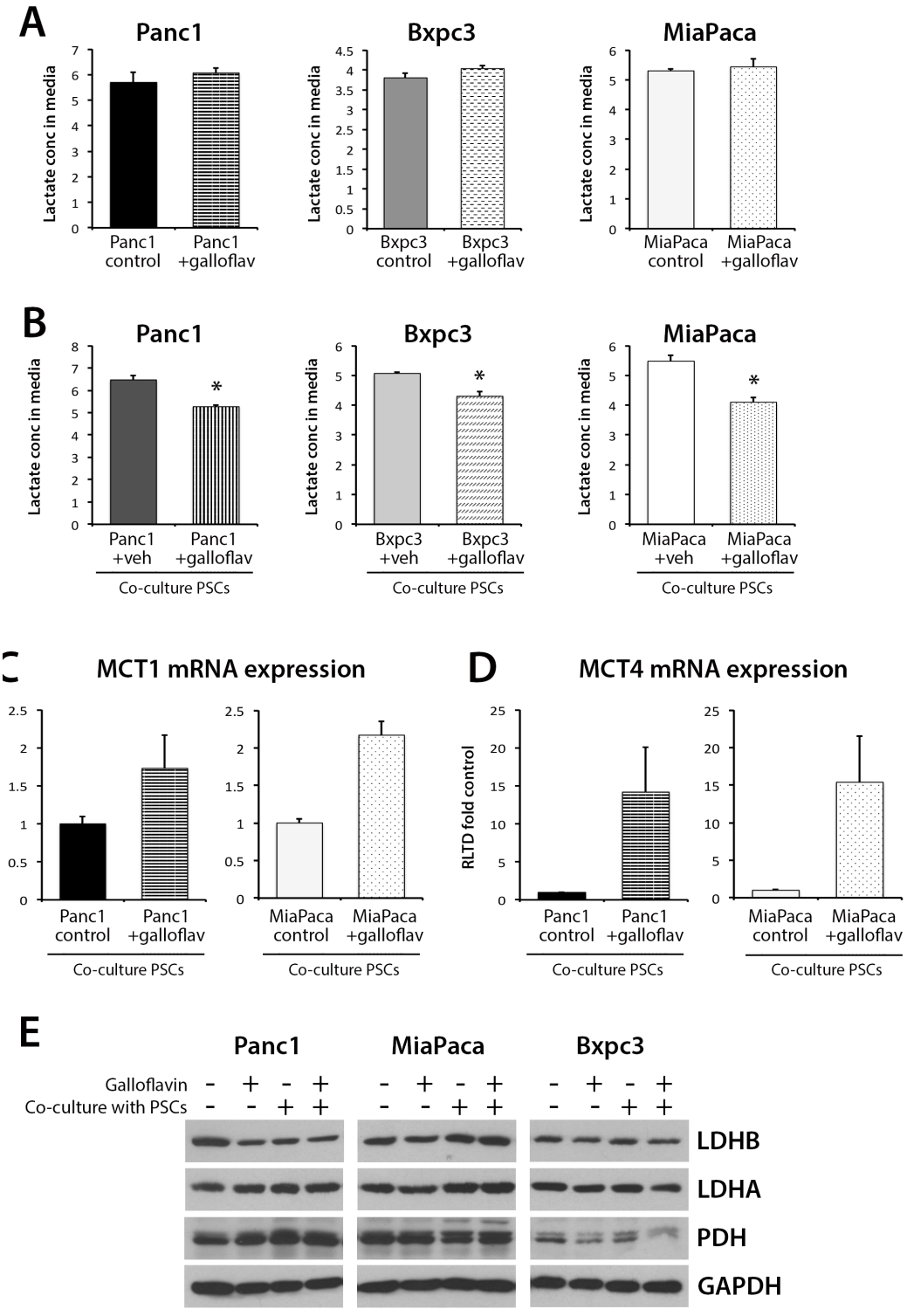
To determine if the reduction in extracellular lactate concentration was a consequence of altered MCT expression we undertook qRT-PCR of RNA from the PDAC cell lines PANC-1 and MIAPaCa-2 co-cultured with PSC's in the presence of Galloflavin or its vehicle control. MCT expression was unchanged (figure 33C-D); however there was a trend towards increased expression of MCT4 in MIAPaCa-2 of 15.4 fold (p=0.08).

In further efforts to explain the differing effects of Galloflavin on the PDAC cell lines we undertook Western blot to determine the impact of treatment on cellular LDHA, LDHB and pyruvate dehydrogenase (PDH). The latter is a glycolytic enzyme which catalyses the conversion of pyruvate to acetyl-co enzyme A, allowing its usage for mitochondrial respiration and diverting the path of pyruvate away from lactate production, therefore any effects on this enzyme as a result of pyruvate/lactate disruption by Galloflavin may be determined.

Galloflavin exerted no effect on LDHA expression (Figure 33E). There was significantly less overall PDH expression in the BxPC-3 cells as compared to PANC-1 and MIAPaCa-2. LDHB protein expression was reduced in the PANC-1 cell line when treated with Galloflavin in

monoculture. When PANC-1 cells were co-cultured with PSCs a similar effect was seen although there was no additive effect on LDHB expression with Galloflavin treatment. In contrast, co-culture of MIAPaCa-2 cells led to an increase in LDHB expression, regardless of Galloflavin treatment, whilst treatment in co-culture resulted in increased expression of PDH. Therefore one may conclude that whilst Galloflavin does not consistently affect LDHB expression, the contrasting effects of PSCs on the PANC-1 and MIAPaCa-2/BxPC-3 cell lines suggests they are exerting differing effects on the metabolic phenotype which in turn will affect the response to glycolytic targeted therapy.

Figure 33 – Effect of galloflavin on extra-cellular (media) lactate in solo (33A) and co-culture (33B); effect on MCT1 (33C) and MCT4 (33D) gene expression and protein expression of LDHA, LDHB and PDH (33E) [290]



## 6.3 Discussion

Many of the key learning points from the results have already been discussed, however I will now present a number of questions to summarise the findings and suggest hypotheses for the findings along with future directions.

### 6.3.1 Anti-oxidative or anti-glycolytic approach?

As discussed, in the infancy of this project I decided upon investigating means of targeting glycolytic mechanism/lactate shuttling, as opposed to anti-oxidant approaches, given the glycolytic nature of PDAC, combined with the fact this avenue of attack had been under-investigated in the literature with respect to MCT or LDH inhibition. Nonetheless it is prudent to consider whether anti-oxidant therapy may be more beneficial given existing evidence within the literature.

N-acetylecysteine is an aminothiol and a precursor of intracellular cysteine and glutathione (GSH), and exerts an anti-oxidant effect through free radical scavenging actions by increased GSH levels.[278] Interestingly it has been shown that NAC induces cell cycle arrest in hepatic stellate cells through modulation of the mitogen-activating-protein (MAPK) pathway.[279] Similarly it has been demonstrated recently that NAC induces G1 cell cycle arrest in the PDAC cell line AsPC-1, with associated reduced growth, metastatic potential and invasiveness.[280] Whilst that study did not demonstrate increased susceptibility to the chemotherapeutic agent gemcitabine, conversely a study using the Miapaca2 cell line found NAC prevents NFkB activation which in turn enhances sensitivity to gemcitabine, with combination treatment decreasing tumour growth by 50% *in vivo*.[281]

Despite these promising results, interestingly more recent data actually demonstrates that pro-oxidant approaches may be most beneficial in combination with other therapies. It has been shown that the redox master regulator NRF2 is necessary for PDAC proliferation, with NRF2 loss disrupting EGFR signaling and reduced growth. By targeting EGFR through AKT inhibition in combination with the pro-oxidant BSO, the anti-proliferative effects of NRF2 loss was mimicked.[282] This emphasizes the fact that other means of targeting metabolism, regardless of their effect in isolation, should be considered in combination with other drugs as synergistic effects may enhance efficacy and provide overall anti-tumour benefits, in this case by enhancing rather than inhibiting oxidation. Hence the reason for performing the combination experiments with AZD3965, albeit this demonstrated no synergy.

In this project, NAC treatment in the presence of PSCs led to an increase in Miapaca2 activity, however no effect was observed towards PSCs as previously seen with HSCs. Whilst no effect was seen with Panc1, and furthermore no effect with BSO, one may conclude that anti-oxidant approaches may not be globally efficacious in vivo due to the effect of stromal elements such as PSCs on any beneficial effect that may be seen in vitro. Nonetheless, it was observed that NAC treatment globally reduced extracellular levels, both in solo and co-culture treatment of all PDAC cell lines and PSCs, and ultimately this should translate into in vivo benefit given the association of lactate with tumour invasion and dissemination.[177, 178] In view of these findings, NAC was investigated in combination with MCT1 inhibition, and determine whether this significantly influences lactate shuttling. Only a synergistic effect was seen when treating Miapaca2 in solo conditions with a reduction in extra-cellular lactate, whilst this was not replicated in co-culture, and no synergistic effect was seen in the other 2 PDAC cell lines. Therefore, again given the

influence of PSCs in vivo, one may conclude that NAC treatment in combination with MCT1 inhibition is less likely to exert a beneficial effect in vivo.

Sodium azide was also used to investigate any anti-proliferative effects in this co-culture model. This compound is typically used in industry and agriculture, and is well known to induce cell death in tissue culture, however is highly toxic nature makes it not applicable to clinical use.[283] Nonetheless, due to its inhibitory effect on respiratory complex IV, its use can impart useful information regarding metabolic reprogramming in PDAC tumourigenesis. However in this project 100uM treatment in co-culture exerted no significant effect with respect to metabolic activity in MTT assays. It has previously been shown that solo treatment of the Miapaca2 cell line with NaN3 (100uM) increases glucose consumption and lactate production, as well as increasing MCT1 and MCT4 expression.[284] With this in mind, combined with the preliminary results and the fact this compound has limited clinical applicability; it was not investigated further in solo or combinatorial treatment regimes.

Given the literary evidence presented, inhibitory approaches towards oxidative processes is still a potentially viable means of disrupting energy production in solid organ tumours. However my experimental data suggested a means of attacking glycolysis may prove beneficial, and therefore the remainder of the project concentrated on this approach.

### 6.3.2 MCT1 inhibition

The difficulty in performing studies to investigate cancer metabolism is due to the fact there is continual flux in the presence/concentrations of metabolites as they are consumed as a fuel and produced as a byproducts, with lactate representing both of these outcomes and therefore making it even more challenging to determine it's passage of utilization within the complex metabolic pathways. This is ideally demonstrated by the lactate assays performed with AZD3965 treatment, whereby the intra- and extra-cellular results do not entirely equate.

Given its strong correlation with many malignancies, MCT1 inhibition has been heralded as a promising therapeutic approach in disrupting lactate metabolism. The concept of MCT1 inhibition is complicated by the fact it transports lactate in both directions. If we consider inhibition towards the efflux function, it has been demonstrated that MCT1 inhibition decreases intracellular pH, thus inducing apoptosis and reversing chemoresistance. However, in contrast inhibition towards the influx capacity of MCT1 can potentially exacerbate extracellular acidification, resulting in detrimental effects through increasing invasiveness and metastasis. Despite this latter effect, studies thus far of MCT1 inhibition has demonstrated largely beneficial results. Well established MCT1 inhibitors such as a-cyano-4-hydroxycinnamate (CHC), stilbene disulfonates (DIDS/DBDS), p-chlorormercuribenzene sulfonate (pCMBS) and phloretin have been shown to reduce lactate influx by approximately 80% in normal hepatocytes.[285] However issues with these inhibitors centre upon the inhibitory effect towards other transporters, thus lacking specificity towards MCTs. For example CHC is twice as potent an inhibitor towards the mitochondrial pyruvate transporter, whilst DIDS and DBDS inhibit the chloride/bicarbonate exchanger AE1. cCMBS and phloretin are equally non-specific. [217]

MCT2 has shown sensitivity to inhibition with CHC, DIDS and DBDS, emphasizing the non-specific inhibitory effect of these drugs. However it is not sensitive to pCMBS, as this drug only targets CD147 (BASIGIN)-associated MCTS such as MCT1, whereas as MCT2 binds to EMBIGIN.[229] DIDS and CHC have limited inhibitory effects against MCT4, whilst achieving >50% inhibition of MCT1. As such further work is required to define the exact structure of this MCT isoform and thus permit improved drug development to specifically target MCT4, which may be a particularly beneficial approach in hypoxic tumours such as PDAC.

Astrazeneca made a significant development more recently with the fabrication of AZD3965, which has high specificity and affinity for MCT1, with Ki values in the nM region. This drug was originally developed to block proliferation of activated T cells, given the role of MCT1 identified in immune biology.[286] However subsequent studies revealed an alternative target of lactate metabolism, whereby the growth and tumourigenicity of Ras-transformed fibroblasts was impaired[273]; given both the KRAS-driven nature of PDAC and fibroblast-rich stroma, this suggests MCT1 inhibition is an intriguing avenue of therapeutic targeting in PDAC, with no studies in the literature investigating this approach, despite the associations of PDAC with hypoxia, acidification and increased uptake of lactate.[177]

The heterogeneity in the results presented comparing the different cell lines support the theory that response to treatment will be dependent on the metabolic phenotype of a specific tumour. Table 15 summaries the key differences between the 3 cell lines when treated in the clinically relevant co-culture condition, along with the key underlying factors of each which may explain the differences in response. In conclusion, I believe a tumour

with a similar genetic signature to Panc1 is more likely to respond favourably to MCT1 inhibition, whilst it is debatable whether treatment of a more glycolytic tumour representative of Miapaca2 would be of benefit given the increase in PSC proliferation observed. However given the latter point, the anti-inflammatory effect of AZD3965 in reducing αSMA and IL6 expression is of promise. As ever *in vivo* studies would be the next step in determining which patients, whether it be all, a select few, or none, would benefit, depending on both response/efficacy and toxicities.

Table 15 – Summary of key differences in response to AZD3965 across the PDAC cell lines

| | | **Miapaca2** | **Panc1** | **Bxpc3** |
|---|---|---|---|---|
| *Confounding factors* | *Phenotype* | *Glycolytic* | *Lipogenic* | *Lipogenic* |
| | *KRAS* | *+* | *+* | *-* |
| | *MCT4* | *+++* | *++* | *+* |
| Response AZD3965 | Metabolic activit | ↓in normoxia hypoxia | ↓in hypoxia | No change |
| | Glycolysis | No change | ↓ECAR+ G-ATP | ↑ ECAR |
| | Oxidation | No change | ↓OCR | ↓OCR |
| | MCT1 | No change | ↓ PSCs | ↓ PSCs |
| | MCT4 | ↑cancer cells ↓ PSCs | ↓in PSCs | No change |
| | PSC proliferatio | ↑ | No change | No change |
| | PSC apoptosis | ↑ | ↑ | ↑ |
| | PSC αSMA | ↓ | ↓ | ↓ |
| | PSC IL6 | ↓ | ↓ | ↑ |

One finding worthy of discussion, and it's potential association with in *vivo* benefit, is the fact an anti-proliferative effect on Miapaca2 was observed in hypoxia however not normoxia, whilst oxygen concentrations did not influence the anti-proliferative effect on Panc1 cells. This is perhaps explained by the fact Miapaca2 is more reliant on MCT4, and when deprived of oxygen the cells are unable to compensate for the lack of MCT1 functionality, leading to the reduction in proliferation. This very much suggests the mechanism of action is dependent on the presence of hypoxia and the associated Warburg Effect, and with this in itself being a key hallmark of cancers, and particularly in PDAC, this adds further weight towards the potential translational promise of this compound.

It is worth noting that when considering MCT inhibitory tactics, it is important to account for the action of other MCTs, which may pick up the workload of lactate movement. It is therefore possible that response to MCT1 inhibition may be dependent on the form of metabolic reprogramming in each particular tumour type, and understanding this process will help determine whether selective or pan MCT inhibition is the most appropriate therapeutic approach. With respect to the results presented, it appears MCT1 inhibition does not consistently affect the gene expression of MCT4 in either PDAC or PSC cell types, with the only notable result being observed with a significant reduction in MCT4 expression in PSCs when treated in co-culture with Miapaca2. Therefore the mechanism of effect may more impact on lactate shuttling, subsequent effects on metabolic processes, and be very much dependent on the onco-metabolic phenotype of the specific tumour cells. Given the IHC results showing positive correlation between tumoural and stromal compartments with respect to MCT1 and MCT4 it may be that in those tumours that are susceptible, pan-inhibition may be the best tactic i.e. if expressing both MCT1 and MCT4 at

a high level. Furthermore it may be postulated that multiple metabolic phenotypes, representative of all cell lines tested, are present within the same tumour.

When considering the underlying metabolic relationship between PDAC and PSCs, with particular respect to lactate shuttling, the results suggest MCT1s may function differently depending on the tumour phenotype involved. This was most represented by the fact MCT1 inhibition led to reduction in lactate (intracellular and extracellular) with Panc1 and Bxpc3, however not so with Miapaca2, which may suggest that MCT1 is more influential as a "lactate effluxor" in the former 2 cell lines, and conversely a "lactate influxor" in Miapaca2. However the fact PSCs maintained their MCT1 expression (whilst a reduction was seen in Panc1) suggests they also play a role in avoiding this neutralizing effect.

Further examination with MCT4 knockdown would certainly help add further understanding not only to the exact function of MCTs in the PDAC microenvironment, but also whether combined MCT1+4 inhibition is a therapeutic strategy worth pursuing. Considering the IHC results showing positive correlation between tumoural and stromal compartments with respect to MCT1 and MCT4 it may be that in those tumours that are susceptible, pan-inhibition of both transporters may be the best tactic in select patients i.e. if expressing both MCT1 and MCT4 at a high level.

Finally, and needless to say, when considering clinical translation, toxicities need to be considered, given targeting MCTs can impact on the inflammatory and angiogenic response, with particular concerns over the effect on cardiac and skeletal muscle fatigue from intra-cellular lactate accumulation. A phase 1 trial of AZD3965 is currently underway

for advanced solid tumours and diffuse large B cell lymphoma (NCT01791595), which will help to determine the safety of this drug in clinical practice.

### 6.3.3 LDH inhibition

Strategies targeting glycolysis such as inhibition of the glucose transporter Glut1 or various glycolytic enzymes have proven efficacious in both *in vitro* and *in vivo* studies but the availability of safe and well tolerated drugs has limited translation into clinical practice [194, 196, 287]. Similarly the glycolytic enzyme LDHA has been targeted in PDAC with the compound FX-11 that suppresses tumour progression *in vivo* [203]. However again it has proven challenging to convert this into a clinically applicable drug.

Previous work by Mohammed et al. has demonstrated high levels of tumoural LDHA expression to be a predictor of poor survival in PDAC [197]. In this study herein we have not observed such an association for LDHB with neither stromal nor tumoural expression being linked with prognosis, although this may represent a type 2 error given the relatively small number of patients in this cohort. Our observation regarding LDHB may however be correct since it is associated with lactate to pyruvate conversion and would therefore not be predominantly expressed in a typically glycolytic PDAC. However given that cells with access to oxygen on tumour peripheries are able to undergo oxidative phosphorylation and utilise lactate for ATP production in greater abundance [219] one may deduce that despite the high rate of negative LDHB expression in tumour and stroma (57% and 73% respectively), strategies towards LDHB inhibition are still worthy of pursuit in order to decrease energy production in the oxidative peripheral tumour cells. The positive correlation between tumour and stromal LDHB expression is also noteworthy, suggesting that the metabolic change in either compartment are duplicated and therefore inhibiting LDH has the potential to target both tumoural and stromal entities.

The concept of targeting both isoforms of LDH in PDAC is further supported by the observation that elevated pre-treatment serum levels of LDH are associated with an adjusted HR of 2.47 for death [288], whilst low levels are associated with prolonged PFS and OS in PDAC patients treated with sorafenib [213]. The pan LDH inhibitor Galloflavin is a promising agent for such an approach with proven efficacy against breast, endometrial and hepatocellular carcinoma cell lines *in vitro* [275-277] although until now its utility in PDAC has not been examined.

In this study we have also demonstrated the ability of Galloflavin to exert a mixed effect on proliferation and apoptosis on PDAC cell lines *in vitro*, with the efficacy of the drug strongly associated with the presence of PSCs in the cell culture system. It is therefore important to determine how PSC's influence the efficacy of Galloflavin treatment so that we are able to predict which patients might benefit from LDH inhibition. We have demonstrated a significant reduction in proliferation in PANC-1 cells treated with Galloflavin both in monoculture and co-culture with PSC's. In contrast the antiproliferative effects of Galloflavin are reversed in both the MIAPaCa-2 and BxPC-3 cell lines when PSC's are added into the culture system. With regard to the MIAPaCa-2 cells this is most likely explained by the increase in PDH protein demonstrated in *in vitro* by co-culture with PSC's, suggesting pyruvate is being diverted towards acetyl CoA production, rather than lactate entering the TCA cycle for ATP production. Alternatively the increase in LDHB expression in co-culture suggests PSCs are facilitating the utilization of lactate in the MIAPaCa-2 cells, allowing for increased ATP production through oxidative phosphorylation. In a similar manner the K-RAS wild type phenotype of BxPC-3 cells may mean they are less reliant on glycolysis and able to utilise oxidative phosphorylation as a means of energy production. These differing treatment responses are supported by the previously referenced work by Daemen et al

which stratified PDAC cell lines into distinct metabolic categories with BxPC-3 displaying a lipogenic as opposed to glycolytic phenotype with a subsequent impact on response to a variety of metabolic inhibitors [251].

In contrast we only see a significant apoptotic effect of Galloflavin when treating the MIAPaCa-2 and BxPC-3 cell lines in the presence of PSCs. These results suggest that some tumours (such as those with a phenotype similar to the Miapca2 or BxPC-3 cell line) may undergo apoptosis with Galloflavin treatment yet retain the ability to maintain proliferation through further metabolic reprogramming alterations potentially related to PSC-interactivity. We can therefore hypothesise that Galloflavin treatment of tumours with lower LDHB expression and a less profound glycolytic phenotype (similar to PANC-1) is likely to have a beneficial impact on proliferation, adding further support towards the need to stratify the metabolic profile of tumours to assist in optimal patient selection in clinical trial design.

Whilst it is possible that not all tumours may respond to Galloflavin, the most promising result is the consistent effect on reduced lactate acidification of the media. This occurs regardless of the impact on proliferation, apoptosis or glycolytic enzyme expression. In keeping with an effect on lactate metabolism, we demonstrated a trend towards significance regarding treatment in co-culture causing upregulation of MCT1 and MCT4. We postulate that through its impact on both LDHA and LDHB, Galloflavin is indirectly affecting MCT expression due to increased lactate flux. This trend towards upregulation is likely to be purely functional and unlikely to incur poor prognostic outcomes concerning MCT4 as previously documented by others [254]. We hypothesise that if this effect is mimicked *in vivo* the over-riding neutralization of the acidic microenvironment could lead to a

significant cytotoxic effect in tumours which the appropriate metabolic phenotype. Furthermore, given LDHA inhibition has been shown to act synergistically with gemcitabine [289], it is certainly feasible that a tumour-personalized approach with Galloflavin (or a clinically translatable derivative) could be used. This could be applicable to both neoadjuvant and adjuvant settings alongside, with the aim of disrupting lactate metabolism thereby exerting anti-tumoural effects through energy starvation and sensitizing tumours to chemotherapy.

# Chapter 7

# Conclusions

## 7.1 Concluding discussion

Within this summarizing discussion, I will chronologically and briefly summarise the thought processes that went about the evolution of this project whilst summarizing potential theories, limitations and ideas on future directions.

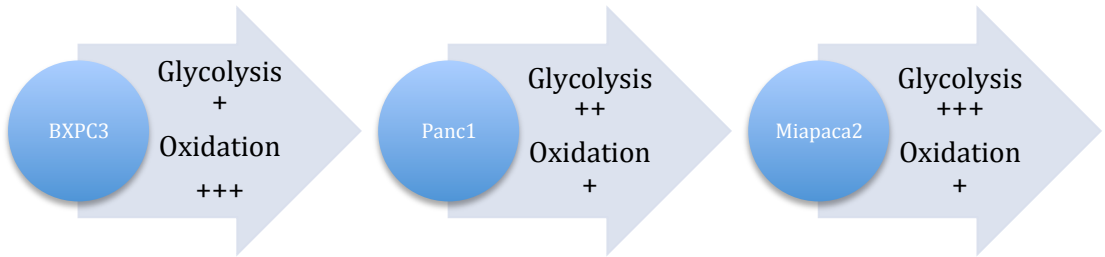
In the initial stages of the project, it was imperative to validate the in vitro experimental methodology being undertaken to confirm it as a reliable means of assessing metabolomics treatment effects with respect to both the impact of different cell types on each other, and the results of drug treatment. The baseline validation of the transwell model confirmed this as a viable approach, with the known pro-inflammatory effect of PSCs being seen to directly impact on each individual PDAC cell line with respect to proliferation, metabolic activity and chemokine expression. Conversely an increase in αSMA was seen in PSCs when in coculture with PDAC, confirming a symbiotic pro-inflammatory relationship, albeit this is dependent on KRAS mutation given the absence of effect seen with Bxpc2.

With the latter finding in mind, it was noteworthy to see at this early stage that the cell lines differed in the degree of PSC treatment effect, and this is highly reflective of the heterogeneous nature of PDAC, which has been increasingly demonstrated upon at a genomic level, however metabolic heterogeneity is a concept less investigated, despite the fact emerging research suggests targeting cancer metabolism can not only inhibit tumour growth but also assist in the delivery and efficacy of other therapeutics. With this in mind it

was prudent to demonstrate the differing metabolic phenotypes in the various cell lines, a concept which has not been published on previously. This was done using the initial seahorse experiments which revealed Bxpc3 to be more oxidative, with Miapaca2 the least so, which is not entirely unsurprising given the influential effect KRAS has on the Warburg effect glycolytic switch, further confirmed by the fact Panc1 and Miapaca2 produced significantly more ATP by glycolysis than Bxpc3; considering the work done by Daemen et al [251], I would therefore postulate that on a spectrum of lipogenic and glycolytic phenotypes, Bxpc3 would sit on the extreme lipogenic (and ultimately more oxidative) end of the scale, with Miapaca2 considered more glycolytic (particularly given the high MCT4 expression exhibited on preliminary baseline analysis) and lastly with Panc1 somewhere in the middle of that spectrum (fig 34). Ultimately this preliminary work on the PDAC lines nicely demonstrated how they differ in metabolic behavior, which would no doubt account for any future observed differences in treatment effects.

Fig 34 – Hypothesised differences in the metabolic phenotype between the PDAC cell lines

OXIDATION>GLYCOLYSIS ⟹ GLYCOLYSIS>OXIDATION




LIPOGENIC ⟹ GLYCOLYTIC

The Warburg effect has been referred to in detail as a classical hallmark of cancer and a theory upon which much of this project was based upon considering the hypoxic-driven nature of PDAC. However when we consider the Warburg effect we are only referring to areas of tumour which are oxygen deficient, and previous studies have suggested that only 30% of the whole tumour are located in significantly hypoxic regions [177]. Therefore we must consider that glycolytic mechanisms are still occurring within cells with access to oxygen, and this is ultimately blurring any attempts to define how metabolites, and most specifically lactate, is being shuttled throughout the microenvironment. Ultimately I believe no tumour can simply be classified as having a single metabolic phenotype such as the Warburg effect and rather multiple subtypes likely exist within the same tumour, and one needs to determine which is the over-riding mechanism of energy production which is most pro-tumourigenic and thus most appropriate to therapeutically target. With that in mind, one may consider that within a single tumour, it is possible that areas of tumour all behave differently depending on its exposure to oxygen, oncogene expression and the degree of glycolytic switch that has occurred. In other words a single tumour could exhibit characteristics of both Miapaca2 and Panc1; considering this heterogeneity in metabolic phenotypes within a tumour is therefore essential when making the step from in vitro to in vivo drug discovery.

With this in mind, and in reference to the IHC analysis, we already know from previously published data that cancer cell properties within the same tumour can vary within different regions of the resected specimen [14, 255, 267] which may in part be influenced by the variability in hypoxia depending on the degree of angiogenesis. As such it may be possible that the MCT/HIF/αSMA expression profiles/ratios examined within the slice of tumour analysed may be different if an alternative slice was examined in the same patient.

The risk of this unreliable representation was limited by the aperio methodology, whereby the entire slice of tumour was analysed for protein expression, rather than smaller areas. However an additional study examining multiple areas of the same tumour for any heterogeneity in metabolic marker expression would have been useful, and if differences were observed then a mean taken as being more representative of the tumour as a whole.

Whilst this initial work examined the inherent phenotype of the various PDAC cell lines for purposes of subsequent experimental analysis, I then wished to introduce PSCs to the equation, and for the first time look at how they may be influencing PDAC metabolism. The key initial result was with respect to the pro-glycolytic effect of PSCs on all PDAC cell lines; in other words PSCs are potentially implicated in the metabolic switch (Warburg effect) conferring survival advantage in vivo and thus poor patient prognosis. This result has never been previously demonstrated, and provided yet another example of how PSC interactions are key to tumour activity and in this instance metabolic reprogramming. Not only is this finding mechanistically novel and intriguing, the presence of this relationship suggests it is possible that PSCs hold the key to cancer metabolism drug discovery in PDAC, as if they are not considered or influenced in an in vitro setting, then significantly different results may be observed *in vivo*. Furthermore in certain instances they may be the key in driving cancer metabolism and conferring a key survival advantage within the hypoxic PDAC environment.

Needless to say there are multiple factors that influence metabolism, whether it be the triggers or enzymes of certain pathways, or how the resultant metabolites affect each other or other mechanisms. However, if one concentrates on the two key processes of producing ATP, glycolysis and OXPHOS, there is an over-arching and significantly converse difference

between the two processes, and that is lactate metabolism, and it was this that I wanted to examine in more detail, whilst accounting for the pro-glycolytic PSCs. Herein I was able to demonstrate that PSCs induce significant upregulation of both MCT1 and MCT4 in all PDAC cell lines *in vitro*, thus adding support to the pro-glycolytic relationship, and adding fuel to the theory that targeting MCTs could be a promising therapeutic approach.

However before going down a drug discovery route it was essential to determine the expression profile of MCTs in PDAC and its associated stroma. Herein the prevalence of MCT1 and MCT4 in the tumour and stroma was intriguing given what we think is known about MCT function, whereby MCT1 was significantly more prevalent in the stroma than MCT4, whilst levels were similar within the tumour itself. In addition, there was a strong positive correlation of MCT1 and MCT4 between the tumoural and stromal compartments, going against the reverse Warburg effect theory. It has been suggested that whilst MCT1 is more prevalent in oxidative cells, and thus more likely to be involved in lactate influx, it still bidirectionally transports lactate. Similarly whilst MCT4 is typically seen in glycolytic cells within a hypoxic environment and expected to be more involved in lactate efflux, it can also be involved in movement in both directions. The fact there was no correlation between the hypoxia marker HIF1a and either MCT1 or MCT4 adds weight to the theory that they are not inextricably linked with oxidation or glycolysis respectively, and therefore do not preferentially import or export lactate, and rather both may bidirectionally be involved in lactate shuttling depending on the method of ATP production being used. This discovery itself was essential in deciphering what we were observing in vitro, and the finding of concurrent MCT1 and MCT4 upregulation in PDAC-PSC coculture added further support to this hypothesis.

Again with respect to the potential *in vivo* significance of MCTs, an additional key finding was of the prognostic influence of MCTs, whereby high tumoural MCT4 and high stromal MCT1 separately conferred poor prognosis, the latter being an entirely novel finding, whilst the former result is in keeping with the published data from Daemen et al, and is consistent with the idea that tumours with an improved ability to survive within the typically hypoxic environment of PDAC will be more aggressive and thus have a detrimental impact on survival. Along with the trend towards reduced OS with high tumoural MCT1 and stromal MCT4, and in combination with other results from Daemen et al, I believe the theory that globally high MCT1 or MCT4 across both tumour and stroma results in poor OS is a reasonable one, and therefore suggest in these instances patients should be therapeutically targeted with inhibitors. Essentially this suggests tumours in a state of high lactate flux are more metabolically active, thus driving tumourigenesis, and hence shutting down lactate shuttling by inhibitor their transport method could garner a beneficial effect.

With respect to the IHC analysis, and specifically that of the stroma, obviously it is noteworthy that there are numerous stromal components other than PSCs that could also be expressing MCTs, and as such the expression profile/ratios observed are not entirely representative of PSCs. The method of analysis used with the aperio system meant various other cell types were excluded, most notably including endothelial cells, and non-ductal cell types such as acinar cells and islets of Langerhans. Nonetheless inflammatory cells in particular may be attributing to the metabolic profile, and as such any mechanistic theories postulated from what we are observing cannot be directly related to the simple PDAC-PSC *in vitro* relationships we are observing. However from an *in vivo* and clinical translation perspective, any prognostic significance seen can still be considered important, regardless

of which stromal cell is expressing MCT, with regards to the possibility that inhibition may be of benefit.

When considering therapeutic approaches towards metabolism, there are significant challenges with respect to toxicity and patient selection. There is a concerning risk of potentially lethal off-target effects. Furthermore, given the lack of understanding regarding the metabolic signature of PDAC, and the high probability it follows the known pattern of genetic heterogeneity, it is unclear which patients would best benefit from the inhibitions or promotion of certain pathways or interactions converging on specific metabolites and energy production mechanisms, and other than specific drug discovery into compounds influencing metabolic reprogramming, identifying which patients might benefit, and perhaps even more importantly those whose prognosis would suffer as a result, is a key question to be answered. However it was certain following the PDAC-PSC co-culture experiments that PSCs needed to be accounted for when using drugs that influence metabolism.

Somewhat fortuitously, it just so happened that the NICR at Newcastle University had established links with AZ and were trialing a new MCT1 inhibitor AZD3965 within a phase 1 trial, being one of only 2 institutions in the world that had access to this compound. Obviously this presented a golden opportunity to utilise this within the co-culture system. Whilst knockdown studies and other compounds inhibiting MCT1 could have been utilised, none of them had the specific mechanism of action that would not only offer a more accurate assessment of treatment, however also offer clinically translatable promise given it was already being trialed on patients. Furthermore these compounds (such as CHC,

DIDS/DBDS and cCMBS) all have off-target effects making it difficult to truly assess translational potential.

The results of MCT1 inhibition with AZD3965 was admittedly varied and challenging to interpret, however the one key fact that emerged was that the 3 cell lines were behaving inherently differently to MCT1 inhibition, and I believe this to be inextricably linked with the underlying metabolic phenotype of each line. I would certainly postulate that a tumour with a similar phenotype to Panc1 is most likely to translate to clinical benefit, given the reduction in both glycolytic and oxidative processes, with associated anti-proliferative effects in hypoxia. The impact on MCT1 in both cell types, when considering the detrimental prognosis seen in patients with high expression in IHC analysis, would also suggest promise. And lastly the effect on the PSCs themselves, and in particular inducing apoptosis, could be of great benefit in shutting down the metabolic relationship between PDAC and PSCs already demonstrated (MCT upregulation, pro-glycolytic/survival advantage), with the additional possibility that any affect on PSC inflammatory functionality (as evidenced by reduced αSMA and IL6) may also impact on other pro-tumourigenic pathways with both PDAC and other stromal cell types such as immune cells in particular. These latter effects on the PSCs being replicated with Miapaca2 suggest additional potential for *in vivo* benefit regardless of tumour phenotype.

In reference to galloflavin, a similar theme was identified with differing results dependent on cell lines and PSC presence. Nonetheless the conclusion would be that the over-riding neutralizing effect of galloflavin holds promise in an *in vivo* setting and warrants further investigation. Furthermore it is again suggested that a patient personalized approach may

be the best method, whereby only tumours with a certain LDHA:B expression profile may benefit from therapy.

## 7.2 Strengths and weaknesses

When I embarked upon this PhD project, I had no previous scientific laboratory experience, and as such had to take some time to learn how to use all the equipment and technology available, the processes involved, and the intricacies of the numerous experimental techniques. Without any specific formal training in this field one may perceive that my undertaking of such a complex array of *in vitro* experimentation was over-ambitious, and even a weakness of the study overall. Nonetheless I was extremely fortunate that the ICM, NICR and CAV were all instrumental in providing me the assistance I required to learn and thrive in this environment. Lab technicians, students and postdocs were all extremely accessible and helpful in this regard, and I am forever indebted to their support in helping to make the project the success it was. In the early stages of the project my supervisors allowed me to use what I had learnt during my systematic review of literature to adapt the project direction and take initiative in guiding the aims of the project, and again I am grateful to have been permitted this influence. Certainly the staff, institutions and supervisors involved all provided this project with a lot of strength.

Additional positives include what I believe to have been rigorous *in vitro* methodology to ensure the results observed were reliable and representative, and I always strived for close attention to detail in this regard. I maintained a high work ethic throughout the study period, resulting in a large amount of experimental data. I always endeavored to investigate

results and attempt to answer the next question that the prior experiment brought to the fore, hence generating a logical and understandable story throughout the process.

With respect to experimental methodology, and as is often the case, the fact the majority of this was *in vitro* no doubt has limitations upon the translational element of the results observed, and other than the *in vivo* element of the IHC work there was no expansion into animal work to support my findings. However time was limited with respect to progressing the project to this level, and regardless I hope that the findings themselves represent exciting potential for the benefit of PDAC patients in future. Furthermore it is known to be particularly challenging to investigate the metabolic impact of therapeutics within the *in vitro* set-up, particularly given how complex cancer metabolism is, with countless metabolites and pathways that require consideration. Nonetheless it is without doubt the Seahorse technology was instrumental in providing more reliable and rigorous data outputs, and I certainly attempted to combine multiple assays to help understand the results being produced.

With reference to hypoxia, one limitation of this study, and that could have been implemented within the project, is that none of the initial *in vitro* experiments were performed or compared to incubation under hypoxic conditions. Whilst this could have conferred some useful information, as previously alluded to a tumour *in vivo* will rarely be completely deprived of higher levels of oxygenation, and as such performing experiments at 5% oxygen could in fact provide misleading information with regards to tumour/PSC activity, and rather making observations in the presence of oxygen would be a more reliable means of garnering data which may be clinically translatable. The only other option would have been to perform experiments at varying levels of oxygen concentrations

and observe trends, however this would have required significantly more work (i.e. replicating all experiments performed in varying degrees of oxygenation) which was not feasible in the given timeframe; nonetheless a potential avenue of future exploration.

Ultimately I believe the numerous outputs that resulted from my studies (appendix 1) represents reasonable and peer-reviewed proof that the data I produced from my studies were of a high quality and in itself I hope portray the strength of my endeavours.

## 7.3 Future directions

There are a number of additional experiments that could be performed to help both improve the reliability and further investigate the results observed. Firstly, the known association between tissue culture surfaces and PSC gene expression means some of the key experiments (such as the PSC-PDAC seahorse pro-glycolytic effect, or the AZD3965 effect on proliferation and PSC activity) could be repeated using both physiological and non-physiological culture surfaces, such as comparing plastic, matrigel and collagen-1 seeded plates. Furthermore, and as already alluded to, all experimentation could be repeated in hypoxic conditions where feasible, and potentially add weight to any translational benefit. Additionally further attempts could be made to primarily harvest PSCs from resected human tissue, potentially with the assistance of other laboratories that have had success in this field, and use these cells to again repeat key experiments and observe if the same results are produced.

Utilisation of the transwell culture system was certainly an indispensible means of observing the effect of therapeutics on the combined PDAC-PSC metabolic relationship.

However a more representative means of replicating this *in vivo* association would have been to use organoid models *in vitro*, which both replicate PDAC tumourigenesis, the desmoplastic reaction, and αSMA aggregation.

Regarding additional therapeutic investigation, it may be of interest to treat cells with AZD3965 in combination with chemotherapeutic agents (e.g. gemcitabine), or MCT4 knockdown. With respect to the latter, whilst no specific MCT4 inhibitor is available at present, this may help explain some of the heterogeneity of results, particularly with reference to the more MCT-4 dependent Miapaca2 cell line, whilst additionally suggesting whether combined inhibition may be of translational promise. Furthermore other therapeutics which influence metabolism could also be examined, for example the investigation of any synergistic effect of AZD3965 in combination with glucose transporter blockade, such as the use of the Glut1 inhibitor WZB117, although it should be appreciated that translational concerns do exist regarding the *in vivo* toxicity of such agents. Alternatively targeting other glycolytic enzymes may be of interest, such as using the pyruvate inhibitor DCA, or inhibiting hexokinase 2, the latter of which is already known to be associated with reduced OS in PDAC. [193]

To further investigate the mechanism behind the PDAC-PSC metabolic relationship, it may be of interest to perform similar experiments using PSC derived exosomes, given there has been evidence that these prove instrumental in some observed CAF-related observations. [252-253]

Ultimately, going forward there is no doubt *in vivo* studies are required to determine which patients are most likely to benefit depending on the underlying onco-metabolic phenotype

of their tumours. Whilst animal models are the logical next step, this has to be done with caution given the differences seen with regard to the tumour microenvironment, and particular PSC functionality, as compared to humans. Nonetheless a project investigating the effect of AZD3965 in a variety of established transgenic or xenograft PDAC animal models would be of great interest.

Finally, and potentially most excitingly, depending on the results of the phase 1 trial with AZD3965, and provided toxicity levels are acceptable, a phase 2 RCT of AZD3965 in palliative metastatic PDAC patients, combined with standard chemotherapy (gemcitabine/gem-abraxane/FOLFIRINOX), whilst correlating outcomes with core biopsies examined for expression of a range of metabolic markers (including MCTs, HIF1a, Glut1 and LDHs in both tumoural and stromal compartments) at the time of diagnosis, could impart essential information regarding both efficacy and optimal patient selection, and this in particular is the project I will most enthusiastically seek out if the opportunity presents itself in future, with the ultimate hope of significantly improving outcomes of patients with this devastating disease.

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# Appendix 1

# Outputs related to thesis

## Presentations

| Date | Location | Oral/poster | Abstract title |
|---|---|---|---|
| Nov 2015 | PSGBI conference, Norwich | Oral (prize session) | The effect of monocarboxylate transporter 1 inhibition in the tumour microenvironment of pancreatic ductal adenocarcinoma |
| Jan 2016 | SARS conference, London | Oral | The effect of monocarboxylate transporter 1 inhibition in the tumour microenvironment of pancreatic ductal adenocarcinoma |
| Jan 2016 | SARS conference, London | Oral (Patey Prize session) | Metabolic relationships within the tumour microenvironment of pancreatic ductal adenocarcinoma - pancreatic stellate cells induce glycolysis in cancer cells |
| Apr 2016 | NESS conference, Newcastle | Oral (Prize session) | Therapeutic approaches to the metabolic relationship between pancreatic ductal adenocarcinoma and pancreatic stellate cells through targeting of glycolysis and lactate shuttling |
| June 2016 | Pancreas 2016, Glasgow | Poster | Metabolic relationships within the tumour microenvironment of pancreatic ductal adenocarcinoma - pancreatic stellate cells induce glycolysis in cancer cells |
| Nov 2016 | NOSTRA meeting, Newcastle | Oral | 3-minute thesis presentation |
| Jan 2017 | SARS, Dublin (+ESSR Amsterdam June 2017) | Oral | Therapeutic approaches to the metabolic relationship between pancreatic ductal adenocarcinoma and pancreatic stellate cells through targeting of lactate dehydrogenase |

## Awards

As a result of presenting work from this thesis, I have received the following awards:

- Ronald Smith Medal - best research presentation at PSGBI (Pancreatic Society of Great Britain and Ireland) conference Nov 2015
- ASGBI (Association of Surgeons of Great Britain and Ireland) award – best research presentation at NESS (North East Surgical Society) meeting Apr 2016
- NOSTRA (Northern Surgical Trainee Research Association) thesis award – best 3-minute thesis presentation at annual NOSTRA meeting Nov 2016
- Burnand Prize – best scientific presentation at SARS (Surgical Academic research Society) conference Jan 2017

Furthermore, I won the following bursary and fellowship that provided consumable and salary funding for my studies:

- CRUK bursary 2013
- CRUK training fellowship 2015

## Publications

Moir JAG, Long A, Haugk B, French JJ, Charnley RM, Manas DM, Wedge SR, Mann J, Robinson SM, White SA. Therapeutic Strategies Toward Lactate Dehydrogenase Within the Tumour Microenvironment of Pancreatic Cancer. Pancreas. 2020 Nov/Dec;49(10):1364-1371

Moir JA, Mann J, White SA. The role of pancreatic stellate cells in pancreatic cancer. Surg Oncol. 2015 Sep;24(3):232-8.

John AG Moir, Steven A White, Jelena Mann, Arrested development and the great escape – The role of cellular senescence in pancreatic cancer, The International Journal of Biochemistry & Cell Biology, Volume 57, December 2014, Pages 142-148