Gene expression patterns of signalling pathways in PDAC: towards inhibiting metastases

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DECLARATION

I, Ntombikayise Xelwa declare that this thesis entitled “Gene expression patterns of signalling pathways in PDAC: towards inhibiting metastases” is my own unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

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Ntombikayise Hendrietta Marcia Xelwa
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Publications

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SUMMARY

PDAC has a poor prognosis, with its prevalence varying by geographical location. In South Africa, PDAC ranked seventh among all cancer-related deaths in 2020 for both sexes. Specifically, it was the seventh leading cause of cancer death among males and the sixth among females. In 2020, an estimated 1,982 cancer deaths in South Africa were attributed to pancreatic cancer, with 1,006 occurring in males and 976 in females. However, the annual reported number of PDAC deaths in South Africa varies. This study aimed to identify potential novel therapeutic targets for PDAC in patients from the African population. Following ethical approval, tissue from fifteen patients (15 tumour and 15 corresponding normal tissues) were obtained during Whipple procedures from PDAC patients who consented to the study.

Despite the development of new treatment strategies, patient outcomes have not significantly improved underscoring the necessity for extensive research to identify novel treatment options. A discovery study was conducted to determine the gene expression profile of signalling pathway-related genes using PDAC tissue samples. Top upregulated pathways included those involved in cytokine signalling, receptor kinase signalling, and PI3/Akt signalling. *SPP1* was one of the most highly expressed genes identified in PDAC patients compared to normal corresponding tissues suggesting its potential role in the progression of the disease. To investigate *SPP1*'s role in PDAC, RNA interference was employed to knockdown *SPP1* in a PDAC cell line, MIA PaCa-2. Knockdown was confirmed by a significant reduction in *SPP1* expression at the mRNA level. Combining *SPP1* knockdown with conventional chemotherapy used for PDAC, gemcitabine, resulted in a synergistic effect, leading to an enhanced early apoptotic response. The study also examined the migratory and invasive capabilities of MIA PaCa-2 cells, revealing a noticeable decline in these abilities upon reduction in *SPP1* expression with gemcitabine treatment. Furthermore, proteomic analyses uncovered the complex network of cellular processes influenced by the downregulation of *SPP1* and the synergistic effects of combination therapy.

Altogether, the findings from this study demonstrate the role of *SPP1 in* PDAC indicating that it could serve as a promising therapeutic target. The synergistic effects observed when *SPP1* knockdown was combined with gemcitabine treatment suggest a potential avenue for developing more effective treatments for PDAC while exploring tumour cell adaptation for survival.

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LIST OF ABBREVIATIONS

Abbreviations	Full meaning
ACSL4	Acyl-CoA synthetase long-chain family member 4
ACTB	Actin, beta
ADM	Adrenomedullin
AJCC	American Joint Committee on Cancer
ATG5	autophagy-related gene 5
ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67)
BAX	BCL2-associated X
BMI	body mass index
CA19-9	carbohydrate antigen 19-9
CAF	Cancer-associated fibroblasts
CDKN1A	cyclin-dependent kinase inhibitor 1A
CEA	carcinoembryonic antigen
CHBAH	Chris Hani Baragwanath Academic Hospital
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Colony stimulating factor
CSIR	Council for Scientific and Industrial Research
CT	Computed Tomography
CTGF/CCN2	Connective Tissue Growth Factor
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition
EPO	Erythropoietin
EUS	Endoscopic ultrasound
FABP1	Fatty acid binding protein 1, liver
FBS	Fetal bovine serum

FDS	flow cytometry standard
FGFs	Fibroblast growth factors
FPC	Familial pancreatic cancer
GADD45	growth arrest and DNA damage-inducible 45
GC	gastric cancer
GDF10	Growth differentiation factor 10
GOI	Gene of interest
GSI	gamma-secretase inhibitors
HA	hyaluronic acid
hCNTs	human concentrative nucleoside transporters
HEY2	Hairy/enhancer-of-split related with YRPW motif 2
hENTs	human equilibrative nucleoside transporters
HGF	Hepatocyte Growth Factor
HMOX1	Heme oxygenase (decycling) 1
ICB	Immune checkpoint blocker
IGF1	Insulin-like growth factor 1
IHC	immunohistochemical
IL2	Interleukin 2
IL4	Interleukin 4
IL13	Interleukin 13
IMRT	intensity-modulated radiation treatment
iNOS	increased nitric oxide synthase
JAG1	Jagged 1
LC-MS	liquid chromatography in conjunction with tandem mass spectrometry
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)
MeCP1	Methyl-CpG binding proteins
MEK	mitogen-activated protein kinase 1
mRNA	Messenger RNA
MRI	magnetic resonance imaging
MS	Mass spectrometry
MTT	4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide

MyCAFs	Myofibroblasts
NF-κB	Nuclear factor-κB
NGF	Nerve Growth Factor
PanIN-1A	pancreatic intraepithelial neoplasia
PBS	Phosphate-buffered saline
PCA	Principal component
PCR	Polymerase Chain Reaction
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PSC	Pancreatic stellate cells
PTMs	post-translational modifications
qPCR	Quantitative Polymerase Chain Reaction
RISC	RNA-induced silencing
RITA	Reactivator of p53 and Inducer of Tumour Cell Apoptosis
RNAi	RNA interference
SBRT	stereotactic body radiation therapy
siRNA	small interfering RNA
SPP1	Secreted phosphoprotein 1
SWATH-MS	Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry
TBR	taxane-based treatment regimen
TDE	tumor-derived exosomes
TDGF1	Teratocarcinoma-derived growth factor 1
T2DM	Type 2 diabetes mellitus
TGF-β	Transforming growth factor-beta
THPO	Thrombopoietin
TME	tumour microenvironment
TNM	tumour node-metastasis
TYMP	Thymidine phosphorylase
VEGF	Vascular endothelial growth factor
siRNA	small interfering RNA

CHAPTER 1: LITERATURE REVIEW

1.1 Structure of the Pancreas

The pancreas is a large, flat leaf-shaped organ located in the upper abdomen that plays a vital role in regulating glucose and facilitating digestion (Ungkulpasvich *et al.*, 2023). The pancreas comprises four main structures: head, neck, body, and tail (Figure 1.1). The head of the pancreas is the broadened area surrounded by the C-shaped bend of the duodenum. The neck lies on the left-hand side of the head, acting as a connecting area between the head and the body of the pancreas, and is distinctly narrower. The body is the central and elongated segment that extends from the neck to the left side of the abdomen. The tail portion extends towards the spleen and is situated in the upper left segment of the abdomen (Suda *et al.*, 2006; Tan, 2014). The human pancreas in adults ranges from 12 – 15 cm long, can be approximately 80 g in weight, and can produce up to 1000 g of pancreatic fluid daily (Schaefer, 1926; Tan, 2014; Walker, 2018).

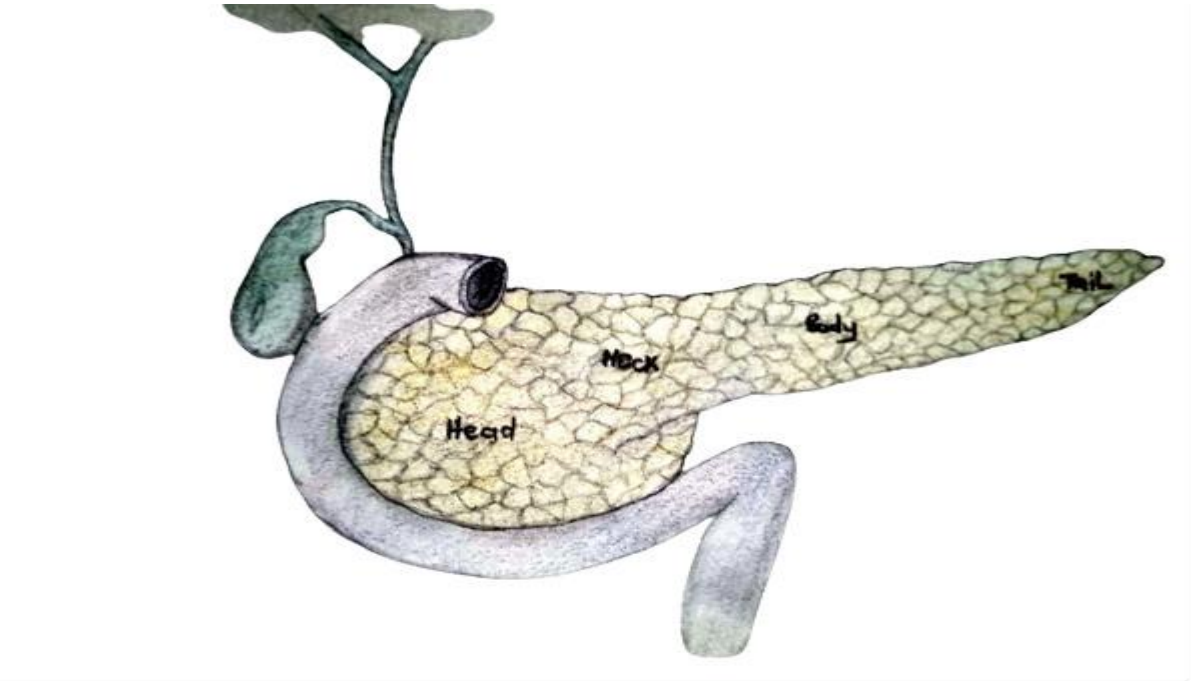


Figure 1. 1: The pancreas structure. The anatomy of the gallbladder consists of four main structures: head (broadened area), neck (connecting area), body, and tail (extends towards the spleen).

1.1.1 Functions of the pancreas

The pancreas has a vital function in the regulation of glucose levels and the facilitation of digestion. Its exocrine feature involves the secretion of digestive enzymes, while its endocrine feature is responsible for controlling blood glucose levels (Karpińska and Czauderna, 2022; Mehta *et al.*, 2022).

The exocrine portion of the pancreas, which constitutes 95% of its anatomy, principally comprises acinar exocrine secretory cells. These cells are characterized by their branched morphology and release digestive enzymes, such as amylase, lipase, and proteases, into the duodenum. These enzymes are essential for the degradation of carbohydrates, fats, and proteins (Walker, 2018). Imbalances in these enzymes can result in detrimental consequences for the absorption and digestion of nutrients (Johansson and Grapin-Botton, 2002). The pancreatic ductal adenocarcinoma (PDAC) subtype predominates among exocrine tumours, comprising over 90% of the total pancreatic malignancies (Kleeff *et al.*, 2016; Skorupan *et al.*, 2023).

Endocrine activity is performed by the Islets of Langerhans which are responsible for secreting hormones like glucagon and insulin into the bloodstream. These hormones are vital in controlling blood glucose levels, with insulin facilitating glucose uptake by cells and glucagon prompting the release of glucose into the bloodstream. The equilibrium between these two processes is crucial in maintaining glucose homeostasis within the body (Karpińska and Czauderna, 2022).

1.2 Pancreatic Ductal Adenocarcinoma (PDAC)

PDAC represent almost 90% of all pancreatic cancers (Skorupan *et al.*, 2023). PDAC is among the top three leading causes of cancer-related deaths in both sexes in the USA (Siegel *et al.*, 2023), with almost same number of new cases and deaths reported annually. In 2020, worldwide 496,000 new cases and 466,000 deaths was reported (Ferlay *et al.*, 2021). The Globocan 2020 statistics database estimated approximately 66 440 newly diagnosed PDAC cases (34 530 males [51.9%] and 31 910 females [48%]) annually, with a mortality rate that has progressively increased by 0.3% each year since 2000 for both males and females globally (Siegel, Giaquinto and Jemal, 2024). The occurrence of PDAC differs substantially across various geographic regions worldwide. Europe has reported the highest rates of PDAC, followed by Northern America, and Australia/New Zealand (Arnold *et al.*, 2020). Regrettably, a limited number of patients qualify for potentially curative surgery. However, a considerable proportion of those undergoing surgical resection encounters recurrence within two years, with an anticipated recurrence rate of up to 97% (Groot *et al.*, 2018). Patients undergoing surgery

with curative intent have a 5-year survival rate that fluctuates between 16-22% and shows considerable variability in the duration of survival. Despite this, survival outcomes for resected pancreatic cancer patients have experienced a modest improvement over time (Aaltonen *et al.*, 2022). A recent study assessing records between 2000-2008, and those around 2009-2016, indicated a significant enhancement in survival following radical surgery, rising from 20 months to 28 months. On the other hand, the median survival for patients who did not undergo surgery increased slightly from 3.1 months to 3.3 months (Aaltonen *et al.*, 2022).

In South Africa, pancreatic cancer, of which PDAC is the most common subtype, presents a significant health burden, being the seventh leading cancer-related death in 2020 in both sexes. In males, PDAC was the seventh leading cause of cancer-related deaths and was ranked sixth, in females. In the year 2020, the estimated number of PDAC deaths in South Africa was 1,982. This figure comprised 1,006 cases among males and 976 among females (Ferlay *et al.*, 2021). Regrettably, a significant number of people are diagnosed at the intermediate or advanced stages of the disease (Liu *et al.*, 2022). The anatomical positioning of the pancreas within the abdomen presents a considerable obstacle due to its deeply situated location, rendering it inaccessible through standard endoscopic techniques. This poses a challenge for the formulation of effective screening strategies. Moreover, its proximity to vital structures, such as the bile ducts and significant blood vessels, exacerbates the difficulties of detection. Furthermore, PDAC cells are known for exhibiting resistance to conventional radiotherapy and chemotherapy treatments (Lowenfels and Maisonneuve, 2005).

1.2.1 Risk factors of PDAC

Several risk factors of PDAC have been identified including smoking, Type 2 diabetes, chronic pancreatitis, family history and genetic predisposition, obesity, alcohol consumption, advanced age and *Helicobacter pylori* infection (Chandana *et al.*, 2024).

1.2.1.1 Smoking

Smoking is the leading risk factor for PDAC, accounting for 20-25% of all cases (Bonelli *et al.*, 2003; Larsson *et al.*, 2005). Unlike the lungs, which are directly exposed to tobacco smoke, the pancreas is indirectly exposed to tobacco products. Carcinogens from tobacco move to the pancreas through the bloodstream or potentially via exposure to duodenal contents or bile. The pancreatic head region, where most PDAC cases occur, is particularly vulnerable to carcinogenic exposure from duodenal juice or bile (Lowenfels and Maisonneuve, 2005). Smokers have a two-fold higher risk of developing PDAC than non-smokers (Silverman *et al.*,

1994). Passive smoking has only a minimal, statistically insignificant impact on PDAC risk (Villeneuve *et al.*, 2004).

1.2.1.2 Type 2 diabetes mellitus (T2DM)

Research has demonstrated that patients who have T2DM, particularly within 5 years of onset, have a risk that is approximately double for developing PDAC (Everhart and Wright, 1995; Coughlin *et al.*, 2004). However, investigating the connection between these two conditions can be complicated by the possibility of reverse causality, as diabetes can sometimes be an early symptom of PDAC. It is estimated that approximately 1% of newly diagnosed cases of diabetes in individuals aged 50 or older are caused by PDAC (Chari *et al.*, 2005). T2DM, which accounts for the majority of diabetes cases at 90-95%, is often associated with obesity and being overweight, leading to hyperglycemia resulting from a combination of insulin resistance and insufficient insulin secretion (Connor and Gallinger, 2015; Menke *et al.*, 2015).

1.2.1.3 Chronic pancreatitis

Chronic pancreatitis is characterized by a progressive and irreversible deterioration of the pancreatic tissue resulting from persistent inflammation, which ultimately leads to the destruction of both the exocrine and endocrine cells (Ungkulpasvich *et al.*, 2023). It is most commonly linked to heavy alcohol consumption in Western countries (Lowenfels and Maisonneuve, 2006). It is worth noting that a significant portion of those at increased risk for PDAC have chronic pancreatitis, which can be caused by, diabetes chronic alcohol dependence, smoking, renal failure, or hypertriglyceridemia. Several studies have confirmed the increased risk of PDAC associated with chronic pancreatitis (Lowenfels *et al.*, 1993; Malka *et al.*, 2002; Duell *et al.*, 2012; Munigala *et al.*, 2014).

Although the connection between chronic pancreatitis and PDAC is well established, the precise molecular mechanisms underlying this relationship remain unclear (Silke and O'Reilly, 2021; Michalak and Małecka-Wojciesko, 2023).

1.2.1.4 Genetic predisposition

Approximately 10%-15% of PDAC cases demonstrate familial or genetic susceptibility. Familial pancreatic cancer (FPC) accounts for 7% of these cases, while individuals with recognizable genetic predisposition syndromes comprise an additional 3% (Klein, 2012). While the practice of screening for patients with germline mutations associated with PDAC is not yet widespread, it is essential to identify those who would most benefit from this approach. The *PMS*2, *CDKN2A, TP53, MLH1*, *MSH*2, *MSH6*, *BRCA1*, *BRCA2*, *ATM* and *PALB2* genes are frequently linked with pathogenic germline alterations (Rainone *et al.*, 2020). Candidates

for screening primarily include individuals with a strong family history of PDAC and those diagnosed with hereditary pancreatitis (Durie *et al.*, 2002; Brand and Mahr, 2005). Additionally, the disease has been linked to other hereditary syndromes, extending beyond those specifically related to PDAC.

1.2.1.5 Obesity

In individuals with obesity, the disease progression is characterized by adiposopathy, a chronic disorder of adipose tissue involving the production of pro-inflammatory cytokines by macrophages and hormonal dysregulation, including elevated levels of leptin and decreased levels of adiponectin (Stolzenberg-Solomon *et al.*, 2015). Importantly, there is a temporal association between increased body mass index (BMI) and the development of cancer, with individuals who have been obese since childhood exhibiting an increased relative risk for PDAC (Stocks *et al.*, 2009).

1.2.1.6 *Helicobacter pylori*

Studies indicate that the presence of *H. pylori* infection may heighten the likelihood of developing PDAC (Principe and Rana, 2020; Chandana *et al.*, 2024). This correlation is plausible, considering the established relationship between *H. pylori* and gastric cancer (Trikudanathan *et al.*, 2011). Although the association between past *H. pylori* infection and PDAC has yet to be conclusively proven, it remains a noteworthy avenue for future investigation.

1.2.1.7 Alcohol consumption

Individuals who consume more than three alcoholic beverages every day have a relative risk of 1.22 to 1.36 for developing PDAC, with a dose-response relationship being observed (Wang *et al.*, 2016; Korc *et al.*, 2017). Consumption of alcohol and its breakdown products could lead to the development of PDAC through persistent inflammation (Tramacere *et al.*, 2010). Over the past two decades, there has been a global rise in average per capita alcohol consumption. Notably, Northern Africa and the Middle East exhibit the lowest levels, while Europe and Russia demonstrate the highest consumption rates (Liew *et al.*, 2023). While excessive alcohol consumption has been established as a risk factor for both chronic pancreatitis and type 2 diabetes mellitus, which are both linked to PDAC, the relationship between alcohol use and the incidence of PDAC remains inconclusive (Nitsche *et al.*, 2011; Wang *et al.*, 2016).

1.2.1.8 Age

Incidence of PDAC rises with age, with approximately 90% of newly diagnosed patients aged 55 years or older (McGuigan *et al.*, 2018). Moreover, the highest prevalence of PDAC is

observed among those aged 70 years or above (Bosetti *et al.*, 2012). The global population aged 60 years and above is projected to double from 11% in 2015 to 22% by 2050 (Kanasi, Ayilavarapu and Jones, 2016). The ageing populations are primarily concentrated in regions such as North America, Eastern Asia and Europe, which are also characterized by high incidence rates of PDAC (Siegel, Giaquinto and Jemal, 2024).

1.3 Clinical presentation

PDAC is often diagnosed at an advanced stage, which renders tumour resection impossible (Tonini and Zanni, 2021). This delayed diagnosis is a result of the slow growth of pancreatic tumours, the lack of sensitivity of conventional radiologic tests for early disease detection, and the absence of specific and sensitive serum markers for diagnosis. While endoscopic ultrasonography can identify smaller pancreatic lesions, the tendency of tumours to metastasize even at a small size, coupled with the resistance of cancer cells to radiotherapy and/or chemotherapy, presents a significant challenge (DiMagno, Reber and Tempero, 1999; Sener *et al.*, 1999; Kornmann, Beger and Link, 2003). Furthermore, the non-specific nature of PDAC symptoms contributes to a median delay of over two months between presentation and diagnosis (Raptis *et al.*, 2010). These symptoms, which include jaundice, hepatomegaly, cachexia, epigastric mass, or ascites (Porta *et al.*, 2005), are less frequent and can make it difficult for physicians to determine when to request specific clinical and diagnostic tests.

1.4 Diagnosis of PDAC

PDAC requires a comprehensive approach that goes beyond relying on clinical signs and symptoms. Patients who present with jaundice or experience epigastric pain should undergo a thorough evaluation, which includes a blood chemistry panel, liver function tests and a complete blood count (Gheorghe *et al.*, 2020; Primavesi, 2021).

1.4.1 Tumour markers

PDAC is often diagnosed using the tumour marker carbohydrate antigen 19-9 (CA19-9), which recognizes the sialylated Lewis a blood group antigen. While its sensitivities and specificities range from 70% to 90% in symptomatic patients, the positive predictive value of elevated CA 19-9 in asymptomatic individuals is as little as 0.9%. Consequently, this marker is not a reliable diagnostic tool for asymptomatic individuals (Kim *et al.*, 2004; Goonetilleke and Siriwardena, 2007). The utility of this diagnostic tool is limited by the presence of elevated levels in benign pancreaticobiliary diseases, as well as other cancers distinct from PDAC. Additionally, it is important to note that 5-10% of the population has a prevalence of Lewis antigen non-expression, which can impact the diagnostic accuracy of this tool (DiMagno, Reber and

Tempero, 1999; Kaur *et al.*, 2017). However, the relationship between serum CA 19-9 levels and tumour size is closely linked, and the extent of CA 19-9 elevation is associated with prognosis. Cancers often exhibit increased levels of certain biomarkers, such as carcinoembryonic antigen (CEA), which may indicate treatment ineffectiveness and recurrence. Nevertheless, these biomarkers are only elevated in a select number of PDAC patients, thereby limiting their utility in diagnosis (Xing *et al.*, 2018).

The accuracy of PDAC detection and staging is greatly influenced by the selection of an appropriate imaging protocol, the application of post-processing techniques, and the expertise of the radiologists involved. The imaging techniques commonly applied for preoperative staging include computed tomography, abdominal ultrasound, endoscopic ultrasonography and magnetic resonance imaging.

1.4.2 Imaging

Computed tomography (CT) is the favoured imaging method for PDAC assessment due to its cost-effectiveness and accessibility (Park *et al.*, 2021). CT is an advanced imaging method that offers higher spatial resolution and is less susceptible to motion artefacts compared to magnetic resonance imaging (MRI), which is critical for accurately showing the essential relationship between tumours and their accompanying blood vessels (Kaissis and Braren, 2019).

MRI is applied for secondary imaging in cases of severe iodinated contrast allergy or renal insufficiency, but it can also provide additional insights in cases with unclear CT findings and help characterize ambiguous liver lesions (Park *et al.*, 2021). Endoscopic ultrasound (EUS) is primarily used to guide needle biopsies for PDAC diagnosis confirmation and can detect small pancreatic masses that may be difficult to visualize on CT or MRI, making it a valuable tool in certain early detection surveillance (Krishna *et al.*, 2017; Canto *et al.*, 2018). Various other techniques are being investigated, such as multi-parametric imaging and functional and molecular markers. Researchers are currently examining functional markers, including stromal changes, microvascular density, and tumour metabolism, as potential indicators of disease progression (Couvelard *et al.*, 2005; Erkan *et al.*, 2008).

1.5 Staging of PDAC

Stage determination is a critical element in delivering the highest quality patient care. The assessment of resectability in PDAC relies on the utilization of the tumour node-metastasis (TNM) classification system to establish tumour staging (Table 1.1). The 8th edition of this system, which was published by the American Joint Committee on Cancer (AJCC) in 2017

(Amin *et al.*, 2017), delineates guidelines for each TNM category and subsequent staging based on these categories.

Table 1. 1: TNM Classification and Staging in accordance with AJCC 8th Edition.

Tumour size or location	Description
T1	Maximum tumour diameter ≤2 cm
T2	Maximum tumour diameter >2 cm, ≤4 cm
T3	Maximum tumour diameter > 4 cm
T4	Tumour involves the celiac artery or superior mesenteric artery
Lymph node status	
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N2	Metastasis in ≥4 regional lymph nodes
Distant metastases	
M0	No distant metastasis
M1	Distant metastasis
Stage	
IA	T1N0M0
IB	T2N0M0
IIA	T3N0M0
IIB	T1-3N1M0
III	T4NXM0 or TXN2M0
IV	TXNXM1

1.6 Treatment Strategies for PDAC

The outlook for patients diagnosed with PDAC is typically dismal, primarily because the majority of tumours have progressed to an advanced stage at the time of diagnosis (Gillen *et al.*, 2010). However, several treatment strategies could be employed which is largely dependent on the stage of the disease (Figure 1.2).

1.6.1 Surgical treatment

The recommended surgical approach for PDAC varies depending on its location. Commonly known as the Whipple procedure, a pancreaticoduodenectomy is often recommended (Khachfe *et al.*, 2021). However, only about 20% of individuals are eligible for surgical resection when

their PDAC presents clinically in its advanced stages (Klaiber *et al.*, 2021). The implementation of novel multi-agent therapies is anticipated to further improve survival rates (Neoptolemos *et al.*, 2017; Conroy *et al.*, 2018). Furthermore, the tumour recurrence rate remains as high as 80%, despite advancements in surgical techniques (Moletta *et al.*, 2019). In contrast, patients with locally advanced unresectable PDAC typically undergo palliative care, which may involve treatment with radiation or chemotherapy, as well as other appropriate disease management approaches (Balaban, Mangu and Yee, 2017; Klaiber *et al.*, 2021).

Obtaining a reliable biopsy for the pancreas is challenging due to its location, which is behind other internal organs. Consequently, extensive surgery is often recommended to diagnose malignancy (Lanki *et al.*, 2023). Even in advanced cases, perioperative mortality after pancreatic cancer surgical resection is estimated to be 3-4% (Hackert *et al.*, 2016). However, reported fatality rates can reach up to 16.5%, which is similar to the total surgical perioperative mortality (Traub, Link and Kornmann, 2021). Thus, exploring alternative therapeutic approaches, such as targeted therapy, is necessary to combat the ineffectiveness of traditional treatments and their associated side effects (Kara, Calin and Ozpolat, 2022).

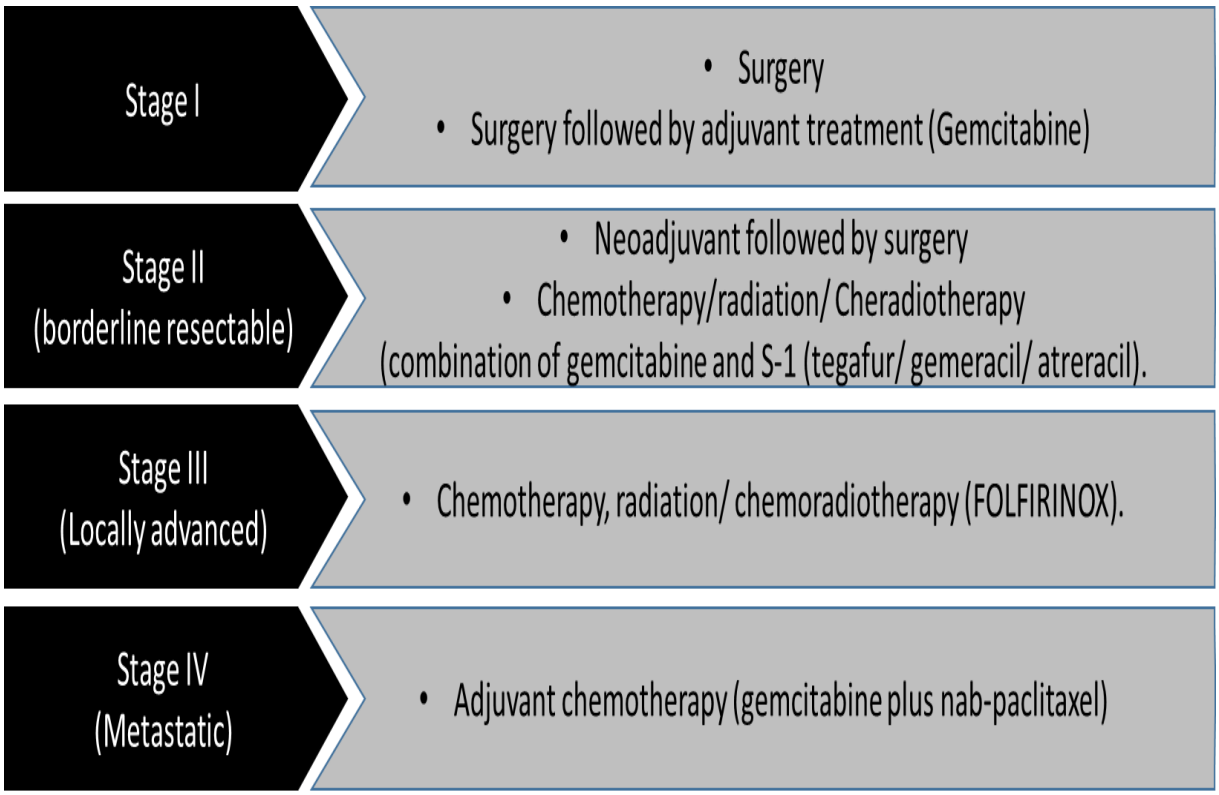


Figure 1. 2: Standardised treatment options for each PDAC stage. Stage I, PDAC tumours are surgically removed, followed by adjuvant treatment. Stage II (borderline resectable) PDAC tumours are treated with chemoradiation in combination with surgical removal. Stage III

(locally advanced) and IV (metastatic) PDAC tumours are subjected to palliative treatment including chemotherapy (self created).

1.6.2 Chemotherapy

Oncological interventions for PDAC typically include chemotherapy or radiotherapy. Resistance to chemotherapy is one of the major challenges in treating PDAC (Jadid *et al.*, 2021; Heiat *et al.*, 2023). Potential chemotherapeutic drugs used in the treatment of PDAC include gemcitabine, with or without capecitabine, 5-fluorouracil with folinic acid, and the combination regimen FOLFIRINOX (fluorouracil, folinic acid, irinotecan, and oxaliplatin) (Petrelli *et al.*, 2023). About one-third of PDAC patients are diagnosed with either local or distant metastases. In such cases, the combination of, nab-paclitaxel gemcitabine, and FOLFIRINOX might be the only effective treatment. However, only a small percentage of individuals respond well to these drugs (Lambert *et al.*, 2019; Ettrich *et al.*, 2022).

Gemcitabine (2',2'-difluoro-2'-deoxycytidine) as shown in Figure 1.3, is one of the first-line treatment for advanced PDAC. Unfortunately, gemcitabine's promise as a therapeutic medication is often limited by chemoresistance and dysregulated intracellular metabolism (Bhoopathi *et al.*, 2023). Gemcitabine is a synthetic form of deoxycytidine nucleoside which demonstrated increased survival benefits in PDAC in 1997 (Burris *et al.*, 1997). Even though it was first observed to be beneficial decades ago, it remains at the forefront of PDAC therapies as well as breast, bladder, ovarian, and non-small lung cancers (Beutel and Halbrook, 2023). However, there are side effects related to gemcitabine treatments that can be categorised into haematological and non-haematological side effects. Haematological side effects include anaemia, thrombocytopenia, and thromboembolism. Side effects include nausea and vomiting, fatigue, and loss of appetite (Verma, Sahu and Bhaskar, 2023). Although these side effects can be managed clinically, potentially fatal treatment-related side effects are rare (Verma, Sahu and Bhaskar, 2023).

The mechanism of action of gemcitabine is to inhibit DNA synthesis within the cells to prevent further cellular proliferation. As a hydrophilic synthetic deoxycytidine nucleoside, gemcitabine utilises the normal cellular nucleoside transporters on the cell surface to enter the cells. Gemcitabine uses endogenous human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs) for cellular entry (De Sousa Cavalcante and Monteiro, 2014). Specifically, gemcitabine utilises hENT1, hENT2, hCNT1, hCNT2, and hCNT3 to permeate the cellular membrane. Once inside the cell, gemcitabine is metabolised via a three-step phosphorylation process to become active. Gemcitabine is phosphorylated to

gemcitabine monophosphate and then converted to the active gemcitabine diphosphate and triphosphate molecules (Mini *et al.*, 2006).

One method of resistance to gemcitabine that cancer cells exhibit is the inactivation of gemcitabine. Cytidine deaminase deactivates gemcitabine by converting it to 2'-deoxy-2',2'-difluorouridine, and gemcitabine monophosphate is inactivated by being converted to 2'-deoxy-2',2'-difluorouridine monophosphate by deoxycytidylate deaminase (Patzak *et al.*, 2019). To avoid potential resistance to gemcitabine, FOLFIRINOX, another chemotherapy therapy is used. FOLFIRINOX exhibits substantial efficacy, but has higher toxicity, making it mostly useful for persons with a good performance status (patient able to be active and undertake self-care). A modified FOLFIRINOX regimen with a lower dosage to decrease toxicity has been developed (Gresham *et al.*, 2014). Traditionally, the efficacy of chemotherapy treatments is typically below ideal levels, with gemcitabine, in particular, being known for its increased chemoresistance (Amrutkar and Gladhaug, 2017).

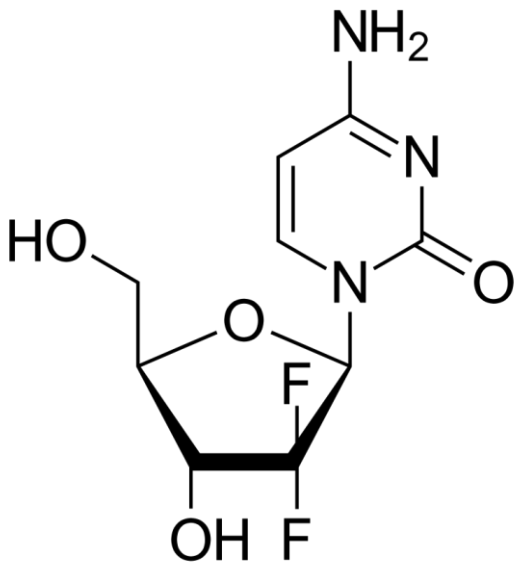


Figure 1.3: Chemical structure of gemcitabine (Venkataramanan *et al.*, 2023).

The FOLFIRINOX regimen was first introduced during the PRODIGE4/ACCORD11 trial in 2011. This regimen consists of 5-fluorouracil, leucovorin, oxaliplatin, and irinotecan (Conroy *et al.*, 2011). This clinical trial indicated that when compared to gemcitabine monotherapy, FOLFIRINOX displayed superior survival rates (Hackert *et al.*, 2016). To bypass this, modifications to the original FOLFIRINOX cocktail have been made whereby at least one of the drugs has been reduced and/or 5-fluorouracil was removed. This modification was termed modified FOLFIRINOX (Tong *et al.*, 2018). The PRODIGE 24 randomised clinical trial was

performed to directly assess the comparisons between modified FOLFIRINOX and gemcitabine in pancreatic patients and was conducted over 5 years. This study reported that patients who received the modified FOLFIRINOX regimen had significantly prolonged overall survival, disease-free survival, cancer-specific survival, and metastasis-free survival when compared to gemcitabine monotherapy (Conroy *et al.*, 2022).

1.6.2.1 Neoadjuvant therapy

Neoadjuvant therapy can address key clinical challenges in PDAC, such as preventing early systemic spread and achieving complete tumour cell-free resection margins (Springfeld *et al.*, 2023). By administering chemotherapy prior to surgery, the percentage of patients receiving optimal doses of these medications may increase, as they are typically better tolerated before undergoing surgical procedures (Seufferlein *et al.*, 2023). Patients diagnosed with Stage III or Stage IV PDAC typically receive palliative chemotherapy. For medically fit patients, appropriate regimens include FOLFIRINOX or gemcitabine/nab-paclitaxel are prescribed, which have been proven to extend the median survival rate to 11.5 months (Conroy *et al.*, 2011; Von Hoff *et al.*, 2013). Moreover, since the implementation of the FOLFIRINOX treatment, there have been instances of conversion rates to resectability in locally advanced cases reaching as much as 60% (Hackert *et al.*, 2016).

1.6.3 Radiotherapy

Radiation therapy has long been a primary treatment option for PDAC, with at least 91 patients receiving it as far back as the 1950s (Nweke *et al.*, 2020). However, there is ongoing debate regarding its efficacy. Radiotherapy utilizes high-energy X-rays that produce ionizing radiation, which damages the DNA of cancer cells and leads to either cell death or cycle arrest (Chen *et al.*, 2021; Liu *et al.*, 2023). Despite this, radiotherapy has been found to be ineffective in treating PDAC due to the immunosuppressive environment produced by the tumour microenvironment (TME), which renders the tumour resistant to conventional targeted and cytotoxic treatments, such as radiation therapy (Dougan, 2017; Quiñonero *et al.*, 2019). In recent years, intensity-modulated radiation treatment (IMRT) and stereotactic body radiation therapy (SBRT) have become more commonly used for administering radiation to PDAC patients (Ng and Koay, 2018; Hill and Herman, 2023). These methods utilize high-dose radiation to induce apoptosis in cancer cells and shrink tumours, but their effectiveness remains debatable and their use depends on the stage of cancer.

While extensive research has been conducted to develop novel cancer drugs and improve existing treatments, finding highly effective therapies continues to be challenging. Traditional

methods like surgery, chemotherapy, and radiation often have serious side effects that can negatively impact patients' quality of life (Tian *et al.*, 2021). This is partly due to resistance to therapy propagated by the dysregulation of several key signalling pathways. In the next sections, some of these pathways are discussed.

1.7 Signalling pathways in PDAC

PDAC is a form of cancer that involves modifications in various fundamental signalling pathways, such as apoptosis, Hedgehog, TGF-β, and KRAS signalling. These changes result in an average of 63 genetic mutations in tumour cells (Jones *et al.*, 2008). In PDAC tumours, mutations in crucial genes, such as *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*, are frequently observed (Jones *et al.*, 2008). The early occurrence of *KRAS* mutations in PanIN-1A (pancreatic intraepithelial neoplasia) lesions indicates that it plays a significant role in the initiation of PDAC (Löhr *et al.*, 2005; Macgregor-Das and Iacobuzio-Donahue, 2013). *CDKN2A* and *TP53* mutations, both of which contribute to PDAC development, are inactivated in about 80% and 50% of tumours, respectively (Scarpa *et al.*, 1993; Attri *et al.*, 2005). Late-stage *SMAD4* inactivation is present in 50-60% of tumours and may be associated with more aggressive disease (Iacobuzio-Donahue *et al.*, 2009). Although the precise mechanisms governing PDAC development are not yet fully understood, genomic analyses of large patient cohorts offer a promising approach for identifying common mechanisms that can inform the development of new therapeutic strategies for the disease.

1.7.1 Nuclear factor-κB (NF-κB)

Over the past several years, substantial evidence has emerged indicating the crucial role of the transcription factor NF-κB in the development of PDAC. The NF-κB signalling pathway is a critical pathway that significantly influences tumour development. This pathway plays a pivotal role in regulating tumour proliferation, migration, invasion, and apoptosis (Gao *et al.*, 2023). NF-κB is a family of inducible transcription factors widely expressed and essential for the rapid activation of cellular responses in innate and acquired immunity (Sartori *et al.*, 2021; Sprooten *et al.*, 2021). Recent research has demonstrated that aberrant activation of the NF-κB signalling pathway is necessary for promoting and maintaining PDAC cell invasion by controlling epithelial-mesenchymal transition (Cui *et al.*, 2022). The importance of NF-κB to pancreatic cancer progression is highlighted by its inhibition which can restrict tumour growth and metastasis, rendering pancreatic tumour cells more susceptible to apoptosis induced by anti-cancer agents (Han *et al.*, 2022).

NF-κB has also been identified as a key factor in chemoresistance to gemcitabine through various mechanisms (Zhang *et al.*, 2016). A study has demonstrated that the concurrent activation of NF-κB and HIF1-α triggers hypoxia-induced epithelial-to-mesenchymal transition (EMT) and resistance to gemcitabine. In PDAC cells, Nexrutine1 was found to downregulate the NF-κB/STAT3 signalling pathway, which ultimately led to the reduction of gemcitabine chemoresistance (Jingjing Gong *et al.*, 2017; Pramanik *et al.*, 2018). LYTAK1, an inhibitor of TAK1, which itself is upstream of NF-κB, resulted in improved cytotoxic responses to oxaliplatin, SN-38, and gemcitabine in PDAC cells (Melisi *et al.*, 2011). These studies highlight the critical role NF-κB has in PDAC progression. GP-2250 is presently in clinical trials for the treatment of pancreatic cancer (Majchrzak-Stiller *et al.*, 2023). Furthermore, tumour growth can be inhibited by targeting the transcription factor NF-κB, indicating their potential application in patient treatment.

1.7.2 Notch signalling

The Notch signalling pathway is critical for cellular proliferation, differentiation, development, and homeostasis (Ranganathan, Weaver and Capobianco, 2011; Zhou *et al.*, 2022). Mammals express three transmembrane Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4) and five classical transmembrane ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged-1, and Jagged-2) (Gao, Long and Wang, 2017). The Notch signalling pathway modulates its target genes and plays a crucial role in the development and progression of human cancers (Gao, Long and Wang, 2017; Li *et al.*, 2023). It is worth noting that the Notch signalling pathway can have either oncogenic or tumour-suppressive effects, depending on the biological environment (Avila and Kissil, 2013). In skin cancer, Notch-1 functions as an oncosuppressive, as demonstrated by Nicolas et al. (Nicolas *et al.*, 2003), while in a different study, Notch1 was shown to inhibit PanIN, the lesions believed to be precursors in the establishment of PDAC in a mouse cancer model (Hanlon *et al.*, 2010). Studies have demonstrated correlations between the Notch signalling pathway and the initiation, progression, and drug resistance of pancreatic tumours in cell lines as well as in genetically altered mouse models (Cook *et al.*, 2012; Mizuma *et al.*, 2012). In preclinical models, small molecules designed to affect HES1 transcriptional function (JI051 and JI130) were used to inhibit Notch signalling (Perron *et al.*, 2018; Kovach *et al.*, 2022). Notch signalling has further been associated with chemoresistance, with a study showing that its inhibition by genistein, a naturally occurring agent, reduces the rate of tumour growth and invasion, while enhancing the effectiveness of chemotherapy (Pan *et al.*, 2012). Genetic inhibition of Notch in myeloid cells resulted in reduced tumour size and decreased

macrophage infiltration in an orthotopic PDAC model. Combining pharmacologic Notch inhibition with PD-1 blockade led to increased cytotoxic T-cell infiltration, enhanced tumour cell apoptosis, and smaller tumour size (Yan *et al.*, 2024).

1.7.3 Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathway

The JAK/STAT signalling pathway plays a crucial role in processes such as growth, proliferation, survival, inflammation, invasion, angiogenesis, and the progression of various tumours (Ashrafizadeh *et al.*, 2020; Fahmideh *et al.*, 2022). In PDAC, the activation of the JAK/STAT signalling pathway is a significant contributor to the progression of the disease by causing the overexpression of proteins involved in cell growth and survival (Toyonaga *et al.*, 2003). Aberrant activation of the JAK-STAT signalling pathway is implicated in the oncogenesis of PDAC (Qiu *et al.*, 2007; Nagathihalli *et al.*, 2015). Elevated STAT3 expression in patients is associated with advanced tumour clinicopathological characteristics and poorer survival outcomes (Nagathihalli *et al.*, 2015). Treatment with Momelotinib, a JAK inhibitor was found to exhibit efficacy in PDAC and has shown promising potential as an adjuvant therapy (Buchert, Burns and Ernst, 2016; Fang *et al.*, 2023). JAK/STAT signalling can also converge with other signalling pathways, including the Notch and NF-κB pathways to enhance chemoresistance in PDAC (Jin, 2020). Understanding the proteins involved in PDAC carcinogenesis is imperative, yet equally essential is elucidating the pathways in which these proteins participate to gain comprehensive insights into the mechanisms driving cancer progression.

1.7.4 phosphatidylinositol 3-kinase/protein kinase B (P13K/AKT)

The PI3K/AKT pathway is a downstream signalling pathway that is activated by *KRAS*, resulting in apoptosis inhibition and promotion of cell proliferation (Taheri, Noroozi and Amoli, 2023). Dysregulation of this pathway has been linked to the development of tumours, as well as resistance to radiation and chemotherapy treatments (Mahajan and Mahajan, 2012). Cancer cells have a heightened dependence on the PI3K/Akt/mTOR signalling pathway, making it a promising target for therapy (Yu, Wei and Liu, 2022). PDAC often exhibits dysregulation of the PI3K/AKT/mTOR pathway, affecting oncogenic signalling and metabolic reprogramming (Hoxhaj and Manning, 2020; Yu, Wei and Liu, 2022). Additionally, activation of the PI3K/AKT pathway is important in promoting the progression of PDAC in response to external stimuli, leading to the development of resistance to cell death and a proliferative phenotype similar to multidrug resistance (Eberle, 2019). EGFR acts as an upstream regulator

of the PI3K-AKT signalling pathway through its tyrosine kinase activity (Sabbah, Hajjo and Sweidan, 2020). As a result, various approaches have been pursued to develop and assess inhibitory strategies that target this pathway for the treatment of cancer (Yu, Wei and Liu, 2022).

1.7.5 Tumour protein 53 (*P53)*

The *p53* gene is frequently mutated in various malignancies, including PDAC, and can be found in approximately 70% of cases (Hu *et al.*, 2021). Under normal conditions, *p53* initiates cell growth arrest or apoptosis in response to oxidative stress or DNA damage (Cicenas *et al.*, 2017). Upon detecting DNA damage, *p53* initiates cell cycle arrest and/or apoptosis through the regulation of genes, such as *MDM2*, growth arrest and DNA damage-inducible 45 (*GADD45*), cyclin-dependent kinase inhibitor 1A (*CDKN1A*), and BCL2-associated X (*BAX*) based on the severity of the damage (Bieging, Mello and Attardi, 2014; Calheiros, Corbo and Saraiva, 2023). The dysfunction of *p53*, either due to mutations or inhibitory interactions with *MDM2*, has been widely recognized as a critical factor in the initiation and progression of cancer (Muller and Vousden, 2013). The p53 protein is subject to tight regulation through a negative transcriptional feedback loop involving MDM2. This regulatory mechanism involves the direct binding of MDM2 to p53, leading to its ubiquitination and subsequent degradation by the proteasome (Momand *et al.*, 1992). In response to DNA damage stress, MDM2 undergoes auto-ubiquitination and proteasome-dependent degradation, allowing p53 to be stabilized. This stabilization enables p53 to execute its crucial role in determining the cell's fate, directing it towards apoptosis or cell cycle arrest and DNA repair (Stommel and Wahl, 2004). *In vitro* studies have demonstrated that mutations in the *TP53* gene can result in gemcitabine resistance of PDAC cells (Fiorini *et al.*, 2015). To counteract this resistance, an therapeutic strategy has emerged that involves manipulating the *p53* signalling pathway through the use of *MDM2* inhibitors. These inhibitors have the potential to enhance the efficacy of chemotherapy and directly target cancer cells bearing *p53* mutations. For instance, PXN822 prevents MDM2 from binding to the p53 binding pocket, which hinders the interaction between MDM2 and p53 (Conradt *et al.*, 2013). A range of compounds, including CP-31398 (Calheiros, Corbo and Saraiva, 2023), Reactivator of p53 and Inducer of Tumour Cell Apoptosis (RITA) (Issaeva *et al.*, 2004), PRIMA-1 and its methylated derivative PRIMA-1^{MET} (APR-246) (Bykov *et al.*, 2002) and COTI (Synnott *et al.*, 2020) have been found to reactivate mutant *p53* (mutp53).

It is essential to reactivate the function of *p53* in PDAC cells for them to effectively respond to a variety of chemotherapy drugs. This implies that *p53* activity may be a necessary component for cancer cell's responsiveness to chemotherapy (Voutsadakis, 2014; Shechter *et al.*, 2023), notwithstanding the tumour cells use multiple mechanisms to ensure progression.

1.7.6 Kirsten rat sarcoma virus (*KRAS)*

KRAS, a member of the RAS subfamily and a key component of the MAPK/ERK pathway, is crucial in the development of PDAC. It has been established that approximately 90% of PDAC cases exhibit abnormal activation of the *KRAS* oncogene, leading to the formation of PanIN and contributing to the progression of the disease to invasive and metastatic cancer (Feig *et al.*, 2012; Kanda *et al.*, 2012). Studies emphasize the crucial function of the *KRAS* signalling pathway in the context of chemoresistance, a major obstacle in the treatment of PDAC. The activation of oncogenic *KRAS* is strongly associated with decreased cell death and increased resistance to drug therapy (Hata *et al.*, 2014), due to the activation of pro-survival pathways and the elevation of anti-apoptotic factors (Möller *et al.*, 2014). As a result, *KRAS* activation is considered a critical event in PDAC, playing a vital role in the initiation and progression of tumours. Directly targeting *KRAS* has been challenging and has resulted in disappointing outcomes. The activation of signalling molecules, such as RAF, MEK, and ERK, is triggered by mutations in the *KRAS* gene and these molecules subsequently drive cell proliferation, and lead to the development of resistance (Zhu *et al.*, 2022). *KRAS* inhibits Raf kinase inhibitory protein (RKIP) through the MAPK-ERK pathway, leading to increased cancer cell growth, metastasis, and drug resistance (Rosner, 2007; Yang *et al.*, 2018).

Approximately 10% of PDAC cases, lacking *KRAS* mutations, activate wild-type RAS through upstream signalling pathways involving receptor tyrosine kinases, particularly the EGFR. Despite extensive research and examination of various RAS inhibitors, the development of drugs targeting RAS has been hindered by the scarcity of well-defined target sites (Wang, Fang and Rudolph, 2012). This mechanism is also required for $KRAS^{G12D}$-driven PDAC (Ardito *et al.*, 2012; Singhi *et al.*, 2019). For example, the inhibition of $KRAS^{G12C}$ mutation by Adagrasib (MRTX849) has shown promising tolerability in early results, with around half of the patients diagnosed with PDAC carrying this mutation experiencing a partial response (Ou *et al.*, 2022). MRTX1133, another $KRAS^{G12D}$ inhibitor, showed promising results in pancreatic cancer-bearing mice by inducing tumour regression and altering the tumour microenvironment (Kemp *et al.*, 2023). It was also observed to increase the presence of tumour-associated macrophages (TAMs) and tumour-infiltrating cytotoxic T-cells (Wang *et al.*, 2022; Kemp *et al.*, 2023). This

suggests that targeting the *KRAS* signalling pathway may prove to be a promising strategy for improving the efficacy of treatments for PDAC patients (Peri *et al.*, 2005; Elhariri *et al.*, 2023).

1.8 Growth Factor Signalling pathways

Growth factors are associated with the progression of several cancers including PDAC, leading to the development of several drugs to target them (Juhász *et al.*, 2003; Taeger *et al.*, 2011). The functions of the various growth factors in promoting chemoresistance in PDAC, current drugs targeting them and clinical trials are further elaborated upon.

1.8.1 Vascular endothelial growth factor (VEGF)

Angiogenesis is required for solid tumour development and progression (Sipos *et al.*, 2002; J. Yan *et al.*, 2023). The heparin-binding glycoprotein, VEGF, functions as an endothelial cell mitogen and is strongly linked to angiogenesis in different tumours, including PDAC (Nusrat *et al.*, 2016). VEGF is highly expressed in PDAC and many studies have also determined that its overexpression is correlated to greater tumour size, increased liver metastases, and reduced patient survival (Itakura, 1997, 2000; Ikeda, 1999; Seo, 2000; Luo, 2001; Niedergethmann, 2002; Doi *et al.*, 2012; Li *et al.*, 2019). Several preclinical and clinical studies have evaluated the efficacy of inhibiting VEGF and its receptors in PDAC (Tsuzuki, 2001; Doi *et al.*, 2012; Costache *et al.*, 2015; Craven, Gore and Korc, 2016). For example, the inhibition of VEGF/VEGFR by foretinib blocked angiogenesis and cell proliferation and resulted in increased apoptosis (Chen, Tsai and Hung, 2015; Malekan and Ebrahimzadeh, 2022). Bevacizumab (an anti-VEGF antibody) and Sunitinib (an anti-tumour and anti-angiogenic tyrosine kinase (TKI) inhibitor) were observed to inhibit PDAC cell motility and migration (Doi *et al.*, 2012). Sunitinib blocks VEGFR, allowing angiogenesis to be inhibited within pancreatic cancer cells (Delbaldo *et al.*, 2012; Skaraitė, Maccioni and Petrikaitė, 2023) (Figure 1.3a). Treatment with cedirinib, a VEGF inhibitor, was also found to reduce the expression of key EMT markers such as *ZEB1*, *Snail* and *N-cadherin* (Momeny *et al.*, 2020), suggesting potential roles in inhibiting cancer cell migration and metastasis. The combination of VEGF inhibitors such as bevacizumab and chemotherapeutic agents like gemcitabine and 5-FU have shown promising potential in treating PDAC (Martin *et al.*, 2012; Barati Bagherabad *et al.*, 2019).

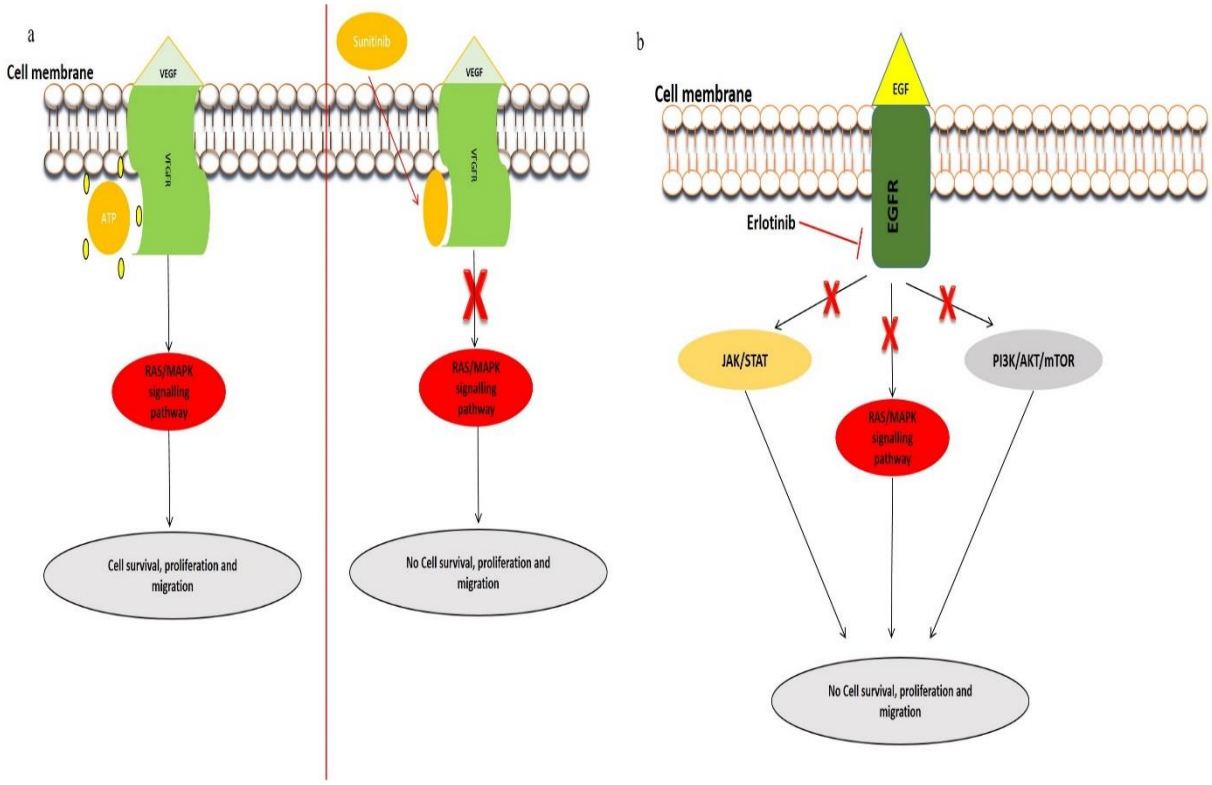


Figure 1. 4: Examples of inhibition of the VEGFR and EGFR signalling pathways (Xelwa *et al.*, 2021) **(a)** Inhibition of VEGFR signal transduction by sunitinib. After the entry of sunitinib into the cytoplasm, it competitively binds at the ATP site of VEGFR, consequently inhibiting the activation of the pathway. **(b)** Mechanism of action of Tyrosine kinase inhibitor, erlotinib. Erlotinib is a small molecule that acts as an ATP analogue and inhibits EGF signalling by binding to receptor tyrosine kinases (RTKs), consequently inhibiting the activation of downstream signalling pathways.

1.8.2 Epidermal growth factor (EGF)

Epidermal growth factor receptor (EGFR) is expressed in up to 60-90% of PDAC and is involved in inducing cell growth and migration (Xie *et al.*, 2019). Targeted anti-EGFR molecular strategies have been employed in the treatment of PDAC especially to circumvent chemoresistance (Bloomston *et al.*, 2006; Oliveira-Cunha, Newman and Siriwardena, 2011). Erlotinib, one of the most studied EGFR inhibitors has been implored to block the EGFR signalling pathway (Figure 1.3b). A clinical trial showed that erlotinib might be an effective and safe treatment option for PDAC (Deiana *et al.*, 2024). Combinational treatment of erlotinib with gemcitabine prolonged survival in patients PDAC patients in a phase III study (Moore *et al.*, 2007; Y.-T. Fang *et al.*, 2023). Simultaneous treatment of erlotinib, gemcitabine and capecitabine also showed efficacy in metastatic PDAC patients (Oh *et al.*, 2012; Singh and O'Reilly, 2020). Additionally, studies have demonstrated the use of statins in combination with anti-EGFR agents in pancreatic cancer treatment (Jun Gong *et al.*, 2017). Statins, known to lower lipid concentrations, block the production of intermediates needed for prenylation and

RAS/mitogen-activated protein kinase 1 signalling activation. They appear to alter resistance to anti-EGFR agents, such as erlotinib or cetuximab, plus gemcitabine and attenuate angiogenesis by inhibiting vascular endothelial growth factor synthesis (Dulak and Jozkowicz, 2005; Hong *et al.*, 2014; Araghi *et al.*, 2023).

1.8.3 Fibroblast growth factor (FGF)

The fibroblast growth factor family (FGF) with about 23 known proteins and their receptors are associated with PDAC playing a role in tissue hyperplasia, transition of EMT, tumour metastasis and angiogenesis (Gnatenko, Kopantsev and Sverdlov, 2017). FGF is overexpressed in PDAC and promotes cell growth, proliferation and invasion (Kornmann, 1997; Kornmann, Beger and Korc, 1998). Similarly, the overexpression of FGF and FGFR can lead to oxidative stress evident through increased nitric oxide synthase (iNOS) (Kang *et al.*, 2019). FGF10 is a molecule involved in mesenchymal-epithelial signalling and is crucial in the development of multiple organs including the pancreas (Itoh and Ohta, 2014). However, when alterations occur, the FGF10 can induce migration and invasion in tPDAC cells. As the mesenchyme is essential for the pancreatic embryonic growth, its absence can result in a lack of islet cells and hypoplasia. Furthermore, the crosstalk between the FGF10 and TGF-β pathway can promote EMT and cancer cell invasion (Clayton and Grose, 2018; Ndlovu *et al.*, 2018). A recent study observed that high expression of FGF8 was independently associated with diminished overall PDAC patient survival, indicative of poor prognosis (Jomrich *et al.*, 2020). The FGF can also play a role in chemoresistance. It was determined that acquired drug resistance observed when tumours were treated with VEGF inhibitors was induced by several factors including hypoxia and FGF expression (Casanovas *et al.*, 2005). FGF2 targeting agents and inhibitors can aid in preventing TAM-associated cell migration and chemoresistance (Ireland and Mielgo, 2018a; Kang *et al.*, 2019). One study evaluated the use of dovitinib, an inhibitor of the FGFR/VEGFR pathway in combination with gemcitabine and capacitabine and determined improved efficacy in patients with advanced PDAC (Ma *et al.*, 2019). Mastinib, another inhibitor of FGFR and PDGFR was shown to decrease inflammation in PDAC patients (Waheed, Purvey and Saif, 2018; Jiménez *et al.*, 2024). Combining mastinib and gemcitabine in both *in vitro* and *in vivo* PDAC models showed that mastinib sensitized tumour cells to gemcitabine (Humbert *et al.*, 2010; Mashayekhi *et al.*, 2021). This was further supported by a phase III clinical trial which indicated the use of the combination of mastinib and gemcitabine in patients that overexpress *ACOX1* (Deplanque *et al.*, 2015; Bararia *et al.*, 2023).

1.8.4 Transforming growth factor-beta (TGF-β)

Mutations of key genes such as SMAD4 are a major characteristic of PDAC initiation and progression. These mutations can be found in more than half of PDAC patients and play an important role in TGF-β signalling (Massagué, 2008; M. Li *et al.*, 2020). In the tumour microenvironment (TME), the TGF-β signalling pathway is involved in the regulation of several cell types. For instance, it promotes the differentiation of myofibroblasts and, the recruitment of immune cells and influences epithelial and endothelial cell differentiation (Ma *et al.*, 2020). This pathway also has contradictory functions as a tumour suppressor and promoter (Q. Zhou *et al.*, 2019). During early tumorigenesis, TGF-β signalling can inhibit the cell cycle and induce apoptosis, but could also promote tumour growth via enhancing EMT, cancer stem cell formation, cellular migration, invasion and immune response evasion by inhibiting Th1 immune response (Vivekanandhan and Mukhopadhyay, 2019). Cancer cells can secrete both PDGF-BB and TGF-β leading to the promotion of fibroblasts transformation thereby resulting in the low expression of the anti-cancer Pigment Epithelium-Derived Factor (PEDF) (Nwani *et al.*, 2016; Avagliano and Arcucci, 2022).

Another study determined that silencing TGF-βRII, the protein that begins the TGF-β signalling, promoted tumour growth and resistance to gemcitabine (Drubay *et al.*, 2018). On the other hand, several inhibitors of the TGF-β signalling pathway such as trabedersen and galunisertib have been developed and tested in PDAC to inhibit tumour growth (Schlingensiepen *et al.*, 2011; Herbertz *et al.*, 2015). LY2109761, an inhibitor of TGF- β receptors I and II, was used alone and in combination with gemcitabine and revealed to inhibit cell survival, migration and metastases (Melisi *et al.*, 2008; Yap *et al.*, 2021). Cisplatin (Platin) resistance can also be promoted through altering TGF- β/SMAD4 signalling and up-regulation of EMT-markers by tumour-derived exosomes (TDEs), known to function in the development and progression of different biological processes in cancer (Mashouri *et al.*, 2019). It was shown that the release of TGF-β and FGF5 from cancer-associated fibroblasts (CAFs) causes myofibroblast reprogramming in cancer stem cells (CSCs), needed to protect the cells from external influences, to acquire a chemoresistance characteristic within the cells (Najafi, Farhood and Mortezaee, 2019).

1.8.5 Connective Tissue Growth Factor (CTGF/CCN2)

Connective Tissue Growth Factor (CTGF/CCN2) is a protein found in the extracellular matrix that functions in regulating diverse cellular processes such as cell survival, proliferation, migration, and apoptosis (Charrier and Brigstock, 2013). CTGF has also been linked to other

growth factor signalling pathways including TGF- β and FGF. Studies showed that TGF-β can prompt CTGF production in PDACcell lines and it can induce the expression of FGFR2 (Kwon *et al.*, 2007; Aoyama, Kubota and Takigawa, 2012). Over recent years, CTGF has been increasingly investigated as a target for PDAC therapies (Chen *et al.*, 2020; Y.-T. Fang *et al.*, 2023). *In vivo* silencing of CTGF resulted in the reduction of tumour growth and its expression was closely associated with hypoxia and density of tumour-surrounding stromal cells (Bennewith *et al.*, 2009). Treatment with an antagonist of CTGF, mAbFG-3019, using murine models of PDAC revealed that gemcitabine-based chemotherapy was enhanced by leading to a reduction of tumor size (Neesse *et al.*, 2013; Zhang *et al.*, 2023). The efficacy of pamrevlumab (FG-3019) against pancreatic tumours was further confirmed by another study that showed its effectiveness in selectively targeting pancreatic tumor cells and inhibiting metastases (Dornhöfer *et al.*, 2006). In a phase I and II study, pamrevlumab, enhanced gemcitabine activity in locally advanced PDAC patients (Picozzi *et al.*, 2018). Similarly, another study also demonstrated the use of peptides targeting CTGF alone and in combination with gemcitabine in reducing tumor size (Resovi *et al.*, 2020).

1.8.6 Nerve Growth Factor (NGF)

Nerve growth factor (NGF) is present in sympathetic and neural crest-derived sensory neurons, as well as in the central nervous system (CNS) (Skaper, 2017; Nemati Mahand *et al.*, 2024). Pancreatic cancer cells can invade surrounding nerve cell spaces leading to perineural invasion. Perineural invasion is common in PDAC correlating to poor prognosis and can be facilitated by nerve growth factors (Hirai *et al.*, 2002; Ceyhan *et al.*, 2008; Gu *et al.*, 2019). Overexpression of NGF and BDNF induces noradrenaline accumulation, consequently inducing pancreatic cancer cell growth (Renz *et al.*, 2018). Due to its role in inhibiting apoptosis, NGF has also been implicated in chemoresistance (Liu *et al.*, 2003). Time-dependent treatment of PDACin mouse models determined that depleting NGF inhibits inflammation and metastasis (Saloman *et al.*, 2018). The inhibition of NGF by blocking STAT3 resulted in decreased PDAC migration and reduced perineural invasion (Guo *et al.*, 2013). Similarly, another study showed that NGF knockdown prevented PDAC cell proliferation, invasion and migration (Bapat *et al.*, 2016; Li, Kang and Tang, 2021).

1.8.7 Platelet-derived growth factor (PDGF)

The PDGF family can bind to the tyrosine kinase receptors, PDGFRα and PDGFRβ, and interact with different cell types within the TME to enhance tumour progression and chemoresistance (Hoffmann *et al.*, 2008; Bartoschek and Pietras, 2018). PDGF regulates

PDAC progression by mediating pathways such as HIPPO/Yes and via its interaction with DUSP28 (Lee *et al.*, 2017; Li *et al.*, 2021). Inhibiting the phosphorylation of PDGFR by using Gleevec (Imatinib) with gemcitabine led to a reduction in pancreatic tumour growth in nude mice models (Hwang *et al.*, 2003). The tyrosine kinase inhibitor (Sunitinib) targets both VEGFR1-3 and PDGFR pathways (Christensen, 2007; Guihong Liu *et al.*, 2021). Sunitinib appears to be a promising drug in instances where patients did not respond well to gemcitabine-based treatments (O'Reilly *et al.*, 2010). In another study, the use of a multi-kinase inhibitor, nintedanib, which simultaneously targets VEGFR, FGFR and PDGFR signalling was investigated alone, and in combination with gemcitabine in xenograft models and this drug displayed a strong antitumour activity (Awasthi *et al.*, 2015; Zhao *et al.*, 2023).

1.8.8 Hepatocyte Growth Factor (HGF)

Together with its receptor, c-MET, HGF is overexpressed in PDAC and has been linked to cancer cell invasion, metastasis and chemoresistance via tumour-promoting pathways such as P13/Akt and neuropilin (Matsushita, Götze and Korc, 2007; Watanabe, Kishimoto and Yokosuka, 2011; Gafarli, Tian and Rückert, 2017; Modica *et al.*, 2018; Pothula *et al.*, 2020). HGF levels predicted overall survival in locally advanced PDAC patients who underwent neoadjuvant therapy (Roberts *et al.*, 2021). One study demonstrated the promotion of PDAC cells by pancreatic stellate cells through the HGF/c-MET pathway; this pathway required surviving expression and was regulated by the p53/p21 pathway (Yang *et al.*, 2017). Several inhibitors such as rilotumumab, ficlatuzumab onartuzumab, crizotinib, tivantinib, foretinib and cabozantinib can be used against HGF/c-MET (Demkova and Kucerova, 2018; Anitha *et al.*, 2023). For example, blocking HGF using rilotumumab *in vivo* led to decreased metastasis compared to treatment with gemcitabine (Jin *et al.*, 2008). Patients treated with the combinatorial therapy of ficlatuzumab nab-paclitaxel and gemcitabine had favourable treatment responses albeit with an observed significant decrease in albumin levels and body swelling (Perez *et al.*, 2020). A synergistic effect was observed when PDAC cell cultures were treated with tivantinib and gemcitabine, suggesting their possible use in patient treatment (Rolfo *et al.*, 2014).

The inhibition of key growth factors is a promising strategy towards PDAC treatment and management which is evident in several studies discussed here including approximately 170 associated ongoing clinical trials (Table 1.2).

Table 1.2: Some clinical trials evaluating growth factor inhibition in PDAC.

Drugs/Inhibitors	**Growth factor target**	**Number of ongoing clinical trials with results***	**Status of clinical trials**	**Clinical trial number**
Nab-Paclitaxel	IGF-1R	142	41 active, not recruiting	Such as NCT03316599, NCT03520790, NCT02827201, NCT02210559, NCT02047513, NCT03086369, NCT02717091, NCT02481635, NCT03252808, NCT02427841
			101 recruiting	Such as NCT04808687, NCT02340117, NCT03929094, NCT03850769, NCT03941093, NCT03885219, NCT03652428, NCT03636308, NCT04498689,
Bevacizumab	VEGF	13	7 active, not recruiting	NCT03387098, NCT03329248, NCT03136406, NCT03586869, NCT03376659, NCT01229943, NCT04299880
			6 recruiting	

				NCT03351296, NCT03597581, NCT03193190, NCT02820857, NCT04430842, NCT03597581
Sunitinib	VEGFR, PDGFR,	4	4 recruiting	NCT02230176, NCT02282059, NCT02465060, NCT03878524,
Nintedanib	VEGFR, FGFR, PDGFR	1	1 recruiting	NCT02902484
Erlotinib	EGFR	6	4 active, not recruiting 2 recruiting	NCT01013649, NCT00878163, NCT01660971, NCT02737228 NCT04136132, NCT03878524
Cetuximab	EGFR	4	3 active, not recruiting 1 recruiting	NCT03992664, NCT03319459, NCT01420874 NCT03785249
Imatinib	PDGFR	1	1 recruiting	NCT03878524
Pamrevlumab (FG-3019)	CTGF	2	1 active, not recruiting 1 recruiting	NCT02210559 NCT03941093
Larotrectinib	NGF	1	1 recruiting	NCT02465060
Ficlatuzumab	HGF/c-MET	1	1 active, not recruiting	NCT03316599
Crizotinib	HGF/c-MET	2	2 recruiting	NCT02465060, NCT02568267

*Studies could involve treatment alone or in combination with other therapeutic strategies

In summary, signalling pathways play a role in tumour initiation and progression in PDAC. Of note, their dysregulation has also been linked to chemoresistance. Efficient elucidation of these

signalling pathways in initiating and propagating chemoresistance is crucial. Novel strategies must be developed to target these pathways to identify effective treatment for PDAC.

1.9 Study Rationale

PDAC is a challenging type of cancer to treat and has a poor prognosis. Although several treatment strategies exist, they have not resulted in any significant improvement in patient outcomes. As such, compared to other cancers, PDAC continues to be a disease with abysmal outcomes. Therefore, it is crucial to identify new therapeutic targets with the potential to improve patient outcomes. Furthermore, individuals of African descent, have a higher risk of developing the disease with the poorest prognosis compared to other groups. There is little information on the molecular profiles of these patients. Therefore, exploring the gene expression patterns of key signalling pathways may provide critical insights into the molecular pathogenesis of this patient group. PDAC is characterised by disruptions in pathways that control cellular proliferation, invasion, and migration. Prior studies, including those conducted in our group, have identified the intricate molecular signatures within these pathways (Nweke *et al.*, 2020). This study aimed to identify novel therapeutic targets in PDAC in patients from the African population, this was achieved by conducting a discovery study looking at the gene expression profile of signalling pathway-related genes using PDAC tissue samples. Subsequently, screening revealed up and downregulated genes, with *SPP1* identified as one of the top upregulated genes. *SPP1* has been demonstrated to play a crucial role in cancer, with its functions established across various cancer types. Specifically, *SPP1* contributes to metastasis by mediating cell adhesion and invasion, enhancing angiogenesis, and facilitating the evasion of apoptosis. *SPP1* was selected as a potential gene target for this study due to its role not being fully validated in PDAC.

Therefore, the current study contained three parts, whereby the first involved a targeted pathway-specific analysis conducted to determine the gene expression profiles of signalling pathway-related genes using PDAC tissue samples.The second part included optimising the downregulation of *SPP1* in the MIA PaCa-2 cells with siRNA, followed by assessing the effect of *SPP1* downregulation and gemcitabine treatment on apoptosis and migration potential. Furthermore, proteomic analysis was conducted to elucidate the pathways affected by *SPP1* downregulation and gemcitabine treatment.

These unique molecular signatures can be explored and targeted against PDAC cells for effective treatment. This research is significant because it has the potential to uncover new therapeutic targets, which could lead to more effective treatment strategies.

1.10 Overall Aim

To identify the gene expression patterns of signalling pathways in PDAC and identify novel potential targets.

Objectives

- To determine differentially expressed genes in pancreatic tumours obtained from South African patients using pathway-specific RT^2 Profiler PCR arrays;
- To verify the identified gene target(s) using real-time Polymerase Chain Reaction.
- To perform a knockdown of the identified target gene using siRNA in MIA PaCa-2 PDAC cells.
- To investigate the effect of the target gene knockdown and combination therapy with Gemcitabine on the phenotype of the MIA PaCa-2 cells via cell-based assays such as apoptosis, cell migration
- To identify key molecular pathways dysregulated with *SPP1* knockdown and combined treatment with gemcitabine using SWATH-MS

The thesis is divided into varying chapters. In Chapter 1, PDAC was introduced and varying key signalling pathways involved in PDAC were described especially in the context of chemoresistance. In Chapter 2, pathway-focused analysis of gene expression was conducted using tumours obtained from South African patients and a gene target was identified and verified. Chapter 3 examines the functional role of the target on the phenotype of PDAC cells and the effect of targeting it in combination with conventional chemotherapy (gemcitabine). Finally, Chapter 4 delves into the molecular underpinnings that are responsible for the phenotype observed.

CHAPTER 2: PATHWAY-FOCUSED GENE EXPRESSION ANALYSIS OF TUMOURS FROM SOUTH AFRICAN PATIENTS.

2.1 The Tumour Microenvironment in PDAC

The TME of PDAC is characterised by a dense extracellular matrix (ECM) (Kalluri, 2016; Jena *et al.*, 2020) and an immune infiltrate that is implicated in creating an immunosuppressive environment. The crosstalk between numerous cell types, including CAFs, myofibroblasts, tumour-associated macrophages (TAMs) and stromal cells plays a key role in tumour progression and in inducing chemoresistance (Shree *et al.*, 2011; Feig *et al.*, 2012; Shibuya *et al.*, 2014; Ireland *et al.*, 2016; Ireland and Mielgo, 2018; Schnittert, Bansal and Prakash, 2019; Jena *et al.*, 2020). Unpacking the heterogeneity of PDAC TME is essential to understand the dynamics of this disease and how cellular interactions, mediated by autocrine and paracrine factors, can induce signalling pathways that confer more aggressive properties onto pancreatic cancer cells.

2.1.1. Cancer-associated fibroblasts

CAFs have been linked to drug resistance in PDAC through various pathways, including impeding drug delivery, elevation of cytokine levels, accelerating cell proliferation, triggering signalling pathways, disrupting cellular metabolism and facilitating metastasis. Novel approaches to target these mechanisms are currently being developed (Sun *et al.*, 2018; Thomas and Radhakrishnan, 2019; Jena *et al.*, 2020; Wang *et al.*, 2020). The extracellular matrix of PDAC is composed primarily of proteins secreted by CAFs (Kalluri, 2016; Jena *et al.*, 2020); therefore, the heterogeneity and dominance of CAFs within the TME ensure their crucial involvement in therapeutic resistance (Öhlund *et al.*, 2017; Jena *et al.*, 2020). The delivery of drugs to tumour cells is crucial for achieving therapeutic efficacy and drug sensitivity. In PDAC, the dense TME architecture, evidenced by high levels of ECM proteins, impedes drug delivery. Our group recently showed using SWATH-MS, the intricate network of signalling pathways within the ECM of PDAC tumours highlighting different known and potentially novel associating proteins (Nweke *et al.*, 2020). Moreover, in mouse models of PDAC, it has been noted that CAFs tend to accumulate gemcitabine, potentially contributing to the development of drug resistance (Hessmann *et al.*, 2018). Also, CAFs are derived from pancreatic stellate cells (PSCs), which can release metabolites like deoxycytidine, potentially granting resistance to PDAC cells (Dalin *et al.*, 2019).

2.1.2. Myofibroblasts (MyCAF)

Myofibroblasts play a vital role in maintaining tissue repair and fibrosis balance in tumours (Nallasamy *et al.*, 2021). These cells have a significant impact on cancer progression, and their presence is closely associated with disease progression. α-SMA-expressing myofibroblasts, or myCAFs, have been found near tumour sites, where they synthesize extracellular matrix components and contribute to the formation of dense desmoplasia. The presence of myCAFs is a significant factor in promoting cancer development and progression (Öhlund *et al.*, 2017). TGF-β is a crucial factor in the development and maintenance of the physical and functional characteristics of myofibroblasts (Das *et al.*, 2020). It appears to foster an immunosuppressive myofibroblast profile in PDAC, which hampers the effectiveness of checkpoint blockade (Dominguez *et al.*, 2020; Grauel *et al.*, 2020). Cases where there is an abundance of TGF-β-induced $LRRC15^{+}$ myofibroblasts have been reported to have decreased responsiveness to anti-PDL1 therapy (Dominguez *et al.*, 2020). Elahi-Gedwillo et al. discovered that administering the anti-fibrotic drug (halofuginone) to target the stromal microenvironment altered the matrix composition, leading to the suppression of the fibroblast activation and enhancement of the drug distribution (Elahi-Gedwillo *et al.*, 2019).

2.1.3 Pancreatic stellate cells (PSCs)

PSCs represent about 7% of PDAC cells and can be activated from a dormant state to become CAFs as a result of various stimuli, such as tumour necrosis factor-α TNF-α, TGF-β, interleukin IL-1, IL-2, IL-10, and PDGF (Gu *et al.*, 2021; Chakkera *et al.*, 2024). The activation of PSCs has been shown to stimulate angiogenesis, ECM synthesis, invasion and drug resistance in PDAC. These cells contribute to cancer resistance by depositing ECM components, such as collagen, fibronectin and hyaluronic acid (HA) which impede the penetration of drugs (Jaster and Emmrich, 2008). PSCs play a crucial role in the fibrotic processes that occur within the pancreatic tissue and have a significant impact on the stromal response observed in PDAC, due to their increased proliferation and collagen synthesis (Apte and Wilson, 2012; HRABÁK *et al.*, 2021). Coetzee et al. investigated the interplay between PSCs and PDACs, revealing that nuclear FGFR1 facilitates the PSC-induced invasion of PDAC cells (Coetzee *et al.*, 2023). These processes are dependent on interaction with ECM components that ultimately drive tumour progression.

Preclinical studies have demonstrated that mebendazole, an FDA-approved drug known for its inhibition of tubulin polymerization, diminishes PSC activity and disrupts stromal desmoplasia in aggressive PDAC models, consequently resulting in decreased tumour size (Williamson *et*

al., 2021). Additionally, vitamin A analogues or vitamin D receptor agonists may potentially be used to attain PSC quiescence, which could lead to a reduction in oncogenic signalling, hinder tumour growth, and improve the efficacy of chemotherapy (Froeling and Kocher, 2015; Carapuça *et al.*, 2016). Inhibition of HGF and its receptor c-Met (HiCi) effectively slowed down PDAC disease progression post-surgery, reduced angiogenesis, and lowered PSC counts in PDAC mouse models (Pang *et al.*, 2021). PSCs also contribute to the development of resistance to gemcitabine by interacting directly with PDAC cells and by communicating through cytokine signalling (Gu *et al.*, 2021).

2.1.4 Immune cells

The recognition of cancer cells, initiation of inflammation, and anti-tumour responses are crucial aspects of TME regulation, and granulocytes macrophages, dendritic cells and cells of the adaptive immune system, play a vital role in these processes (de Visser, Eichten and Coussens, 2006; Hinshaw and Shevde, 2019). The tumour microenvironment is a complex interplay of various elements that leads to the proliferation of connective tissue and the suppression of anti-tumour immune responses (Hessmann *et al.*, 2020). This contributes significantly to various immune evasion techniques, thereby enabling the advancement of invasion, progression, and metastasis (Karamitopoulou, 2019). The PDAC microenvironment is known to display localised inflammation, which is a result of interactions between tumour cells and immune cells that have invaded the area (Zhu *et al.*, 2023).

In the PDAC microenvironment, T cells exert both anti-tumour and pro-tumour immune responses (Hinshaw and Shevde, 2019). The TME of PDAC is composed of multiple T cell subtypes, such as $CD8^+$ cytotoxic T cells (CTLs), also known as effector T cells (Teff), $CD4^+$ T cells, including helper T cells (Th) like Th1, Th2, and Th17 subsets, regulatory T cells (Tregs), and natural killer (NK) T cells (Ostios-Garcia *et al.*, 2021).

Extensive research has been conducted on the role of $CD8^+$ T cells within the PDAC TME and immunotherapy studies. These cells are essential to the immune system and play a critical role in evaluating the PDAC microenvironment and the efficacy of immunotherapy. Their secretion of IFN-γ, TNF, and cytotoxic molecules is crucial to the elimination of cancerous cells (Ostios-Garcia *et al.*, 2021). Enhanced numbers of CD8+ T cells correlate with treatment efficacy and survival rates (Liu *et al.*, 2021; Yu *et al.*, 2021). Teffs in the TME may have reduced function, leading to weaker cytotoxicity and reinforcing immunosuppressive features of PDAC's microenvironment (Balli *et al.*, 2017; Ahmed and Neidle, 2020).

$CD4^+$ T cells recognize specific proteins presented by MHC II molecules on antigen-presenting cells such as macrophages and dendritic cells. This recognition causes their conversion into helper T cells (Aykut, Chen and Miller, 2020). Th1 cells are recognized for their ability to exhibit anti-tumour properties by secreting IFN-γ and cytotoxic molecules. Conversely, Th2 cells produce cytokines like IL-4, IL-5, IL-3, and IL-13, which encourage M2 polarization of TAMs, leading to immunosuppression and ultimately promote tumour growth (Leinwand and Miller, 2020).

Tregs are a distinct type of $CD4^+$ T cells that express forkhead box P3 (FOXP3) and are instrumental in maintaining self-tolerance in healthy tissues. However, in certain disease states, Tregs can exert a significant impact on the immune system. By secreting IL-10 and TGF-β, Tregs can suppress both $CD8^+$ and $CD4^+$ T cell subsets, and they can also compete with other immune cells for antigens presented by DCs. This can create an immunosuppressive environment that fosters tumour progression (Aykut, Chen and Miller, 2020; Bhatia *et al.*, 2022). PDAC's resistance to therapies is well-known due to significant tumour heterogeneity and a highly desmoplastic, immunosuppressive TME (Kleeff *et al.*, 2016). The inhibition of JAK/STAT signalling pathway by Ruxolitinib has been demonstrated to alleviate immunosuppression and improve the efficacy of anti-PD-1 therapy (Ajina *et al.*, 2021). Such immune checkpoint inhibitors act to enhance T- cell mediated elimination of tumour cells. A recent study using live-cell imaging has explored combination therapies for treating PDAC. The study has revealed that a combination of trametinib (a mitogen-activated protein kinase 1 (MEK) inhibitor) and palbociclib (a CDK4/6 inhibitor), along with anti-PD-L1 therapy, can significantly enhance the effectiveness of the treatment. These drugs have the potential to improve immune activity through CD8+ T cell-dependent and unconventional Rb-dependent pathways, thereby increasing T cell infiltration and modifying the myeloid population (Knudsen *et al.*, 2021; Lesinski, 2021). Immune checkpoint blockers (ICBs) have been approved for the treatment of various types of cancer (Mahoney, Freeman and McDermott, 2015; Galluzzi *et al.*, 2023), however, ICB monotherapy is ineffective for PDAC treatment (Kabacaoglu *et al.*, 2018), indicating a multiplicity of pathways that drive PDAC tumour progression.

In the TME, TAMs have dual roles in tumourigenesis, acting as M1 inflammatory macrophages with tumor-inhibiting properties and M2 macrophages promoting tumour growth. Macrophage diversity in PDAC, contributes to its complexity (Ruffell, Affara and Coussens, 2012; Murray *et al.*, 2014; Noy and Pollard, 2014). PDAC chemoresistance has been associated with the

presence of CSCs, which exhibit an increased capacity to undergo EMT , altered metabolism, altered key genes (such as *TP53*, *KRAS*, *CCND1*, *BCL-2* and *BIRC5*), dysregulated signalling pathways (including NF-kB, Hedgehog , PI3K/ AKT and Notch) increased cell cycle and reduced apoptosis (Di Carlo, Brandi and Cecconi, 2018; Gzil *et al.*, 2019).

2.2. The intersection of signalling pathways: the role of the TME

The TME as illustrated, is diverse, with multiple cell types using cytokine and growth factors to engage signalling pathways that drive tumour progression. Key molecules and pathways that we have identified in PDAC progression (Section 1.7), bear noting. Tumour cell interactions with immune cells for example, can not only prevent immune cell elimination of tumour cells themselves, but also subvert the immune response for tumour progression. This is seen particularly, with for example IL-4 and IL-13, which not only polarises TAMs to M2 phenotypes that promote tumourigenesis (Leinwand and Miller, 2020), but limits the cytotoxic T cell response (Aykut, Chen and Miller, 2020). The network of signalling pathways that are thus induced in tumour cells incrulde activaton of the JAK/Stat pathway that is a major contributor to progression of PDAC by inducing overexpression of factors involved in cell growth and survival (Toyonaga *et al.*, 2003). Similarly engagement of the PI3K/AKT/mTOR pathway promotes the differenetiation of a variety of cell types into CAFs (Z. Fang *et al.*, 2023), which in turn secrete factors that create a pro-tumorigenic ECM, permitting cellular migration and consequently, creating an ECM that is implicated in impeding drug delivery (Öhlund *et al.*, 2017; Jena *et al.*, 2020).

The complexity of the interplay between the cell types of the TME and tumour cells also accounts for variability in functional outcomes of major signalling pathways. For example, the Notch signalling pathway can have either oncogenic or tumour-suppressive effects, depending on environmental influences (Avila and Kissil, 2013). Given the impact that the convergence of cellular-interactions and induced signalling pathways can have on tumour progression, chemoresistance and ultimately survival, this study profiled tumours obtained from South African PDAC patients to identify key differentially expressed pathways and identify potentially new therapeutic target genes.

2.2 Materials and Methods

2.2.1 Study population

The inclusion criteria included both male and female patients between the ages of 18 and 90 years and black South African patients who had been clinically and histologically diagnosed

with only PDAC. Patients with acute inflammation, sepsis, organ failure, and those undergoing therapy were excluded from the study.

2.2.2 Patient recruitment and sample collection

After informed consent, biopsy samples were collected from fifteen patients (15 tumour and 15 corresponding normal tissues) during Whipple procedures (surgical resection of cancerous tumours located on the head of the pancreas) from the Hepatopancreaticobiliary (HPB) units at Chris Hani Baragwanath Academic Hospital (CHBAH). The recruited patients were aged between 53 to 95 years and included 7 females and 8 males (Nweke *et al.*, 2020). The study was carried out in compliance with the Declaration of Helsinki. Patients were all diagnosed with PDAC through abdominal screening using a contrast-enhanced triple-phase CT scan. Histopathological diagnosis was obtained either endoscopically or surgically. These specimens were promptly submerged in RNA later™ stabilisation solution (2857138, Invitrogen™, Waltham, MA, USA) for preservation and subsequent analysis. Figure 2.1 shows a flow diagram of the sampling methods used in this chapter. Laboratory analysis of the tissue samples was conducted at the Department of Surgery laboratories, Faculty of Health Sciences at the University of the Witwatersrand.

2.2.3 Ethics approval and consent

Ethics approval was obtained from the medical Human Research Ethics Committee (HREC) of the University of the Witwatersrand (Ethics number: M190735) (see appendix A for certificate). Before enrolling the participants, the participant information sheet detailing the research was provided to the patient and explained in a language they understood and in the presence of a registered health practitioner. Written informed consent was then obtained before the Whipple procedure was performed. Volunteers who were recruited received an information sheet explaining the study.

2.2.4 RNA extraction and cDNA synthesis

Tissues weighing between 15 to 25 mg were dissected into small fragments using sterile surgical blades. These fragments were then placed into 15 ml corning Falcon tubes (339659, ThermoFisher Scientific, Waltham, MA, USA) containing 700 µl of TRIzol reagent (T9424, Sigma Aldrich St. Louis, Missouri, USA), and subsequently homogenised using a Tissue Ruptor (9002755, Qiagen, Hilden, Germany) then incubated at room temperature for 5 minutes. About 150 µl of chloroform (02487, Sigma Aldrich, Missouri, USA) was added to each sample, mixed vigorously, and centrifuged at 12 000 x g at 4 ºC for 15 minutes, forming three layers. The transparent aqueous layer was transferred into a new labelled 1.5 ml centrifuge tube, with

350 µl of isopropanol (I9516, Sigma Aldrich, St Louis, MO, USA) added to the sample. The samples were resuspended, allowed to stand for 8 minutes at room temperature and centrifuged at 12 000 x g at 4 ºC for 8 minutes. The resulting supernatant was discarded, and the pellets were washed with 700 µl of 75% ice-cold ethanol (E7148, Sigma Aldrich, St Louis, Missouri, USA), and centrifuged at 7500 x g at 4 ºC for 5 minutes. Supernatants were discarded, and pellets were air-dried for 5 minutes. Pellets were resuspended in 30 µl of nuclease-free water (3098, Sigma Aldrich St. Louis, Missouri, USA), inserted into a heating block at 55-60 ºC for 5 minutes, and stored on ice. RNA purity was confirmed using the Nanodrop® 1000 (ND2000CLAPTOP, Thermo Fisher, Waltham, Massachusetts, USA) ensuring that the A260/A280 and A260/230 of the samples were above 2.0.

cDNA (complementary DNA) synthesis using the RT cDNA synthesis kit: Tumour and normal tissue samples were pooled (15 tumours pooled into one sample, normal tissues pooled into one sample), and adjusted to a concentration of 500 ng/µL. The RT^2 First Strand kit (330401, Qiagen, Hilden, Germany) was used for cDNA synthesis from two micrograms of total RNA per the manufacturer's instructions.

2.2.5 Differential gene expression analysis using Targeted PCR arrays.

The RT^2 Profiler PCR Human Growth Factor (PAHS-014Z 330 529, Qiagen, Hilden, Germany) and Signal Transduction arrays (PAHS-014Z 330 529, Qiagen, Hilden, Germany) were used to perform a pathway-focused gene expression analysis of the signalling pathway genes. Each of these arrays contains 96 embedded genes: 84 target genes, five reference genes (*ACTB, B2M, GAPDH, HPRT1, RPLP0*).

The PCR mix was prepared according to the manufacturer's instructions and processed on the QuantStudio1 real-time thermocycler (A40427, ThermoFisher Scientific, Waltham, MA, USA). The Ct values were then exported, and fold changes were analysed using the QIAGEN RT^2 Profiler PCR analysis software tool (https://www.qiagen.com/us/product-categories/instruments-and-automation/analytics-software, accessed 12 September 2019). A fold change of Log2 was applied for differential analysis comparing the upregulated and downregulated genes, profiled on 2 sample groups and normalised using the normal control group. This is done relative to the expression levels of the gene of interest in the corresponding normal tissue samples. The data analysis web portal calculates fold change using the delta-delta Ct method. In this process, delta Ct is determined between the gene of interest (GOI) and the average of reference genes. This is followed by delta-delta Ct calculations (delta Ct of the

test group minus delta Ct of the control group). Fold change is then calculated using the formula 2^ (-delta-delta Ct).

2.2.6 Selection of target gene

Following the screening of genes from using the RT^2 Profiler PCR Arrays, *SPP1* was selected. This selection was made because it was one of the most upregulated genes. *MRPL-19* was used as the reference gene for the quantification of *SPP1* gene expression in tissue samples. An extensive literature search conducted also showed little information on the role of *SPP1* in PDAC especially in the context of chemoresistance, although it has been identified to be involved in key processes linked to carcinogenesis (Chen *et al.*, 2022).

2.2.7 Verification of target gene using real-time polymerase chain reaction (qRT-PCR)

SPP1 was selected as the gene of interest as it was the most upregulated gene and has not yet been fully functionally validated in PDAC. Verification of *SPP1* gene expression in tissue samples was accomplished by synthesising cDNA followed by real-time PCR, adhering to MIQE guidelines (Bustin *et al.*, 2009). The Invitrogen™ SuperScript™ VILO™ cDNA synthesis kit (11754050, ThermoFisher Scientific, Waltham, MA, USA) was utilised, followed by real-time PCR. Validated pre-designed TaqMan Gene Expression assays were employed for *SPP1* (Assay ID: Hs00959010_m1, 4331182, ThermoFisher Scientific, Waltham, MA, USA) and mitochondrial ribosomal protein L19 (*MRPL19*) (Assay ID: Hs00608519_m1, ThermoFisher Scientific, Waltham, MA, USA). The protocol included adding 5 µL of TaqMan® Gene Expression Master Mix (2X) (4369016, ThermoFisher Scientific, Waltham, MA, USA), 0.5 µL of 20X TaqMan® Gene Expression Assay for the target and reference genes, 3 µL of cDNA template, Human Male Raji cDNA template (control), and 1 µL of nuclease-free water to the PCR plate, followed by brief vortexing, centrifugation, and execution per the manufacturer's instructions as shown in Supplementary Table 2. The samples were run on a QuantStudio 1 real-time PCR system using the set-up described in Supplementary Table 3. *SPP1* was selected as the target gene, with *MRPL-19* used as the reference gene. Fold changes were calculated using the $2^{\wedge-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Experiments were performed once for both assays.

2.2.8 Functional and Network analyses

Functional analysis was performed by employing the search tool for retrieval of interacting genes, namely the STRING database v2.0.1 (Doncheva *et al.*, 2019) on Cytoscape and the Reactome pathway browser (Fabregat *et al.*, 2017). A confidence cut-off score of 0.4 (lower

confidence score was used to get a bigger network of proteins) and zero additional interactors. Single non-interacting proteins were excluded from the interaction network.

2.2.9 Statistical analysis

Data analysis was conducted using the GeneGlobe software (https://geneglobe.qiagen.com/za/analyze/ software accessed 12 September 2019) (QIAGEN, Hilden, Germany). Fold-change (2^ (-Delta Delta CT) was the normalized gene expression $2^{\wedge-\Delta\Delta CT}$ in the tumour samples divided by the normalized gene expression $2^{-\Delta\Delta CT}$ in the normal tissue control samples. For each array, the data analysis included a normalization step which involved the automatic selection of the most stably expressed reference genes in both sample groups. The statistical analysis for confirmation of *SPP1* knockdown was carried out using Microsoft Excel 365 and GraphPad Prism Software (GraphPad, San Diego, California, USA), followed by pairwise comparisons using a two-tailed student's t-test. All experiments were conducted with three technical and biological replicates, and the error bars depicted the standard deviation. The results were reported at a 95% confidence interval, and p-values less than 0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

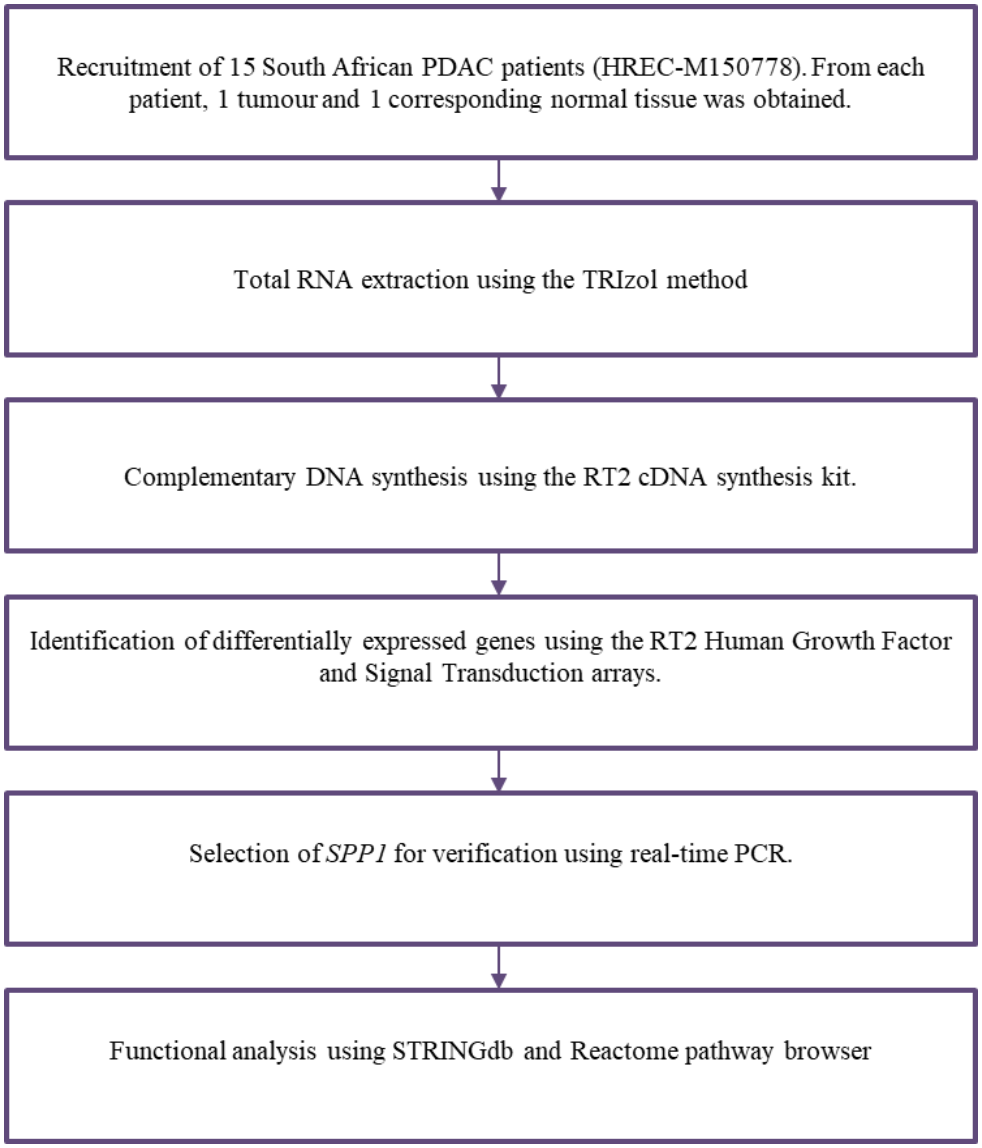


Figure 2. 1: Flow diagram of methods. Total RNA was extracted from each sample using the TRIzol method, complementary DNA was synthesised, and the study of Human growth factors and Signal Transduction genes was performed using the RT^2 Profiler Array. Real-time PCR was used to verify the over-expression of *SPP1*. Functional analysis using STRING and the Reactome pathway browser.

2.3 Results

2.3.1 Dysregulated genes in pancreatic tumours

To study the differentially expressed genes in PDAC tissues, two RT^2 PCR array panels were used to get a comprehensive gene profiling. The results showed that from the growth factor array, 48 were upregulated and 10 were downregulated in tumours compared to adjacent normal tissues (Supplementary table 4 (a and b). This included the following top 5 upregulated genes *B2M* (Fold change = 190.39), *RPLP0* (Fold change = 122.00), *SPP1* (Fold change 73.73), *JAG1* (Fold change = 60.65) and *ACTB* (Fold change = 41.30). The top 5 downregulated genes included *CSF2* (Fold change = -7802.06), *HGDC* (Fold change = -6.84), *GDF10* (Fold change

= -3.86), *IL2* (Fold change = -3.29) and *THPO* (Fold change = -3.10). The plate layout is indicated in Supplementary fig. 2a.

For the signal transduction panel, a total of 56 genes were found to be upregulated and 10 genes were downregulated (Supplementary table 5 (a and b)). Among the genes that were upregulated, *HMOX1* displayed the highest level of upregulation (Fold change = 261.74). This was followed by the top 4 upregulated genes including *MCL1* (Fold change = 162.02), *ADM* (Fold change 91.77), *B2M* (Fold change = 68.93) and *RPLP0* (Fold change = 65.21). The top 5 dowregulated genes included *HEY2* (Fold change = -5.66), *FABP1* (Fold change = -4.50), *EPO* (Fold change = -3.71), *TNF* (Fold change = -3.65) and *HGDC* (Fold change = -3.57). The plate layout is indicated in Supplementary fig. 2b.

After a rigorous screening (as highlighted in section 2.2.7) of the dysregulated genes from both panels, *SPP1* was selected as a gene of interest. *SPP1* was selected because its functional role in PDAC has not yet been fully validated. Consequently, qRT-PCR was applied to verify the expression of *SPP1* in PDAC tumour tissues and corresponding adjacent normal tissues. The expression of *SPP1* was significantly elevated in tumour tissues compared to normal tissues ($p < 0.0001$).

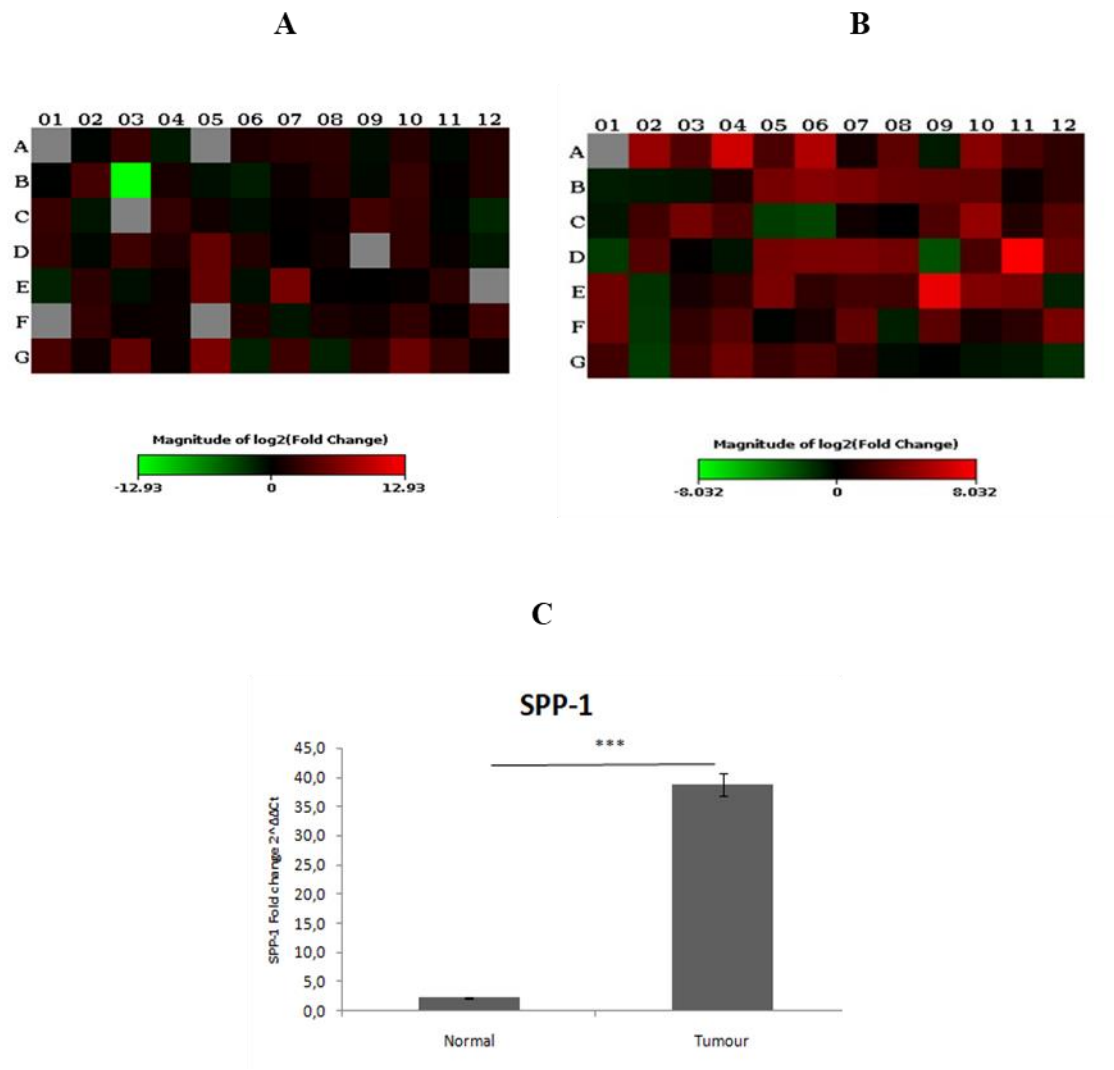


Figure 2. 2: *SPP1* overexpression in tissues of PDAC patients using real-time PCR. **(A)** 48 were found to be upregulated, and 10 were downregulated in tumours compared to adjacent normal tissues in the Growth factor array. **(B)** 56 genes were upregulated, and 10 were downregulated in tumours compared to adjacent normal tissues in the Signal Transduction array**.** Heat maps represent the fold changes in expression for every gene in the array (colour-coded from green to red), where green represents downregulated targets and red represents upregulated genes with positive values **(C)** Quantification of *SPP1* gene expression in tissue samples was performed using real-time PCR. p values less than 0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

Table 2.2: List of differentially expressed genes in tumours.

Growth Factor array panel				Signalling pathway array panel			
Upregulated		Downregulated		Upregulated		Downregulated	
Gene	Fold regulation	Gene	Fold regulation	Gene	Fold regulation	Gene	Fold regulation
B2M	190.39	CSF2	-7802.06	HMOX1	261.74	HEY2	-5.66
RPLP0	122.00	HGDC	-6.84	MCL1	162.02	FABP1	-4.50
SPP1	73.73	GDF10	-3.86	ADM	91.77	EPO	-3.71
JAG1	60.65	IL2	-3.29	B2M	68.93	TNF	-3.65

ACTB	41.30	THPO	-3.10	RPLP0	65.21	HGDC	-3.57
TYMP	39.44	TDGF1	-3.04	ATF4	45.73	GATA3	-3.49
INHBA	33.11	CXCL1	-2.63	ACSL4	26.06	OLR1	-3.17
IGF1	32.42	BMP10	-2.36	FTH1	25.32	IFNG	-2.96
PTN	28.32	IL1B	-2.32	BCL2	19.13	WNT6	-2.70
GAPDH	16.74	NRG2	-2.09	CCL5	18.77	NOTCH1	-2.09
PGF	11.95			ACTB	17.98		
CSF1	10.30			MMP7	16.61		
FGF7	9.42			HES1	14.94		
PI	8.31			JAG1	14.57		
TGFB1	8.15			SQSTM1	14.53		
PDGFC	7.00			CCND1	14.31		
FGF14	6.71			MYC	12.91		
BMP1	6.58			CA9	12.89		
VEGFA	6.34			EGFR	12.59		
FGF1	6.04			HERPUD1	12.43		
MSTN	5.70			HEY1	11.93		
FGF2	5.67			ID1	11.00		
GDF11	5.41			TXN	10.43		
NTF3	5.33			NQO1	10.20		
IL18	4.90			ICAM1	9.90		
FGF9	4.89			CCND2	8.83		
TNNT1	4.59			SERPINE1	8.08		
IL3	4.36			CDKN1A	8.06		
LIF	4.28			BAX	7.96		
BMP5	4.12			CDKN1B	7.38		
BMP4	3.71			SLC2A1	7.06		
CECR1	3.63			GADD45B	6.67		
BMP7	3.62			GCLC	6.08		
ERAP1	3.49			PPARD	5.87		
FGF13	3.48			ACSL5	5.80		
IGF2	3.40			VEGFA	5.54		
NRG1	3.22			FOSL1	5.40		
HBEGF	2.92			GAPDH	5.32		
NODAL	2.81			ARNT	5.10		
MDK	2.81			BCL2A1	5.07		
LTBP4	2.81			EMP1	4.98		
IL12B	2.81			HEYL	4.92		
FGF19	2.81			LFNG	4.25		
BMP2	2.81			STAT1	4.11		
AMH	2.81			DAB2	4.08		
BMP3	2.49			TNFSF10	3.94		
NRG3	2.44			LRG1	3.91		
CSF3	2.35			TXNRD1	3.44		
				PCNA	2.81		
				BCL2L1	2.78		
				CPT2	2.72		
				WISP1	2.71		
				LDHA	2.70		
				IRF1	2.68		
				SORBS1	2.54		
				GADD45A	2.10		

2.3.2 Pathway and Network analyses of dysregulated genes

To shed light on the network and pathway interactions present in the dataset, the STRING database and the Reactome pathway browser were used to analyze the dysregulated interrogate the dysregulated genes, respectively. The purpose of STRING analysis is to determine how the dysregulated genes interact with each other. This further allows one to understand the molecular mechanisms of the cancer. The genes upregulated and downregulated from both panels were combined to identify key upregulated and downregulated pathways. The top upregulated pathways included those associated with interleukin signalling (IL4 and IL13), cytokine signaling, receptor kinase signalling, and PI3/Akt signalling. The downregulated pathways included IL-10 signalling, immune system and several transcription regulation pathways. Furthermore, network analysis using String demonstrated an intricate network for the upregulated and downregulated genes (Figure 2.3a and b) suggesting the genes interact together and are involved in similar biological processes and pathways.

Enrichment analysis of SPP1-related genes-Expression data was utilised to investigate potential proteins that may bind to or influence SPP1 function. Using the STRING database, we analysed related genes and their reported or experimentally confirmed interactions, which were represented by lines connecting the protein nodes. Figure 2.3 presents the protein interaction network, where the lines between nodes represent evidence of interactions. Results indicated that most of these genes were involved in signalling by receptor tyrosine kinase pathways. Proteins such as the EGFR, IGF1 and VEGFA were linked to SPP1. Growth factors are associated with the progression of various cancers, including pancreatic cancer (Xelwa *et al.*, 2021). EGFR is expressed in approximately 60-90% of PDAC cases and contributes to cell growth and migration (Xie *et al.*, 2019). VEGF is highly expressed in PDAC and its overexpression has been linked to larger tumour size, increased liver metastases, and reduced patient survival (Hosein, Brekken and Maitra, 2020). FGF is overexpressed in PDAC and promotes cell growth, proliferation, and invasion (Carter *et al.*, 2021).

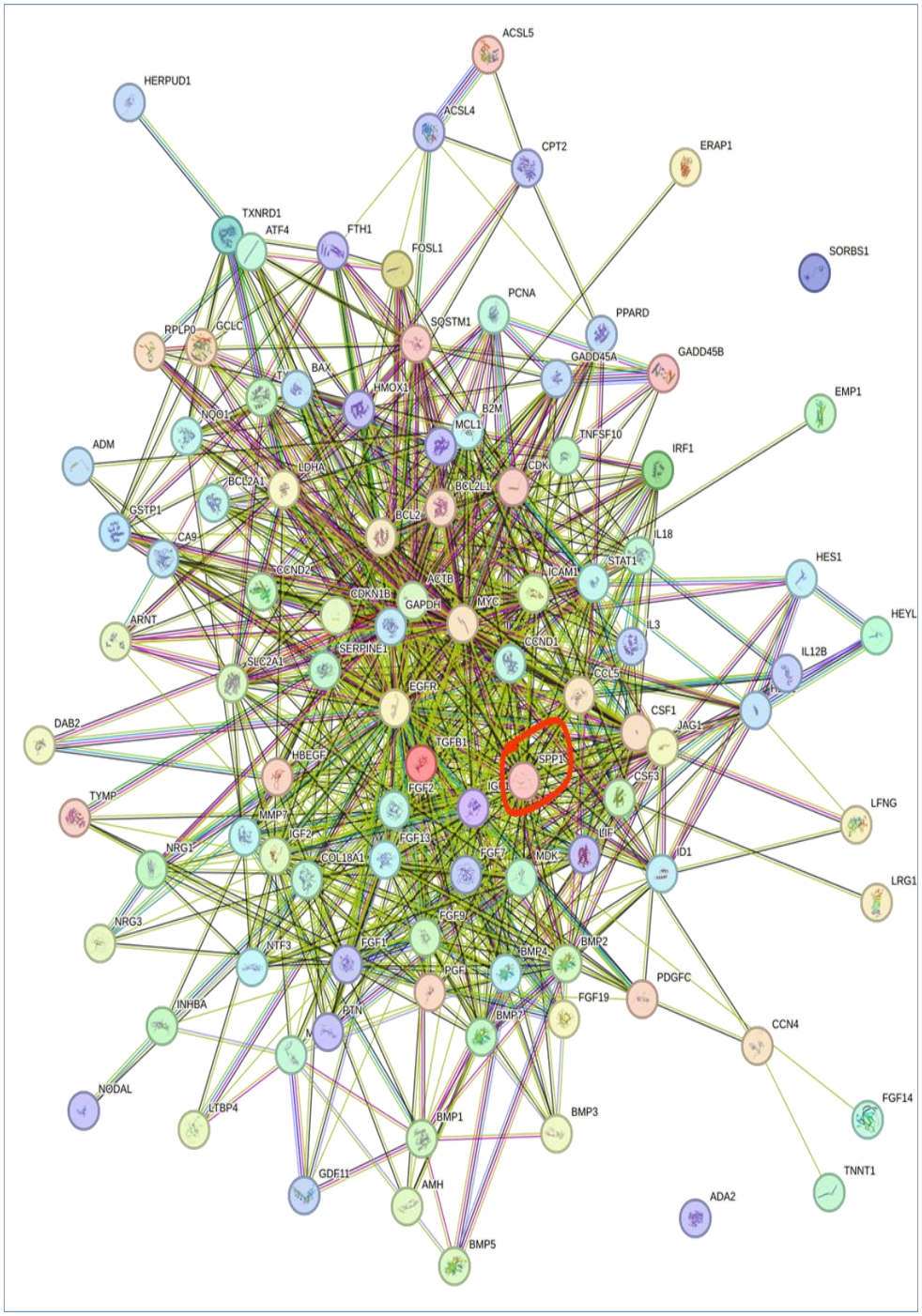
Table 2. 1: Pathways in which dysregulated genes are involved.

Upregulated			Downregulated		
Pathway name	p-value	Proteins enriched	Pathway name	p-value	Proteins enriched

Interleukin-4 and Interleukin-13 signalling	$2.74x10^{-14}$	CDKN1A;TGFB1;STAT1;LIF;IL18;FGF2;VEGFA;ICAM1;CCND1;MYC;BCL2;IL12B;HMOX1;BCL2L1;MCL1	Interleukin-10 signalling	$5.77x10^{-11}$	CSF2;IL1B;CXCL1;TNF
Cytokine Signaling in Immune system	$2.74x10^{-14}$	CDKN1A;CSF3;TGFB1;CDKN1B;CSF1;STAT1;RPLP0;LIF;IL18;FGF2;VEGFA;ICAM1;IL3;CCND1;MYC;CCL5;IRF1;BCL2;IL12B;HMOX1;B2M;SQSTM1;BCL2L1;MCL1	RUNX1 and FOXP3 control the development of regulatory T lymphocytes (Tregs)	$3.35x10^{-6}$	IFNG;IL2
Diseases of signal transduction by growth factor receptors and second messengers	$1.64x10^{-13}$	CDKN1A;JAG1;TGFB1;CDKN1B;BCL2A1;STAT1;NRG1;FGF1;FGF2;ACTB;EGFR;HEYL;FGF7;FGF9;HEY1;NRG3;MYC;FGF19;HES1;SQSTM1;BCL2L1;MCL1;HBEGF	CLEC7A/inflammasome pathway	$2.69x10^{-5}$	IL1B
Nuclear events mediated by NFE2L2	$4.9\ x10^{-13}$	NQO1;GCLC;TXNRD1;MYC;BCL2;HMOX1;TXN;SQSTM1;BCL2L1;ATF4	Transcriptional regulation by RUNX1	$4.28x10^{-5}$	CSF2;IFNG;GATA3;IL2
KEAP1-NFE2L2 pathway	$2.01x10^{-12}$	NQO1;GCLC;CDKN1A;TXNRD1;MYC;BCL2;HMOX1;TXN;SQSTM1;BCL2L1;ATF4	Immune System	$4.23x10^{-4}$	CSF2;IFNG;EPO;IL1B;OLR1;CXCL1;GATA3;TNF;IL2
NFE2L2 regulating anti-oxidant/detoxification enzymes	$1.04x10^{-10}$	NQO1;GCLC;TXNRD1;HMOX1;TXN;ATF4	Signalling by NOTCH	$4.2x10^{-3}$	NOTCH1;HEY2
FOXO-mediated transcription of cell cycle genes	$2.28x10^{-9}$	CDKN1A;CDKN1B;MSTN;GADD45A	Generic Transcription Pathway	$4.22x10^{-3}$	CSF2;NOTCH1;IFNG;HEY2;GATA3;IL2
Cellular response to chemical stress	$1.4\ x10^{-8}$	NQO1;GCLC;CDKN1A;TXNRD1;MYC;BCL2;HMOX1;TXN;SQSTM1;BCL2L1;ATF4	Regulation of gene expression by Hypoxia-inducible Factor	$4.38x10^{-3}$	EPO

Signalling by Receptor Tyrosine Kinases	$1.91x10^{-8}$	STAT1;IGF2;NRG1;IGF1;PTN;FGF1;FGF2;PGF;ACTB;EGFR;VEGFA;FOSL1;FGF7;FGF9;NRG3;MDK;MYC;FGF19;ID1;PDGFC;NTF3;SPP1;BAX;HBEGF	NFE2L2 regulating tumourigenic genes	$4.91x10^{-3}$	NOTCH1
PI3K/AKT Signalling in Cancer	$8.96x10^{-7}$	CDKN1A;FGF7;CDKN1B;NRG3;FGF9;FGF19;NRG1;FGF1;FGF2;EGFR;HBEGF	Formation of the nephric dust	$1.1x10^{-2}$	GATA3

A


ACSL5
HERPUD1
ACSL4
CPT2
ERAP1
TXNRD1
ATF4
FTH1
FOSL1
SORBS1
PCNA
PPARD
RPLP0
GCLC
SQSTM1
GADD45A
GADD45B
BAX
HMOX1
EMP1
NQO1
B2M
MCL1
TNFSF10
IRF1
ADM
LDHA
CDK
BCL2A1
BCL2L1
GSTP1
BCL2
IL18
CA9
STAT1
HES1
CCND2
ICAM1
ACTB
CDKN1B
GAPDH
MYC
HEYL
ARNT
IL3
CCND1
SERPINE1
IL12B
SLC2A1
CCL5
EGFR
CSF1
DAB2
JAG1
TGFB1
HBEGF
SPP1
CSF3
FGF2
TYMP
LFNG
MMP7
IGF2
FGF13
LIF
NRG1
COL18A1
FGF7
MDK
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FGF9
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BMP4
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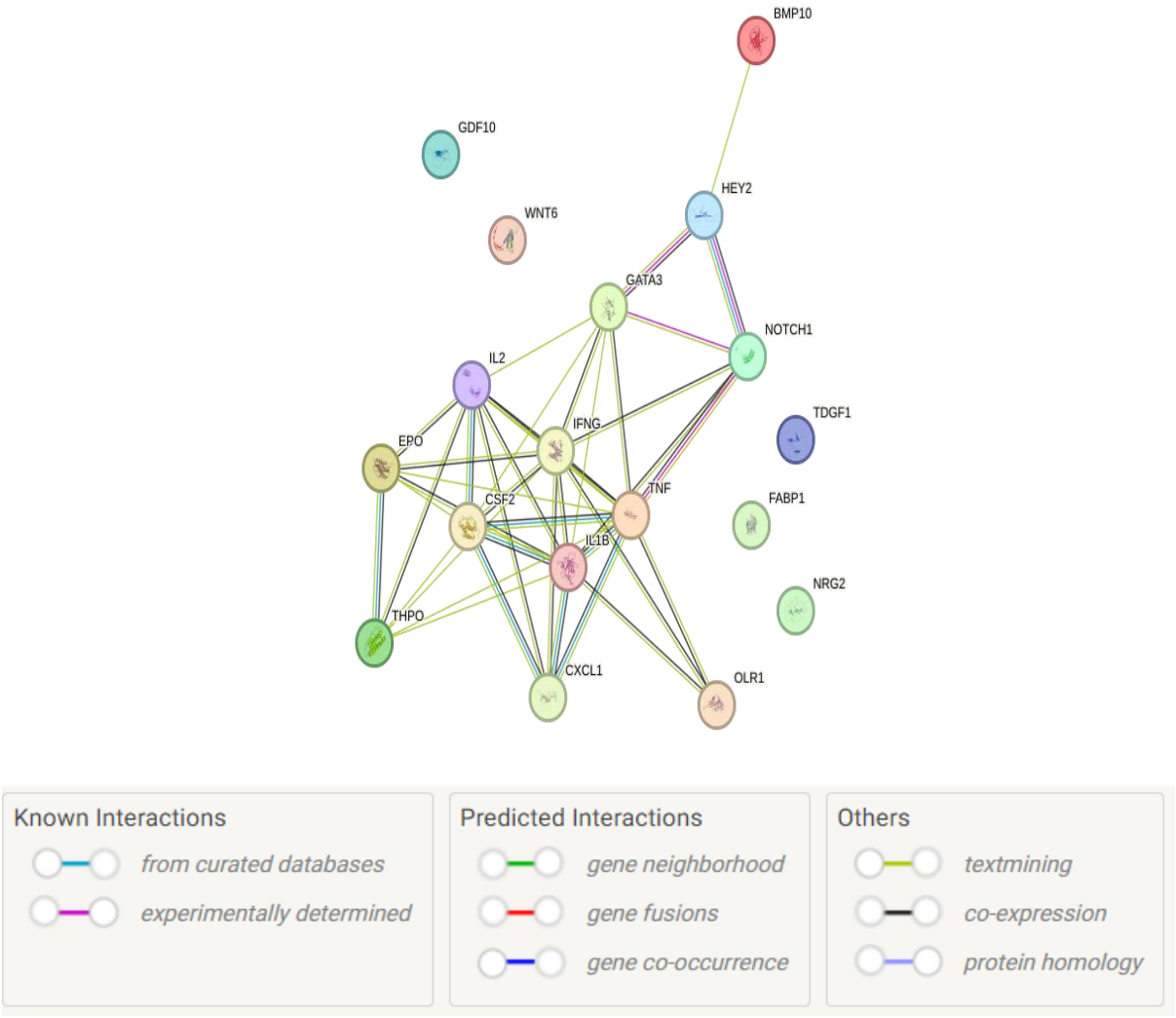


Figure 2. 3: Network analysis of dysregulated genes. The protein-protein interaction network corresponds to all dysregulated proteins associated with SPP1 upregulation. **(A)** The construction of the network involved utilising the STRING database, unveiling potential protein interactions with a moderate level of confidence, *SPP1* (circled in red). **(B)** Circular bubbles, called nodes, symbolise the query proteins. The colours of the nodes signify distinct inputted genes and are not indicative of up- or downregulation.

2.4 Discussion

SPP1 is upregulated in pancreatic tumours

In this study, for the Growth factor panel, 48 genes were shown to be over-expressed in 15 PDAC tumour samples when compared to corresponding adjacent surrounding normal tissues.

For the Signal Transduction panel, a total of 56 genes were found to be upregulated and 10 genes were downregulated. Amongst them, *SPP1* (FC =73.73) was one of the most highly expressed genes. To verify this, real-time PCR was utilized to compare the heightened expression levels of *SPP1* with those in adjacent normal tissues. Comprehensive studies on the precise mechanisms and roles of *SPP1* in the development and progression of PDAC are lacking, as far as we currently know. Research conducted by Liu et al. suggests that *SPP1* may hinder apoptosis and facilitate the advancement of lung cancer cells. Nonetheless, the mechanisms that regulate *SPP1* upstream remain a mystery (Liu *et al.*, 2019). Increased *SPP1* expression in tumour tissue and plasma has been found to be a predictive marker associated with poor outcomes in a variety of cancer types (Cao *et al.*, 2012). In addition, the role of *SPP1* in other cell types (prostate cancer) is noteworthy: *SPP1* activation in stromal cancer cells promotes cell survival and enhances invasion, indicating that *SPP1* directly affects tumour cells (Pang *et al.*, 2019). Elevated expression of *SPP1* was found to be associated with poor clinical outcomes in patients with breast and ovarian cancer (Tu, Chen and Fan, 2019). The study by Tu et al. (2019) suggests that *SPP1* may promote tumour growth and progression. Based on the findings of this study, the potential therapeutic targets for PDAC may include *SPP1*. The precise function of *SPP1* as a tumour promoter or suppressor is still not fully understood in PDAC and cannot be determined by analyzing expression levels alone. Therefore, the next goal of this study, presented in Chapter 3, was to examine the expression levels of *SPP1* and its implications in PDAC.

Dysregulation of Cytokine signalling in PDAC

Using the data generated by the qRT-PCR arrays for Human growth factors and Signal Transduction factors, pathway enrichment analysis was undertaken to gain an understanding of the mechanisms involved in PDAC, especially in our patient group. In the present study, several genes were identified in association with dysregulation of cytokine signalling. Communication in the TME, and thus immune evasion and tumour progression, are heavily reliant on cytokines, small signalling molecules (Bhatia *et al.*, 2022). In the TME cytokines have a dual function, where they can both control the immune response and directly impact cancer cells. The different types of cytokines can influence cancer development and progression in different ways (Roshani, McCarthy and Hagemann, 2014; Padoan, Plebani and Basso, 2019).

These cytokines are also implicated as inflammatory factors that link chronic inflammation to pancreatic tumour development. These molecules, when produced either by tumour cells or cells of the TME, cause the secretion of IL-6 from myeloid cells and pancreatic stellate cells, which ultimately leads to the proliferation and invasiveness of PDAC cells (Nagathihalli *et al.*, 2016; Okabe *et al.*, 2023).

Studies have shown that high levels of IL-4 and IL-13 activity, pathways of which were upregulated substantively in the present study, in PDAC are strongly associated with tumourigenesis and metastasis (Kornmann *et al.*, 1999; Gabitass *et al.*, 2011). Research has demonstrated a significant increase in the levels of IL-4 in the plasma of individuals with PDAC compared to healthy individuals (Chen *et al.*, 2009; Yako *et al.*, 2017). The link between elevated levels of IL-4 and the development and progression of PDAC is significant (Piro *et al.*, 2017). This is possibly underwritten by the induction of the JAK/STAT pathway (Shi *et al.*, 2021) and tyrosine kinase pathway, the latter also upregulated in this study, that promote PDAC progression by enhancing cell growth and survival processes (Toyonaga *et al.*, 2003). The importance of IL-4 in PDAC progression was shown by the inhibitory effect of IL-4 neutralizing antibody on the basal growth of PANC-1, MIA PaCa-2 and COLO-357 cells (Prokopchuk *et al.*, 2005). Notably, IL-13 is also a major player in the TME whereby it is capable of inducing a mitogenic effect on pancreatic stellate cells, which in turn is implicated in TAM polarisation into the M2 phenotype (Ginhoux and Jung, 2014; Little *et al.*, 2019). Moreover, IL-13 is also involved in enhancing the density of the ECM (Shi *et al.*, 2014), together with contributions from growth factors including TGF-β and FGFs (Wu *et al.*, 2021), which were also upregulated in the present study.

PI3K/AKT/mTOR pathway is upregulated in PDAC tumours

Another critical pathway that demonstrated upregulated genes was the PI3K/AKT/mTOR pathway. This pathway can be impaired by germline mutations, leading to hereditary disorders that increase the risk of several cancer types, including PDAC (Burris, 2013; Polivka and Janku, 2014). The PI3K/AKT/mTOR signalling pathway regulates cellular processes, such as survival, proliferation, motility, and metabolism (Noorolyai *et al.*, 2019; Hoxhaj and Manning, 2020). A study by Massihnia has indicated that phospho-AKT might serve as a prognostic biomarker in PDAC (Massihnia *et al.*, 2017). Additionally, numerous studies have elucidated the link between alterations in the PI3K/AKT/mTOR signalling pathway and the emergence of PDAC (Edling *et al.*, 2010; Sun *et al.*, 2011; Murthy, Attri and Singh, 2018). According to the

study conducted by Thibault et al, the intrinsic PI3K/Akt signalling in PDAC is accountable for promoting metastasis and modifying the composition of macrophages. By inhibiting this pathway, the number of macrophages surrounding high-grade lesions decreases, leading to a reduction in metastasis and ultimately improving the patient's prognosis (Thibault *et al.*, 2021).

The activation of the PI3K/AKT pathway by NGF/TrkA facilitated invasion and proliferation in PDAC cells when co-cultured with PSCs (Jiang *et al.*, 2020). Inhibitors targeting the PI3K/AKT/mTOR pathway, including everolimus, have demonstrated therapeutic advantages in PDAC cells by limiting the activity of this pathway (Mortazavi *et al.*, 2022). The PI3K/AKT/mTOR pathway is a critical downstream effector of RAS signalling. Therefore, targeting this pathway may represent a more effective therapeutic strategy for PDAC (Nollmann and Ruess, 2020). In the present study, the upregulation of genes associated with cell cycle (including cyclin d1, cyclin d2, PCNA and BCL2L1) (Loo *et al.*, 2020), and the dysregulation of genes associated with FOX0 transcription family (Ling *et al.*, 2019), also highlighted PDACs aggressiveness by illustrating the intricate interplay between pushing cells through the cell cycle, avoiding the induction of apoptosis, and ensuring survival (Vahabi *et al.*, 2023).

Immune response pathways are downregulated in PDAC

The innate immune system responds to numerous stimuli, such as infections and tissue damage, and plays a critical role in the development and management of diseases (Rivera *et al.*, 2016; Paludan *et al.*, 2021). The function of innate immune cells, like macrophages and dendritic cells, in initiating inflammatory responses is crucial for maintaining organismal balance, including the elimination of pathogens (Rivera *et al.*, 2016). The distinct feature of the TME in PDAC is the absence of an immune response and the low level of immune activation, which leads to increased immune suppression (Uzunparmak and Sahin, 2019; Bear, Vonderheide and O'Hara, 2020; Ho, Jaffee and Zheng, 2020). Research has shown that irregularities in the tumour microenvironment, generated by tumour cells, hinder the functioning of immune cells (Tabe, Lorenzi and Konopleva, 2019). Infiltration of immune cells plays a crucial role in shaping the tumour immune microenvironment of PDAC and has a significant effect on patient survival (Ino *et al.*, 2013; Huang *et al.*, 2023). The degree of metastasis can also play a role in the occurrence of T-cell-depleting immunosuppression (O'Leary, 2021). PDAC is typically associated with a hypoxic tumour microenvironment, which in turn triggers a hostile inflammatory response and decreased immunogenicity (Zhao and Subramanian, 2017; Ho,

Jaffee and Zheng, 2020). This environment may restrict the infiltration of pro-inflammatory cells, including natural killer cells, $CD4^+$ T cells, $CD8^+$ T cells, M1 macrophages, and dendritic cells (Padoan, Plebani and Basso, 2019).

In the present study, downregulated genes were identified that are associated with the innate immune system function including IFN-γ, IL-1β and TNF-α. PDAC is known to be promoted by the inflammatory cytokines TNF and interleukin-1 beta (IL-1β) (Egberts *et al.*, 2008; Ling *et al.*, 2012). Cytokine IL1β, a proinflammatory mediator, is overexpressed in various types of cancer and has been linked to adverse prognosis (Apte *et al.*, 2006; Jiang *et al.*, 2015; Bent *et al.*, 2018). IL1β plays a role in the development and progression of PDAC, whereby higher levels of IL1β in the pancreas are linked to pancreatitis; a risk factor for developing PDAC (Mayer *et al.*, 2000). A study by Das et al. has demonstrated that the activation of quiescent PSCs by IL-1β is a critical factor in the development of a tumour-promoting microenvironment in PDAC. Thus, highlighting the crucial role played by IL-1β-mediated modifications in the tumour microenvironment in the progression of PDAC (Das *et al.*, 2020). The finding of reduced IL-1β gene expression and IL-10 signalling in this study may reflect changes in the immune infiltrate of the TME. Specifically, phenotypic changes in the TME may point to a switched Th2 response (seen as upregulated cytokines in this study), that may, in turn, reflect a more tolerogenic TME that facilitates tumour-immune cell evasion (Herremans *et al.*, 2022).

Lung adenocarcinoma has been demonstrated to derive advantages from the immune-evading features facilitated by *SPP1*, which participates in the polarization of macrophages (Zhang *et al.*, 2017). *SPP1*, which was significantly raised in the present PDAC study, can function as an immune checkpoint, contributing to host tumour immune tolerance by suppressing T-cell activation (Klement *et al.*, 2018). One study proposed that in the hypoxic microenvironment, increased *SPP1* expression fosters the formation of a tumour immune barrier by cancer-associated fibroblasts and *SPP1* macrophages. This interaction prompts extracellular matrix remodelling, impacting the effectiveness of immune checkpoint blockade by restricting immune infiltration into malignant regions (Liu *et al.*, 2023). The proposed reduction in T cell function is further highlighted by the downregulation of IL-2 in this study, necessary for T cell activation (Ross and Cantrell, 2018). Moreover, the upregulation of TGFβ, identified in the present study also highlights its critical role in immunosuppression by irectly suppressing the functions of various effector T cells (Principe *et al.*, 2014; Ho, Jaffee and Zheng, 2020). Cytotoxic T lymphocytes recognize and bind to antigenic peptide-MHC-I complexes using their TCR receptors, initiating anti-tumour immune responses. However, downregulation of

MHC-I can hinder $CD8^+$ T cells' ability to identify tumour antigen peptides, which is closely linked to immunosuppression and a poor prognosis in many malignant tumours (Koelzer *et al.*, 2014). In PDAC, MHC-I can be degraded during autophagic cell survival responses by interacting with NBR1, leading to immune evasion (Yamamoto *et al.*, 2023). Dysregulation of B2M, a subunit of MHC1 which is required for its stabilisation may also affect the presentation of MHC1 in its functional form (Wu *et al.*, 2023), which is of particular interest since overexpression of *B2M*, as identified in this study, is also associated with poor survival in PDAC (Liu *et al.*, 2015).

Other downregulated key signalling pathways

The current study found a reduction in genes associated with the Notch signalling pathway. While this may be surprising given its role in tumour progression, specifically regulating the self-renewal of stem cells and angiogenesis (Wu *et al.*, 2014; Ordaz-Ramos, Tellez-Jimenez and Vazquez-Santillan, 2023). Notch signalling can have either oncogenic or tumour-suppressive effects, depending on the TME (Avila and Kissil, 2013). Studies by Benedito et al. suggested that dysregulated activation of the Notch signalling family disrupts the balance necessary for functional angiogenesis (Benedito and Hellström, 2013); notwithstanding that dysregulated angiogenesis is a hallmark of cancer itself. Further investigations into the tempospatial expression of genes associated with Notch signalling are required to unpack its variable role in PDAC tumourigenesis.

2.5 Conclusion

This study emphasized the importance of *SPP1* expression in the tumourigenesis of PDAC. It clarifies its role in the process of tumour development, shedding light on its contribution to promoting tumour growth and progression. Moreover, pathway analysis highlights the significance of cytokine signalling pathways and their intersection with major signal transduction pathways in the development of PDAC, providing potential targets for future therapeutic interventions, taking into account the impact of the TME.

CHAPTER 3: INSIGHTS INTO APOPTOSIS AND MIGRATION IN PANCREATIC CANCER CELLS FOLLOWING DOWNREGULATION OF *SPP1*

3.1 Introduction

In the previous chapter, it was identified that *SPP1* expression is elevated in individuals with PDAC. The treatment of PDAC typically involves surgical intervention, although, in later stages of the disease, additional therapies such as radiotherapy, chemotherapy, or a combination of these may be necessary (Kolbeinsson *et al.*, 2023). Regrettably, these therapeutic methods often come with significant side effects and may demonstrate less than optimal effectiveness, resulting in relapse (Hu and O'Reilly, 2024). The exploration of alternative therapeutic strategies for PDAC has gained momentum due to the limitations and adverse effects associated with conventional treatments, which often result in significant resistance and toxicity (Liu *et al.*, 2023). As a result, there is an increasing urgency to explore alternative therapeutic strategies, such as targeted therapy (Volpini *et al.*, 2023).

Targeted therapies could address the limitations of conventional chemotherapy by targeting specific biomarkers related to genomic mutations during PDAC metastasis. Focusing on crucial somatic target genes, such as *TP53, CDKN2A, KRAS, SMAD4, BRACA1/2* and DNA repair signalling pathways, offers potential for effective PDAC treatment (Campbell *et al.*, 2010; Pramanik *et al.*, 2024). RNA interference (RNAi) has emerged as a compelling modality for cancer treatment due to its capacity for the downregulation or silencing of genes implicated in tumorigenesis. RNAi works directly inside cancerous cells, boosting the effectiveness of cancer therapy compared to traditional, long-term treatments. Thus the potential of RNAi technology in fighting cancer has recently gained renewed attention (Bobbin and Rossi, 2016; Tian *et al.*, 2021), although the precision and efficacy of regulated small interference RNA (siRNA) delivery pose challenges, attributed to the inherent heterogeneity of the tumour microenvironment (Chen *et al.*, 2018). siRNA is designed to be complementary to the mRNA of the target genes. By associating with the RNA-induced silencing complex (RISC), siRNA leads to mRNA degradation and cleavage pre-translation (Ferguson *et al.*, 2020; Tian *et al.*, 2021). This process effectively hinders protein expression of the molecular target, leading to its ultimate downregulation (Afrin *et al.*, 2023).

siRNA shows promise in combination therapies with chemotherapeutics for sensitizing drug-resistant cancer cells, as well as targeting intracellular and previously difficult-to-treat protein targets (Mahmoodi Chalbatani *et al.*, 2019; Henley and Koehler, 2021; Khan, Budamagunta and Zhou, 2023). This approach eliminates the need to find a binding site on the target protein, which is a significant advantage when reducing mRNA levels (Henley and Koehler, 2021).

Studies by Huang et al. have shown that siRNA can downregulate KRAS protein expression by silencing upstream mRNA encoding it (Huang *et al.*, 2023). The approach discussed here provides a promising solution for reducing the production of mutant KRAS protein, which is a significant contributor to PDAC (Huang *et al.*, 2023). This method has high potential as a therapeutic option. One of the main advantages of siRNA over small molecule pharmaceuticals is its superior specificity, lower level of cytotoxicity, and reduced susceptibility to drug resistance (Ngamcherdtrakul and Yantasee, 2019; Zhang *et al.*, 2022). siRNAs demonstrate a remarkable ability to target specific mRNA sequences of individual genes. Their precise binding affinity leads to effective repression of gene expression (Zuckerman and Davis, 2015). Additionally, siRNA can be highly effective in reducing the adverse effects of traditional drug treatment caused by pathogenic gene mutations (Wang *et al.*, 2021).

Although siRNA has demonstrated efficacy in cancer treatment, tumour resistance presents a substantial obstacle. Consequently, comprehending the underlying mechanisms of cancer resistance is essential to enhance the efficacy of siRNA therapy (Deng *et al.*, 2014). The use of chemotherapeutic agents in conjunction with molecular targeted therapies, such as siRNA, presents a promising strategy for the treatment of PDAC (de la Fuente *et al.*, 2015), the poor prognosis of which necessitates novel therapeutic approaches. One such strategy is to enhance the efficacy of gemcitabine by using targeted therapies that selectively silence specific genes with siRNAs. This approach may result in reduced doses while maintaining therapeutic benefits by improving the destruction of tumour cells (Simonenko *et al.*, 2020). The study conducted by Azorsa et al. used RNAi screening to identify genes that when silenced would improve the response of PDAC cell lines to gemcitabine (Azorsa *et al.*, 2009). Azorsa et al. identified the checkpoint kinase, *CHK1* gene, which when silenced permitted an enhanced effect of gemcitabine on pancreatic cancer cells *in vitro* (Azorsa *et al.*, 2009). Fredebohm et al. demonstrated that inhibition of *CHK1*, which plays a crucial role in the DNA damage checkpoint as part of the apoptotic pathway, results in cell cycle arrest thereby enhancing the toxicity of gemcitabine. Furthermore, they identified various other targets that, when inhibited, can improve the efficacy of gemcitabine (Fredebohm *et al.*, 2013). The combination of gemcitabine, aptamer, and siRNA has demonstrated a moderate improvement in overall survival for individuals at advanced PDAC stages, as indicated by various meta-analyses of randomized controlled trials (Robatel and Schenk, 2022). Three siRNA drugs, including patisiran (Wood, 2018), givosiran (Scott, 2020), and lumasiran (Scott and Keam, 2021), have been approved by the FDA. Despite the encouraging outcomes observed in preclinical studies

involving siRNA for cancer detection and therapy, clinical trials have largely progressed only to the initial phases of I and II (Kanungo *et al.*, 2024).

Developing targeted therapies for cancer treatment is a critical element, particularly in the progression of PDAC. By examining the underlying mechanisms and signalling pathways that contribute to the development of PDAC, valuable insights can be obtained to develop targeted therapies and clinical intervention strategies that make use of pathway inhibitors. Current approaches to suppressing *SPP1* primarily involve the use of small-molecule inhibitors, particularly those that have not been authorized by the Food and Drug Administration, such as 1,2,4-trioxanes (Pasupuleti *et al.*, 2020). As well as MPIIIB10, neutralizing MPIIIB10 antibodies (Soldan *et al.*, 2021), and RNA interference, siRNA (Yan *et al.*, 2010).

The co-administration of RNAi with chemotherapy and radiation therapy may reduce the resistance that PDAC cells display to these treatment modalities (Tian *et al.*, 2021). It was discovered that proNGF-siRNA significantly inhibits PDAC cell growth and promotes the induction of anoikis. Apoptosis induction and autophagy suppression can be triggered by proNGF-siRNA, as demonstrated by the inhibition of autophagy inducers such as autophagy-related gene 5 (ATG5) and Beclin-1 following proNGF down-regulation by siRNA (Xu *et al.*, 2019). RPL1, a ribosomal protein, has reportedly been the focus of PC treatment. Furthermore, apoptosis, cell cycle arrest at the G1 phase, and suppressed DNA replication are caused by siRNA's downregulation of RPL1 (Li *et al.*, 2020). These studies highlight the fundamental need for pancreatic cancer treatment, with a focus on identifying various tumour-promoting factors as a crucial step.

Therefore, it would be beneficial to develop siRNAs that specifically target tumour cells to inhibit the growth of PDAC and target specific pathways (Yu *et al.*, 2019). Nevertheless, given that siRNA technology represents a relatively recent innovation in cancer treatment, the majority of siRNAs under investigation as potential therapeutics are currently undergoing clinical testing and have not yet progressed to market availability.

In the previous chapter, it was identified that *SPP1* expression is elevated in individuals with PDAC, suggesting its significant diagnostic value and indicating the potential utility of SPP1 as a molecular biomarker. SPP1 is a glycoprotein that is secreted by a variety of cell types, including osteoblasts, macrophages, T cells, fibroblasts, and dendritic cells (Del Prete *et al.*, 2019), and it has been linked to several physiological processes, such as bone resorption, wound healing, immune function, angiogenesis, cell survival, and cancer biology (Luo *et al.*,

2015). In particular, *SPP1* has been implicated in the development of tumours, and it has been shown to play a role in the proliferation of cancer cells and their resistance to chemoradiotherapy. *SPP1*'s ability to enhance these processes is thought to be due to its ability to induce epithelial-mesenchymal transition (EMT), autophagy, aberrant glucose metabolism, epigenetic modifications, and reduced drug absorption. When *SPP1* binds to integrin αvβ3 and *CD44*, it triggers signaling pathways that activate the PI3K/Akt and MAPK pathways, which are known to play crucial roles in carcinogenesis (Hao, Lane and Jiang, 2023; Matsubara *et al.*, 2023). AKT, in particular, has been shown to interfere with the cell cycle, making it an important player in the development of cancer (Handra-Luca, 2023).

A study in 2018 reported a close association of the *SPP1* expression levels and patient responsiveness to drug interventions. Specifically, *SPP1* expression levels have demonstrated a significant association with the effectiveness of platinum-based treatments in individuals diagnosed with advanced non-small cell lung cancer (Ouyang *et al.*, 2018). However, it was revealed that there was no correlation between the *SPP1* expression levels and the response to a taxane-based treatment regimen (TBR) in patients with prostate cancer (Aksoy, Artas and Sevindik, 2017). Poor treatment responses across a range of cancer types are positively correlated with abnormal *SPP1* expression in recent studies (Hao, Lane and Jiang, 2023). Drug resistance may also be reversed by regulating *SPP1* expression (Hao, Lane and Jiang, 2023).

Overall, improved tools, such as the incorporation of siRNA with chemotherapy are essential for tailoring treatments to individual needs more effectively (Previdi *et al.*, 2017; Abulsoud *et al.*, 2023). This study aimed to assess *SPP1* function in drug resistance, invasion, and cell cycle progression in MIA PaCa-2 cells employing siRNA-mediated knockdown, thereby contributing to the identification of novel potential biomarkers and therapeutic strategies.

3.2 Materials and Methods

The current study is comprised of two components: The initial part focused on examining the impact of siRNA-mediated knockdown of *SPP1* in MIA PaCa-2 pancreatic cancer cells. This involved optimizing the knockdown of *SPP1* in MIA PaCa-2 cells using *SPP1*-targeting siRNA, followed by evaluating the effects of *SPP1* knockdown on cell morphology, migration apoptosis (Figure 3.1). Additionally, proteomic analysis was conducted to gain insights into the pathways implicated after *SPP1* knockdown and gemcitabine treatment.

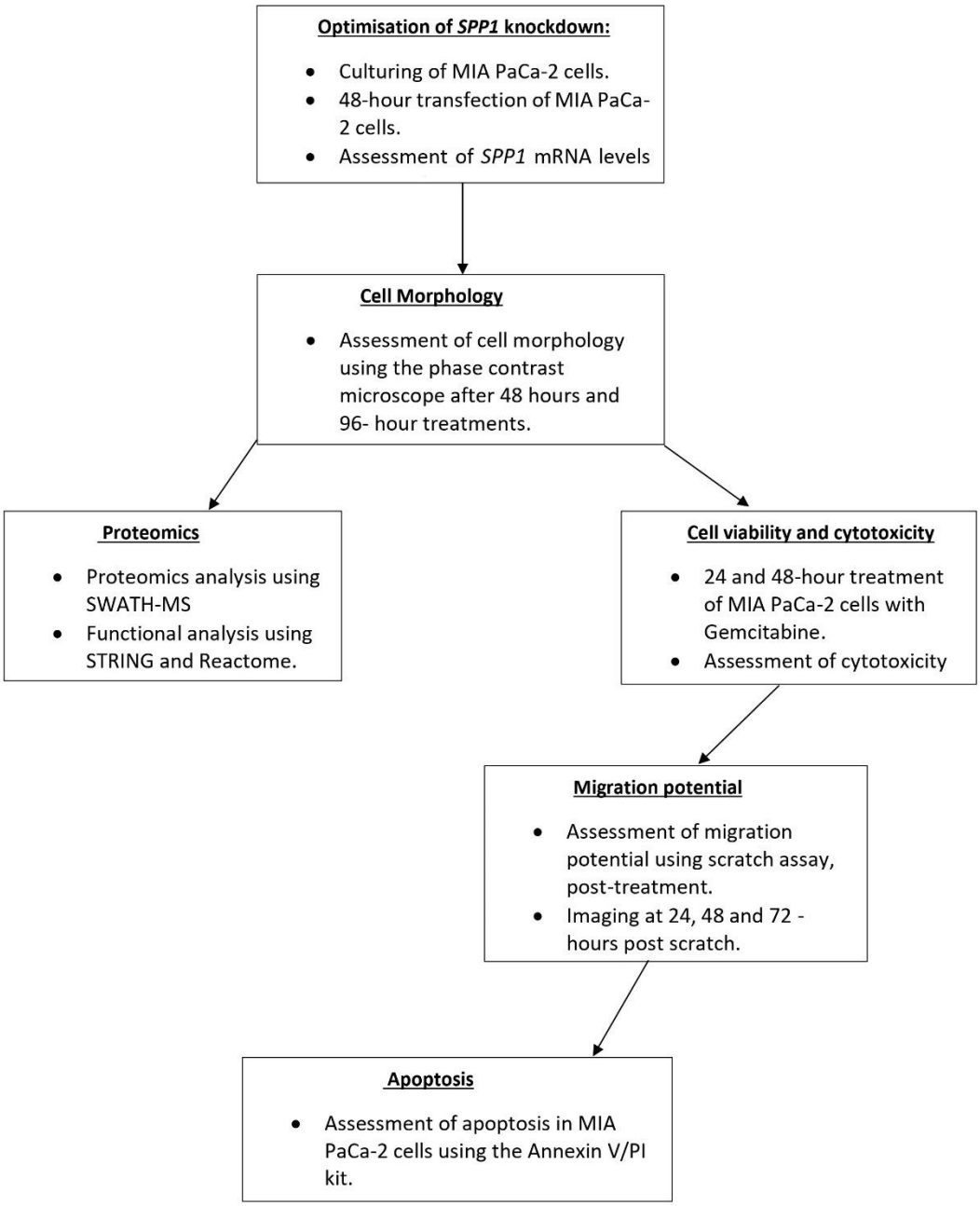


Figure 3. 1: Brief overview of the study design. A brief outline of the experimental procedures performed to assess the effect of *SPP1* knockdown and gemcitabine treatment in MIA PaCa-2 cells.

3.2.1 Cell culture

Tumourigenic Human Pancreatic Carcinoma Cells (MIA PaCa-2) obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank (JCRB0070, Japan), passage 2 (P2) was used in this study. Mycoplasma detection was conducted, yielding negative results (indicating no presence of mycoplasma).

The cell line was cultured under standard physiological conditions, maintaining a humidified atmosphere at 37 °C with 5% CO_2. Cells were cultured in sterile T75 cell culture flasks (156499, ThermoFisher Scientific, Waltham, MA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (D8900, Sigma Aldrich, St Louis, MO, USA) with high glucose (4.5 g/l) containing 4 mM L-glutamine, supplemented with 10 % fetal bovine serum (FBS) (12107C, Sigma Aldrich, St Louis, MO, USA) and 1% penicillin/streptomycin (P4333, Sigma Aldrich, St Louis, MO, USA). Passaging of cells occurred upon achieving a confluency level of approximately 70-80%. Cells were passaged by washing them in 3 ml of phosphate-buffered saline solution (PBS). Following the removal of the PBS, the cells underwent incubation in 3 ml of trypsin/EDTA (25300054, ThermoFisher Scientific, Waltham, MA, USA) at 37 °C for 5 minutes to facilitate cellular detachment. Subsequently, the cells were exposed to 3 ml of fresh complete growth medium to neutralise the trypsin/EDTA. Following this, the cells underwent quantification and appropriate dilution for subsequent treatments. The sub-cultured cells were subsequently sustained in a final volume of 15 ml of fresh complete growth medium.

The trypan blue exclusion test was used for the quantification of viable cells within a cell suspension. Trypan blue (15250061, ThermoFisher Scientific, Waltham, MA, USA) was added to the cell solution in a 1:1 ratio with 10 µl of cells, using a TC20^{TM} automated cell counter (1450102, Bio-Rad Laboratories, Hercules, CA, USA).

3.2.2 Cryo-preservation and resuscitation

Post-pelleting, the cells were resuspended in a 1 ml solution comprising 70% (v/v) FBS and 30% (v/v) dimethyl sulfoxide (DMSO) (589569, Sigma Aldrich, St Louis, MO, USA). Subsequently, the suspension was transferred into cryovials (5000-1020, ThermoFisher Scientific, Waltham, MA, USA) and stored at -80 °C. Upon requirement of cell stocks, cells were thawed (90%) at room temperature, followed by the addition of 1 ml of pre-warmed complete growth medium in a drop-wise manner. The resulting 2 ml suspension was then transferred to 15 ml tubes and centrifuged at 400 x g for 4 minutes to eliminate DMSO and the old medium. The resulting cell pellet was gently resuspended in 1 ml of fresh complete growth medium and introduced into a T25 flask (156367, ThermoFisher Scientific, Waltham, MA, USA) containing 9 ml of complete growth medium.

3.2.3 Transfection with *SPP1* siRNA

Transfection of MIA PaCa-2 cells with *SPP1*-specific siRNA was performed to explore the potential of *SPP1* downregulation as a therapeutic strategy for targeting PDAC. Oligos used for this assay were pre-designed and all subsequent assays were conducted post-transfection of MIA PaCa-2 cells with *SPP1*-specific siRNA (4392420-s13377, ThermoFisher Scientific, Waltham, MA, USA), along with non-targeting control (NTC) siRNA (4390843, ThermoFisher Scientific, Waltham, MA, USA), which showed no off-target effects and was therefore used for all further experimentation. Additionally, *ACTB*-specific siRNA (439420-s230682, ThermoFisher Scientific, Waltham, MA, USA) was employed as a positive control for the transfection. Transfections were executed using the Lipofectamine 3000 Transfection reagent (L 3000015, ThermoFisher Scientific, Waltham, MA, USA), adhering to the manufacturer's protocol. Briefly, MIA PaCa-2 cells were seeded into 12-well plates (150628, ThermoFisher Scientific, Waltham, MA, USA) and incubated overnight until they reached 60-70% confluency. The transfection reagent was mixed with pre-warmed DMEM only in a volume equivalent to that of the *SPP1* siRNA: (10nM siRNA: 10nM Lipofectamine 3000 transfection reagent), and NTC siRNA was then mixed separately with pre-warmed DMEM serum-free culture media under the same conditions.
The transfection reagent mixtures were combined with the corresponding siRNA mixtures, (10nM siRNA: 10nM Lipofectamine 3000 transfection reagent) and (10nM NTC siRNA: 10nM Lipofectamine 3000 transfection reagent) and they were subjected to a 15-minute incubation at room temperature to facilitate the formation of complexes. The complete solution of siRNA and transfection reagent were subsequently introduced into the respective wells, achieving a final volume of 1000 µl in the growth medium. The cells were then incubated for 24 and 48 hours (6 hours post-transfection; the transfection media was removed and replaced with complete DMEM). The transfected cells were imaged using the Olympus iX51 phase contrast microscope (Wirsam Scientific & Precision Equipment Ltd, Johannesburg, South Africa). Laboratory analysis was conducted at the Department of Surgery and Anatomical Sciences laboratories, Faculty of Health Sciences at the University of the Witwatersrand.

3.2.4 Treatment of cells

Cells were allowed to adhere for 24 hours before being exposed to control and test compounds diluted in DMEM. Briefly, MIA PaCa-2 cells were exposed to different concentrations of gemcitabine (Y0000675-50 mg, Sigma Aldrich, St Louis, MO, USA) (85, 42.5, 21.25, 10.63, 5.31, 2.66, 1.33, and 0.66 µM) for 24 and 48 hours. Approximately 0.33 % DMSO was used

as the vehicle control, and 0.4 µM doxorubicin (DOX) was used as the positive control drug, these concentrations were previously determined and employed in our laboratory.

After establishing the optimal concentration (IC_{50}) as shown in Supplementary fig. 3, the cells were treated with gemcitabine alone (10 µM) and in combination *SPP1* knockdown (10 nM si*SPP1)* at different time points (24 hours, 48 hours, 72 hours and 96 hours) in a humidified incubator (humidity, 37 $^{\circ}$C and 5% CO_2). Each treatment was performed in triplicate for each experiment and repeated three times for this assay.

3.2.5 Verification of gene targets using PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted to confirm the expression of *SPP1* in MIA PaCa-2 cells. cDNA was synthesised using the Invitrogen™ SuperScript™ VILO™ cDNA synthesis kit (11754050, ThermoFisher Scientific, Waltham, MA, USA) as shown in Supplementary Table 2, followed by Reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR was performed using primers designed in-house (Table 3.1). Thermal cycling parameters were set as follows: Initial denaturation at 94°C for 30 seconds, followed by 30 cycles of 94°C (30 seconds), 60°C (60 seconds), and 68°C (30 seconds). Finally, the product was subject to extension at 68°C for 5 minutes.

The PCR products were run, alongside a 1kb DNA ladder (SM 0311, ThermoFisher Scientific, Waltham, MA, USA), on a 1% agarose gel containing 0.5 µg/ml of ethidium bromide (E1510, Sigma Aldrich, St Louis, MO, USA) at 110 volts for one hour. The gels were imaged using the ChemiDoc™ MP imaging machine (170-8170, Bio-Rad Laboratories, Hercules, CA, USA), as shown in Supplementary fig.4.

Table 3. 1: Primer sets and sequences.

Gene name	**Primer sequence (5'---3')**
MRLP-19	Forward primer: CCTAGGCCGGAGTTTCCAA Reverse primer: GACTCAACCTGCGTTCC
SPP1	Forward primer: AGCAGAATCTCCTAGCCCCA Reverse primer: TGGTCATGGCTTTCGTTGGA

qRT-PCR: 24 and 48 hours post-transfection, total RNA was extracted according to section 2.2.4 and cDNA was synthesised according to section 2.2.7. Validated pre-designed TaqMan

Gene Expression assays were employed for *SPP1* (Assay ID- Hs00959010_m1, 4331182, ThermoFisher Scientific, Waltham, MA, USA) and *ACTB* (Assay ID-Hs99999903_m1, ThermoFisher Scientific, Waltham, MA, USA) as described in section 2.2.7. *ACTB* was used in our study, as it was pointed out as one of the most stable genes in the pancreas (Erkoç Kaya *et al.*, 2023).

3.2.6 (4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) cell viability assay

The MTT (11465007001, Sigma Aldrich, St Louis, MO, USA), assay was employed to evaluate the viability of MIA PaCa-2 cells. This assay was performed on MIA PaCa-2 cells that were treated with varying concentrations of gemcitabine (0.66- 85 µM) at 24 and 48 hours. The assay aimed to determine whether gemcitabine affected cell viability and to identify the optimal concentration that was toxic to the MIA PaCa-2 cells. The IC_{50} value, representing the concentration at which compounds inhibit cell viability by 50%, signifies the half-maximal inhibitory concentration (Aykul and Martinez-Hackert, 2016). The assay relied on the colorimetric response generated by the presence of NAD(P)H-dependent oxidoreductases within the cells, which served as an indicator of metabolic activity and cell viability. Mitochondrial Succinate Dehydrogenase, a component of viable cells, reduced the tetrazolium dye, MTT, to its insoluble form, leading to the formation of purple formazan crystals. The quantity of crystals formed directly indicated cellular viability and provided a means of quantifying cell viability relative to an untreated sample. The MIA PaCa-2 cells were seeded into 96-well plates (260887, ThermoFisher Scientific, Waltham, MA, USA) and incubated overnight in a humidified incubator (95% humidity, 37 ºC and 5% CO_2). MIA PaCa-2 cells were then treated as described in the methodology, section 3.6.4, using DMSO as a diluent control. Following incubation with the specific treatment concentration, 100 µl of 1 mg/ml MTT was added to each well and incubated at 37°C for 3 hours. The formazan crystals were dissolved in 200 µl of DMSO/well and the absorbance was measured at 450 nm using a spectrophotometer (MULTISKAN Sky, A51119500C, ThermoFisher Scientific, Waltham, MA, USA) with a reference wavelength of 690 nm. The percentage cell viability was determined by using the treated values as a percentage relative to the corresponding untreated values for each biological triplicate.

Equation: $Viability = \frac{mean\ absorbance\ of\ sample}{mean\ absorbance\ of\ control} \times 100$

3.2.7 Morphological assessment of nuclear structure

To evaluate the nuclear morphology of MIA PaCa-2 cells treated with gemcitabine, DAPI staining was utilized. The cells were seeded at a density of 1 x 10^5 cells/well in a 12-well plate in a complete medium at 37°C for 24 hours. Following 48 hours of treatment with 10 µM gemcitabine, the cells were washed twice with PBS and then fixed with 450 µl of 4% formaldehyde for 5 minutes. Next, the cells were permeabilized with 450 µl of 0.01% Triton for 5 minutes and then stained with five µg/ml of DAPI for 15 minutes in the dark. The cells were rewashed with PBS and examined under the Olympus iX51 fluorescent, inverted microscope (Wirsam Scientific & Precision Equipment Ltd, Johannesburg, South Africa). The images were visually scrutinized for morphological nuclear alterations suggestive of apoptosis.

3.2.8 Scratch assay

The scratch motility assay was performed to evaluate the influence of *SPP1* knockdown, gemcitabine and combinational treatment on the migration capacity of MIA PaCa-2 cells. The cells were transfected as described in section 3.2.3 at 1 x 10^5, in 96 well plates, and allowed to reach a confluency of 90-95%, ensuring the formation of a confluent monolayer. A vertical scratch was created across the cell layer using a P10 pipette tip, and markings were placed along the scratch on the underside of the plate for uniformity in the subsequent imaging procedure. A medium change was subsequently performed, replacing it with 100 µl/well of fresh complete growth medium containing 5 µg/ml Mitomycin C. This treatment inhibits cell proliferation, ensuring that the observed cells in the scratch assay are due to migration rather than proliferation. The individual wells were imaged with the Olympus iX51 inverted microscope with CellSens Software v3.2 (Olympus, Tokyo, Japan) at (0 hour) and the cells were further imaged at 24, 48, and 72 hours post-scratch. The ImageJ software v2.9.0 (National Institute of Health, Bethesda, MD, USA) was used for image analysis of the scratched cells (Schindelin *et al.*, 2012). The rate of cell migration was calculated using the formula: (Dinitial – Dfinal) × 100 / Dinitial, D represents the distance.

3.2.9 Cell apoptosis assay

The apoptosis assay was carried out to investigate the effects of *SPP1* knockdown, gemcitabine treatment, and the combination of both on apoptosis in MIA PaCa-2 cells. The Annexin V-FITC apoptosis kit (BD Biosciences, San Diego, CA, USA) was used to detect early apoptosis, when phosphatidylserine is translocated to the outer leaflet of the plasma membrane is tranlocated. MIA PaCa-2 cells were seeded in 12-well plates at a density of 2 x 10^5 cells/well for 24 hours and were then transfected with siRNA targeting *SPP1*. The cells were further

treated with 10 µM gemcitabine for 48 hours, after which they were washed with PBS and trypsinised. DOX (0.4 µM) was used as a positive control for apoptosis induction. Next, 200 µl of complete media was added to inactivate the trypsin. The cells were then centrifuged at 1500 x g for 5 minutes at 4 °C, after which they were resuspended in 2 ml of complete medium and incubated for 30 minutes to allow recovery from trypsinisation. The cells were washed with 2 ml of PBS, centrifuged at 1200 x g for 5 minutes, and resuspended in 100 µl of 1X binding buffer. The suspensions were transferred into flow tubes and stained with 2 µl of Annexin V and 2 µl of propidium iodide (PI). Brief vortexing and incubation of the cells for 15 minutes at room temperature in the dark followed. Subsequently, 250 µl of 1X binding buffer was added to each tube, and the cells were analysed using a flow cytometer. Data acquisition was undertaken at low flow rates (less than 300 events per second) using the BD LSRFortessa™ Analyser and FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA). This was done to achieve enhanced resolution and precise DNA quantification. A minimum of 30,000 events were recorded per sample, and the fluorescent signal from the samples was detected using a 610/20 band-pass filter. The generated flow cytometry standard (FCS) files in FACSDiva were then imported and analysed using FlowJov10.8.1 (FlowJo, LLC, Ashland, KY, USA).

3.2.10 Statistical analysis

To evaluate the normal distribution of the data, normality tests like the Shapiro–Wilk test were conducted. Subsequently, an unpaired Student t-test was employed for comparison between the two groups. The non-parametric Kruskal–Wallis Anova by Ranks was performed, followed by multiple comparisons using the Benjamini–Hochberg test. All experiments were performed with a minimum of three technical and biological repeats with error bars representing standard deviation. A p-value of less than 0.05 was considered statistically significant.

3.7 Results

The initial steps of this investigation were to carry out a transient transfection using *SPP1*-specific siRNA, followed by an examination of the consequences of *SPP1* knockdown on apoptosis, and migratory capacity. The absence of mycoplasma was confirmed in MIA PaCa-2 cells (Supplementary fig. 1).

3.7.1 Validation of *SPP1* knockdown

As the effect of targeting *SPP1* for downregulation in MIA PACA-2 cancer cells had not yet been assessed, it was imperative to optimise this process before conducting any experiments aimed at determining the downstream effects of *SPP1* downregulation.

To optimise the downregulation of *SPP1* levels in MIA PaCa-2 cells, transfection was conducted using *SPP1*-specific siRNA and NTC, followed by a 48-hour incubation period. Subsequently, *SPP1* mRNA levels were quantified using real-time PCR. NTC siRNA was employed to mitigate potential off-target effects. Additionally, a mock transfection control (transfection reagent only) was included to account for any reagent-induced effects. There were no effects on *SPP1* levels in either the NTC siRNA or mock-transfected cells compared to untreated cells. In contrast, *SPP1* levels in the siRNA transfected cells demonstrated a significant downregulation of 81%, as demonstrated in Figure 3.2. These conditions were therefore applied to all subsequent experiments.

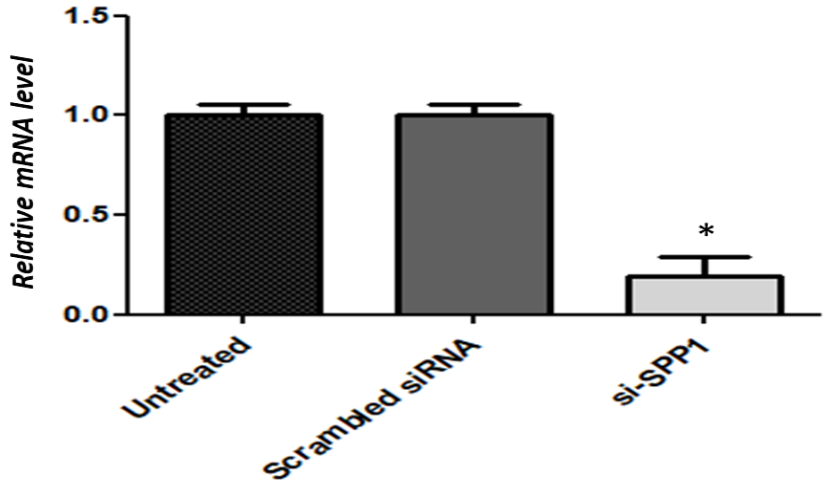


Figure 3. 2: Validation of *SPP1* knockdown. *SPP1* knockdown of MIA PaCa-2 cells was performed for 48 hours. Real-time PCR detected *SPP1* mRNA expression levels. β-Actin was used as a positive control for normalisation. As expected, the non-targeting control (NTC) siRNA did not significantly affect *SPP1* levels. *SPP1* levels were significantly decreased by 81% in the MIA PaCa-2 transfected cells. All values are given as the mean ± SD of three replicates. * denotes a p-value of less than 0.05.

3.7.2 MIA PaCa-2 morphology post-gemcitabine treatment

To determine the impact of 10 µM gemcitabine (optimal concentration) treatment on MIA PaCa-2 nuclear condensation after 48 hours, DAPI staining was utilised to confirm any observed morphological alterations associated with a reduction in cell viability. As shown in Figure 3.3a, it was observed that nuclei of untreated cells were round and intact, whereas apoptotic cells displayed an irregular nuclear shape, with nuclear condensation. These characteristic changes are commonly observed in cells undergoing apoptosis. Additionally, a reduction in cell number under gemcitabine treatment was visualised. A quantitative graph presenting the results of the cytotoxicity assessed using the MTT assay is depicted in Figure 3.3c. The findings indicate that, in contrast to untreated cells, minimal cell rounding and

clumping were observed with 5 and 7 µM gemcitabine. However, the majority of the plated cells remained viable even after 48 hours, displaying 6.22 ± 1.92% and 5.12 ± 3.02% cytotoxicity, respectively. At the highest concentration tested (10 µM), a slight increase in cytotoxic effect was observed (14.62 ± 4.62%). Therefore, the optimal concentration for gemcitabine was established as 10 µM (Supplementary fig.3). Based on these observations, it appears that 10 µM of gemcitabine can induce apoptosis in MIA PaCa-2 cells.

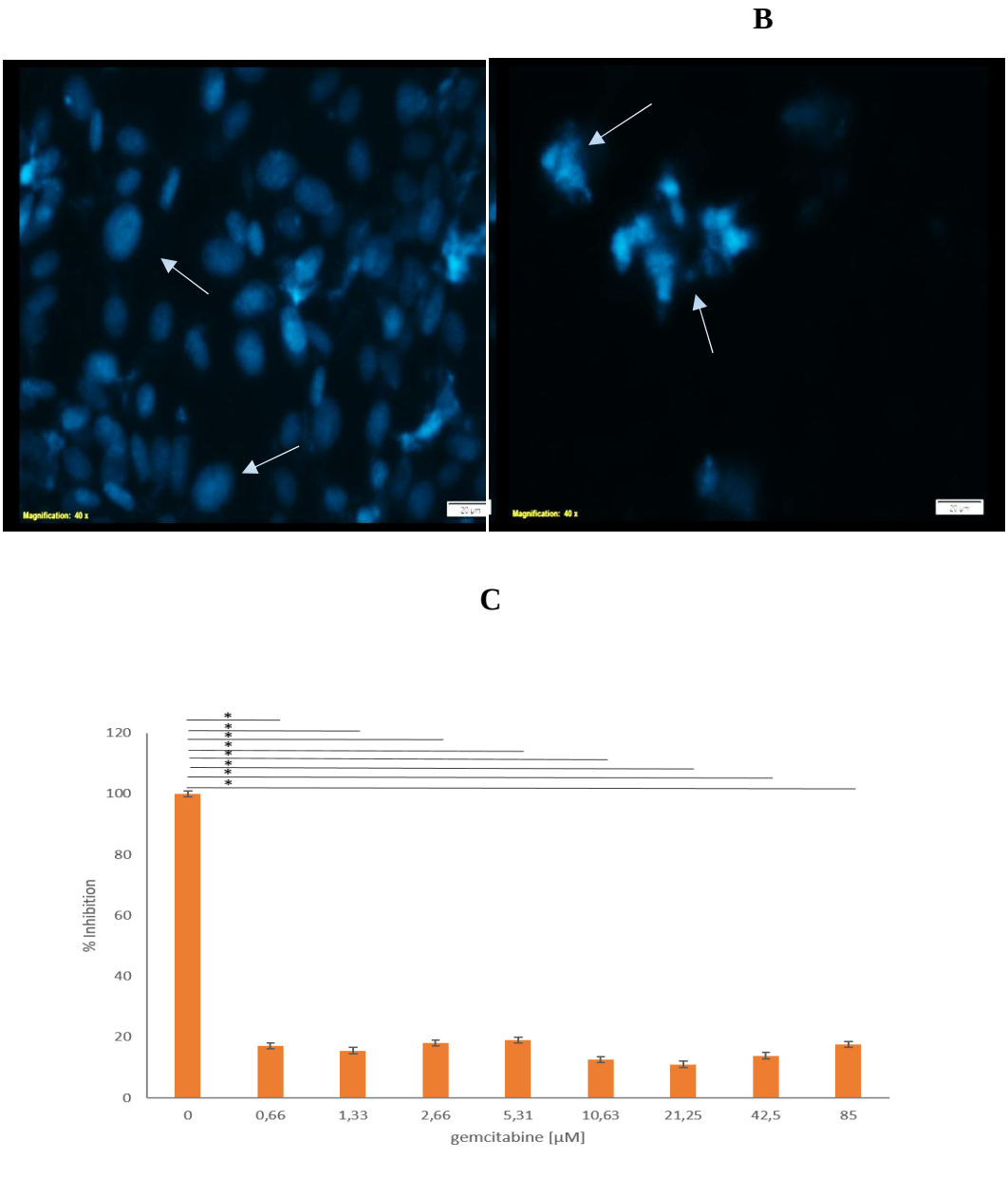


Figure 3. 3: DAPI nuclear stain in MIA PaCa-2 cells. Magnification: 40X. **(A)**: shows untreated cells, arrows showing round intact nuclei and **(B)** shows cells treated with 10 µM of gemcitabine for 48 hours. Note nuclear condensation and the reduction in cell number, indicated by the arrows. **(C)** Effect of gemcitabine on the viability of MIA PaCA-2 cells. Gemcitabine reduced the viability of the MIA PaCa-2 cells. * denotes a p-value of less than 0.05.

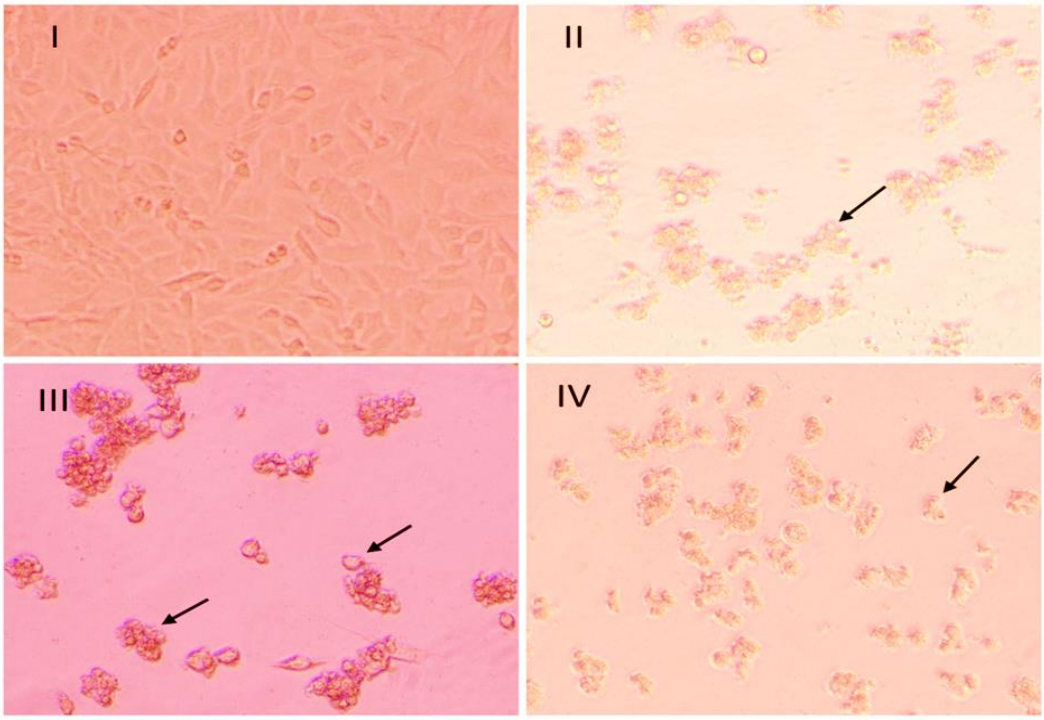


Figure 3. 4: *SPP1* knockdown induces an apoptotic morphology in MIA PaCa-2 cells. A phase contrast microscope was used to assess cellular morphological changes following *SPP1* knockdown, gemcitabine treatment, and a combination of *SPP1* knockdown and gemcitabine treatment. Representative phase-contrast images (I- IV). MIA PaCa-2 cells that were (I) untreated, (II) 48-hour treatment with 10 µM of gemcitabine (III) *SPP1* knockdown for 48 hours (IV) Combinational treatment of *SPP1* knockdown and gemcitabine treatment at 96 hours.

Thereafter, cell morphology was assessed using brightfield, phase contrast microscopy (Olympus iX51 inverted microscope), following a 48-hour treatment with 10 µM of gemcitabine (Figure 3.4), as well as after *SPP1* knockdown (Figure 3.4). From a typical PDAC cell line morphology, showing elongated cells with clear cell boundaries forming a sheet, under *SPP1* knockdown and treatment, cells showed a drastic change in morphology. The observation of cell shrinkage and detachment, which are characteristic of apoptosis (Ziegler and Groscurth, 2004), but not conclusively, was made following these treatments. To confirm these findings and determine the mode of cell death following treatment, an apoptosis assay was performed.

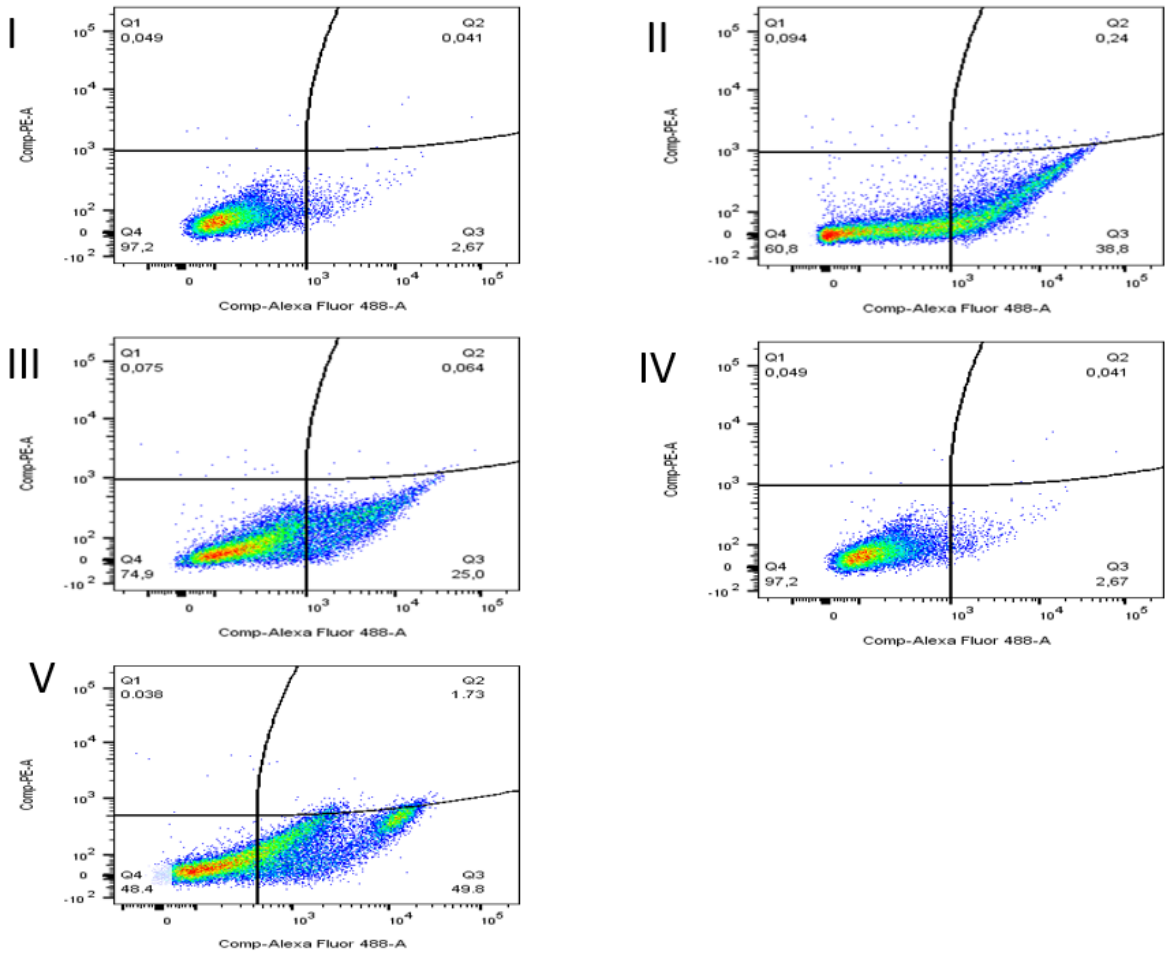
3.7.3 Synergistic effects of *SPP1* knockdown and gemcitabine treatment induce apoptosis

To investigate the potential synergistic induction of apoptosis, MIA PaCa-2 cells underwent *SPP1* knockdown combined with gemcitabine treatment for 96 hours. Thereafter flow cytometry analysis was conducted to determine whether apoptosis was a prime cause of cell death in MIA PaCa-2 cells. The experiment involved subjecting the cells to *SPP1* knockdown

and gemcitabine treatment to determine if cell death resulted from *SPP1* knockdown or co-treatment. The combined treatment demonstrated a significantly heightened apoptotic effect compared to the application of each treatment individually.

Cells were assessed for apoptosis (Annexin-V+/PI+) (Figures 3.5(a) and 3.5(b)). As expected gemcitabine induces early apoptosis in MIA PaCa-2 cells (24.5%), being the first line of treatment for PDAC, only marginally less than the positive control. Interestingly, *SPP1* alone did not show any significant apoptosis induction in MIA PaCa-2 cells following knockdown, with 5.7% of cells being in early apoptosis compared to 2.5% of untreated cells. More importantly, *SPP1* knockdown significantly increased gemcitabine's apoptosis-promoting impact, demonstrating a synergistic effect (37%). Consequently, knocking down *SPP1* could cause the MIA PaCa-2 cell line to sensitise more to gemcitabine.

A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Q3
25,0
Comp-PE-A
Comp-Alexa Fluor 488-A
IV
Q1
0,049
Q2
0,041
Q4
97,2
Q3
2,67
Comp-PE-A
Comp-Alexa Fluor 488-A
V
Q1
0.038
Q2
1.73
Q4
48.4
Q3
49.8
Comp-PE-A
Comp-Alexa Fluor 488-A

B

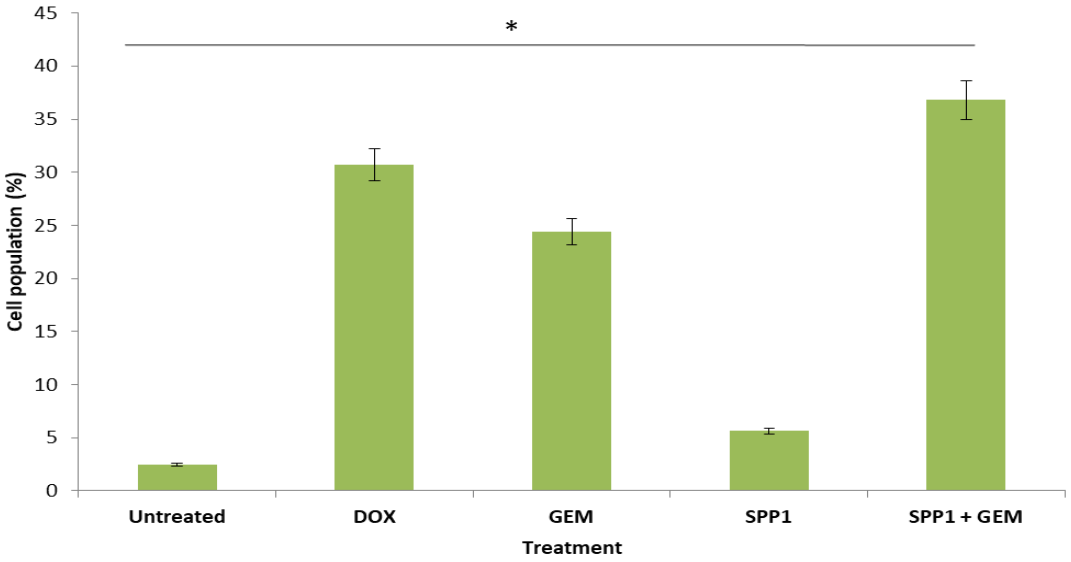


Figure 3. 5: Effect of *SPP1* knockdown only, gemcitabine treatment only, and the combination on apoptosis using PI and Annexin V. (**A**) Cell apoptosis analysis demonstrated with the quadrants (Q1: cells undergoing secondary necrosis (PI+); Q2: cells undergoing late stage apoptosis (Annexin V+ PI+); Q3: cells undergoing early apoptosis (Annexin V+); Q4: live cells. All subsequent graphs show cells stained with Annexin-V and PI. (I) Untreated cells. (II) DOX (positive control) (III) gemcitabine treatment only. (IV) *SPP1* knockdown only. (V) *SPP1* knockdown and gemcitabine treatment. (**B**) Graph representation of cells in early apoptosis (Annexin V positive and PI negative double-staining). *SPP1* knockdown significantly enhanced the apoptosis-promoting effect of gemcitabine. * denotes a p-value of less than 0.05.

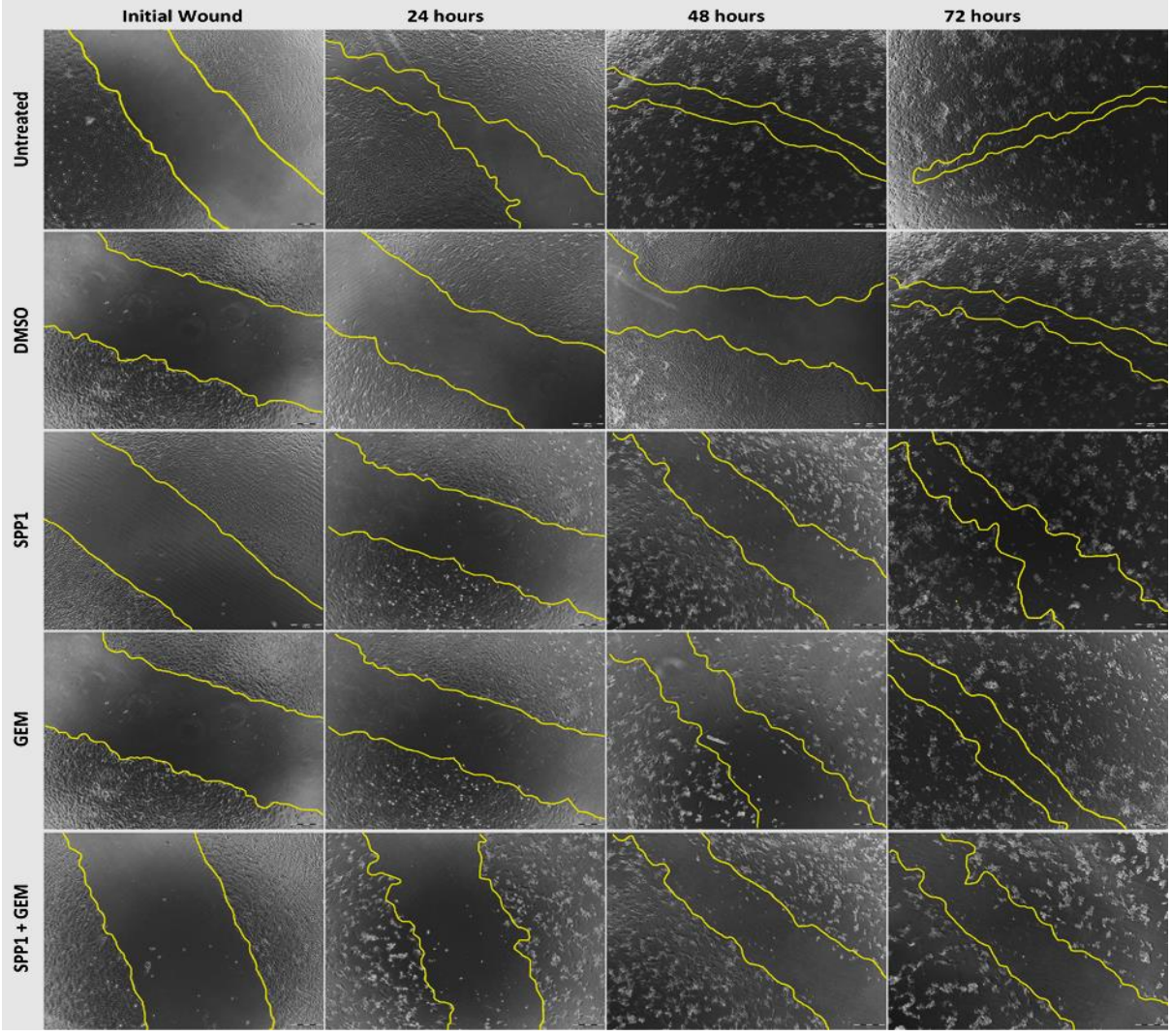
3.7.4 Downregulating *SPP1* decreases cell migration potential in MIA PaCa-2 cells

To investigate the influence of *SPP1* knockdown on cell migration of MIA PaCa-2 cells, the wound healing assay or scratch assay was used. As per section 3.2.8 a 'wound' was created in cells with a confluence of 90-95%, which were then treated with either *SPP1* knockdown alone, gemcitabine treatment alone, or a combination of both *SPP1* knockdown and gemcitabine treatment. The closure of the wound was then observed at 24, 48 and 72 hours post-treatment (Figure 3.6).

The migration of MIA PaCa-2 cells was found to decrease over time. At 24, 48, and 72 hours, the untreated and diluent control exhibited an increase in wound closure. This conclusion is supported by the fact that the wound closure rate increased significantly at 72 hours post-treatment with gemcitabine. Therefore, the combination of *SPP1* knockdown and gemcitabine treatment appears to reduce the migratory potential of MIA PaCa-2 cells.

The migration of MIA PaCa-2 cells was markedly inhibited upon *SPP1* knockdown, as demonstrated by the reduction in cell migration observed in Figure 3.6A, at all time points. Furthermore, the combination of *SPP1* knockdown and gemcitabine treatment resulted in a inhibition of MIA PaCa-2 cell migration, confirmed through relative quantification in Figure 3.6B. These results suggest that *SPP1* plays a role in regulating MIA PaCa-2 cell migration, although the underlying mechanism remains unclear.

A



B

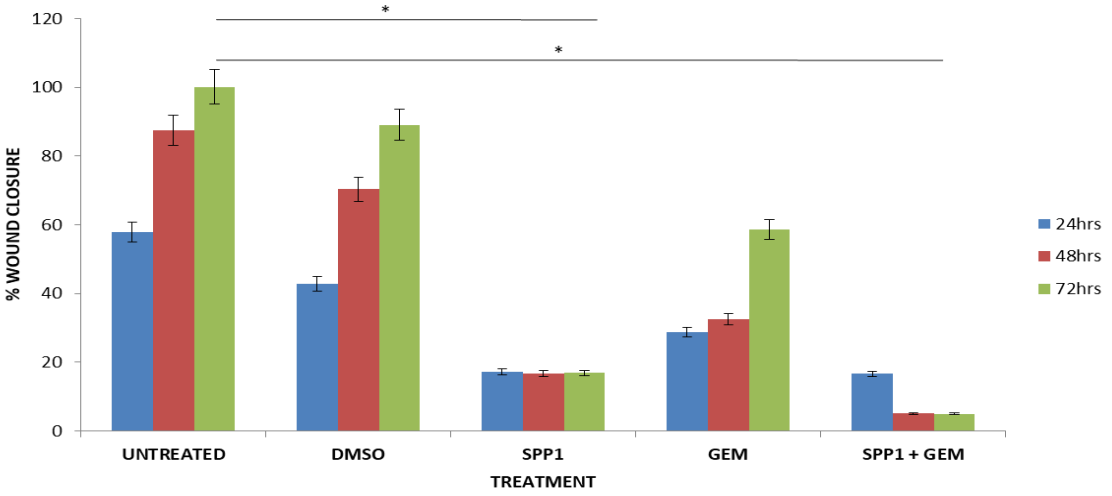


Figure 3.6: Combination of *SPP1* knockdown and gemcitabine treatment significantly decreases cell migration of MIA PaCa-2 cells. MIA PaCa-2 cells were subjected to various treatments for 24, 48 and 72 hours. (**A**) The migration abilities of MIA PaCa-2 cells were determined by scratch assay, the regions highlighted in yellow represent the wound area. (**B**) Graph representation of measured wound closure area. * denotes a p-value of less than 0.05.

3.8 Discussion

SPP1, a protein with multiple cellular functions, has gained considerable interest in the development of cancer therapeutics due to its overexpression in various types of cancer. SPP1 is involved in several areas, including bone metabolism, immune regulation, and wound healing, as well as in the survival and progression of cancer cells (Zhang *et al.*, 2020; Kiss *et al.*, 2021). Therefore, the overexpression of *SPP1* in cancer cells enables these cells to exploit certain functions and thereby participate in the formation of tumours. This study therefore served to validate and build upon previous findings by exploring the effects of *SPP1* knockdown on additional cancer hallmarks in the MIA PaCa-2 cell line.

To effectively downregulate the expression of *SPP1* in MIA PaCa-2 PDAC cells, a transfection process was employed using *SPP1*-specific siRNA as well as a universal negative control siRNA. The success of the downregulation was confirmed by a considerable 81% decrease in the mRNA levels of *SPP1*. This result provided validation for the effective downregulation of *SPP1* in MIA PaCa-2 cells, and these conditions were applied to all subsequent experiments. To explore the potential synergistic effect of *SPP1* inhibition with gemcitabine, a crucial anticancer drug for PDAC chemotherapy, we conducted experiments on MIA PaCa-2 cells to evaluate the combined impact of *SPP1* knockdown and gemcitabine treatment.

Synergistic effects of *SPP1* knockdown and gemcitabine treatment induce apoptosis

PDAC is commonly diagnosed at an advanced stage, which limits the effectiveness of curative treatments. Furthermore, chemoresistance poses a significant challenge in managing PDAC (Yu, Zhang and Xie, 2021), and its complexity is compounded by the invasive and metastatic nature of the disease, which is further exacerbated by its high molecular heterogeneity, making therapeutic interventions more complex (Connor and Gallinger, 2022).

One of the key characteristics of cancer lies in the way its cells resist cell death mechanisms, including apoptosis. This is a basic process that allows cancer cells to survive in environments where healthy cells would normally die (Hanahan and Weinberg, 2011). We assessed the potential synergistic effect of *SPP1* knockdown in combination with gemcitabine, an essential chemotherapeutic agent for PDAC. The results of the Annexin-V-propidium iodide assays analysis indicated a substantial increase in early-stage apoptosis in MIA PaCa-2 cells upon administration of combinational treatment, as compared to *SPP1* knockdown or gemcitabine treatment alone, although there was a definitive response to the chemotherapy drug. In castration-resistant prostate cancer overexpression of *SPP1* has a significant influence on the regulation of apoptosis, as studies by Pang et al., have shown that a decrease in *SPP1* levels can inhibit apoptosis through the PI3K/Akt signalling pathway (Pang *et al.*, 2021). In certain cancers (prostate and colon), an increased correlation between activated PI3K/Akt and poor patient outcomes has been observed. Therefore, it is anticipated that reducing *SPP1* expression might decrease the activation of the PI3K/Akt signalling pathway, preventing the activation of survival pathways and promoting the initiation of apoptosis (Pang *et al.*, 2021).

This study also shows that the combination therapy using *SPP1* knockdown and gemcitabine treatment has superior outcomes compared to gemcitabine alone, indicating a synergistic effect that enhances apoptosis and improves sensitivity to gemcitabine in MIA PaCa-2 cells. This is consistent with *SPP1*'s described role in mediating chemoresistance in lung adenocarcinoma (Matsubara *et al.*, 2022) and in prostate cancer (Pang *et al.*, 2021). Moreover, in prostate cancer cells, *SPP1* elevation of the expression of p-glycoprotein has been linked to the emergence of multidrug resistance (Hsieh *et al.*, 2013). Similarly, the levels of *SPP1* expression in a cohort of breast cancer patients who underwent adjuvant chemotherapy were discovered to predict the effectiveness of neoadjuvant chemotherapy in certain cases (Insua-Rodríguez *et al.*, 2018).

Although research has suggested a relationship between dysregulated total *SPP1* expression and drug resistance, the influence of *SPP1* and its splice variants on chemotherapeutic response

remains unexplored (Li *et al.*, 2014; Nakamura *et al.*, 2015, 2016). Moreover, recent studies have revealed a connection between lung adenocarcinoma's resistance to chemotherapy and the expression of *SPP1* in TAMs, which is associated with a poor prognosis (Matsubara *et al.*, 2022). This could suggest that *SPP1* knockdown can be used to enhance the effectiveness of chemotherapy drugs, particularly gemcitabine by triggering pathways involved in apoptosis, notwithstanding a role in controlling inflammatory cell infiltration and function in the TME.

Synergistic effects of *SPP1* knockdown and gemcitabine treatment on cell migration

The inhibitory effects of *SPP1* knockdown and gemcitabine treatment alone or in combination on the migration of MIA PaCa-2 cells were determined with the wound healing assay. Gemcitabine alone had modest effects on the migration of MIA PaCa-2 cells while the combination of *SPP1* knockdown and gemcitabine treatment had more potent inhibitory effects on cell migration than either compound alone. The activation of invasion and metastasis is one of the most important hallmarks of cancer that is currently known, and it is crucial in the progression and spread of the disease (Joshi, Gutierrez Ruiz and Razidlo, 2023). The involvement of *SPP1* extends to various aspects that influence the biological behaviours of malignancies, encompassing their development, progression, and metastasis (Lindahl, Rzepecka and Dabrosin, 2019; Yan *et al.*, 2023).

The results of this study revealed that decreasing the expression of *SPP1* via knockdown led to a decline in the migratory and invasive capabilities of highly aggressive PDAC cells. This is consistent with findings by Xu et al. suggesting that inhibiting the expression of *SPP1* through siRNA significantly hampers the ability of MIA PaCa-2 cells to migrate (Xu *et al.*, 2017). Specifically, diminishing *SPP1* expression impedes migration, and invasion, while elevated *SPP1* expression is strongly associated with a dismal prognosis in various forms of cancer (Liu *et al.*, 2020). This has been shown in breast cancer studies, with Hao et al.'s findings which suggest that *SPP1* expression is associated with heightened cell migration and adhesion in breast cancer (Hao Zhang *et al.*, 2014); and Rizwan et al. who observed that the knockdown of *SPP1* in MDA-MB-231 breast cancer cells resulted in a significant decrease in migration during *in vitro* transwell migration assays (Rizwan *et al.*, 2018). According to a study by Zeng et al., inhibiting *SPP1* was shown to decrease cell proliferation, migration, and invasion capacities through the β1/FAK/AKT signalling pathway (Zeng *et al.*, 2018).

These findings demonstrate the synergistic effectiveness of the combined interventions and suggest a potential therapeutic strategy for treating PDAC.

Conclusions

Reducing the levels of *SPP1* has a significant impact on essential features of PDAC, including its ability to evade apoptosis and migrate. The results of this study suggest that *SPP1* knockdown can be used to enhance the effectiveness of chemotherapy drugs, particularly gemcitabine by triggering pathways involved in early apoptosis and reducing the migratory capacity of PDAC cells. Therefore, targeting *SPP1* could be a critical intervention for inhibiting these fundamental cancer characteristics in PDAC cells, potentially improving patient outcomes.

CHAPTER 4: PROTEOMIC PROFILING REVEALS PATHWAY ALTERATIONS IN PANCREATIC CANCER CELLS FOLLOWING *SPP1* DOWNREGULATION AND COMBINATION TREATMENTS.

4.1 The use of proteomics in unravelling PDAC molecular landscape

Protein biomarkers offer a greater degree of unique information. CA 19-9 is currently the most widely used and accepted biomarker for diagnosing PDAC, and treatment monitoring with a sensitivity of 82% and specificity of 90% (Wu *et al.*, 2013; Sattar *et al.*, 2019; Luo *et al.*, 2021). However, due to the limitations of current screening techniques, it is crucial to discover new biomarkers that can reliably differentiate PDAC from other conditions with similar symptoms and reduce the death rate from PDAC (Herreros-Villanueva and Bujanda, 2016). Various techniques have been developed for the detection of biomolecules and biomarkers, providing comprehensive information for the characterisation of these markers in complex protein mixtures (Ahmad, Imran and Ahsan, 2023). Proteins are the fundamental building blocks of all living organisms, serving as primary effectors in cellular processes and essential for maintaining cellular homeostasis (Frantzi *et al.*, 2018). Disruption of these processes is frequently associated with a range of disease phenotypes, making the investigation of proteins and their functions crucial for understanding cellular mechanisms and organizational structures (Aebersold and Mann, 2016).

Furthermore, drugs function at the proteome level, and protein expression variations and alterations, particularly post-translational modifications (PTMs), can be better understood through the use of proteomics (Tannu and Hemby, 2006; Mischak, 2015). This approach leads to a better comprehension of physiological and pathological pathways and is essential for both foundational and translational research (Faa *et al.*, 2016; Anjo, Santa and Manadas, 2017). Protein biomarkers have been widely investigated for determining disease type or stage, evaluating disease progression, and determining responsiveness or resistance to therapeutic therapies.

Mass spectrometry (MS) serves as the fundamental method for most protein biomarker initiatives (Rifai, Gillette and Carr, 2006; Anderson, Ptolemy and Rifai, 2013; Olsen *et al.*, 2020). Proteomic analysis is a well-researched method for thoroughly elucidating biological linkages and pathways through the simultaneous examination of many proteins in one assay. Proteomics tools and technologies encompass a diverse range, enabling systematic, comprehensive, and targeted analyses of protein structure, function, expression, interactions, and modifications (Aslam *et al.*, 2017). Proteomics technologies have been widely applied to

PDAC research to find potential PDAC-associated proteins for use in prognostic, staging, diagnostic, tumour resectability assessment and treatment response prediction (Jimenez-Luna *et al.*, 2018). To achieve this goal, diverse clinical specimens such as pancreatic juice (Agrawal, 2017), tumour tissues (Song *et al.*, 2018), and plasma (Saraswat *et al.*, 2017) have been extensively employed in previous studies. Similarly, the use of PDAC cell lines presents an advantageous opportunity to investigate mechanisms associated with tumour growth and can therefore enable researchers to target them effectively (Ansari *et al.*, 2019). In this study, the MIA PaCa-2 cells were used as a model for elucidating key pathways associated with *SPP1* knockdown and combination treatment with gemcitabine.

4.2 Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS)

Proteomic technologies such as MS enable the characterization of numerous proteins in a single label-free experiment (Neilson *et al.*, 2011; Lombard-Banek and Schiel, 2020). SWATH-MS is a proteomic platform based on data-independent acquisition (DIA) that allows for the simultaneous identification and measurement of proteins (Krasny *et al.*, 2018). SWATH-MS has emerged as the preferred approach for proteomics-based cancer research, offering several advantages in protein quantification (Guo *et al.*, 2021). It combines the high throughput of shotgun proteomics with the sensitivity and reproducibility of SRM proteomics (Wang, Sheng and Shyr, 2015). This label-free method can quantify thousands of proteins, providing a comprehensive overview of complex samples (Simbürger *et al.*, 2016). It has been successfully used to identify and quantify proteins involved in specific pathways (Gao, Chen and Zhu, 2022). Trypsin digestion of unlabeled protein samples is the first step in the SWATH-MS process. The resulting peptides are then analyzed using liquid chromatography in conjunction with tandem mass spectrometry (LC-MS). This novel LC-MS-based method uses windows in consecutive mass spectra to identify proteins (Ludwig *et al.*, 2018). It is noteworthy that SWATH-MS reliably produces quantitative assessments of peptides, including thousands of proteins, which is a significant benefit. Additionally, this technique is effective in samples that require accurate and repeatable quantification of the majority of the expressed proteome in a particular sample (Ludwig *et al.*, 2018). The SWATH-MS technique has been shown to be highly effective in detecting and measuring more than 4000 proteins with accuracy. As a result, it has emerged as a promising approach for large-scale protein quantification, as outlined by Collins et al. in 2017 (Collins *et al.*, 2017). In cancer research, quantitative proteomics has made significant progress by providing reasonably precise measurements of protein expression, according to Nusinow et al. (Nusinow *et al.*, 2020). The integration of mass

spectrometry, which involves ionization techniques, and mass analyzers that utilize quantitative labeling strategies, has been demonstrated to enhance protein detection and quantification (Lindemann *et al.*, 2017). Several studies have demonstrated that in complex mammalian samples, single-shot analyses provide proteome coverage of approximately 50% of the mass spectrometry-measurable proteome in a particular sample (Ludwig *et al.*, 2018). For example, Nweke et al. validated the utility of SWATH-MS in the proteomic profiling of solid tumours to identify potential biomarkers (Nweke *et al.*, 2020).

4.3 *SPP1* knockdown in MIA PaCa-2 cells

In Chapter 2, *SPP1* was identified to be significantly upregulated in tumours compared to corresponding normal tissues suggesting its potential role in PDAC. Elevated *SPP1* was able to successfully identify PDAC patients from healthy controls with an 80% sensitivity and a 97% specificity but failed to perform superiorly to the currently accepted standard, CA 19-9 (Koopmann *et al.*, 2004). Furthermore, *SPP1* could also distinguish between healthy patients and chronic pancreatitis but once again failed to improve the diagnostic success rates when compared to CA 19-9 (Poruk *et al.*, 2013). In another study, *SPP1* levels were significantly correlated with tumour stage and aggressiveness in cancers, with *SPP1* mRNA overexpressed in PDAC (Tu, Chen and Fan, 2019). However, it remains to be identified if *SPP1* could be a potential biomarker for PDAC patients in an African patient cohort.

Thereafter, in Chapter 3, the potential role of *SPP1* in PDAC was investigated by using siRNA to knockdown the gene in MIA PaCa-2 cells. Functional assays showed that *SPP1* played a significant role in mediating survival and migratory processes, and chemoresistance in a PDAC cell line. This ties in with the cellular functions of SPP1 as an extracellular matrix, integrin-binding protein secreted from various types of cells in the TME including macrophages, endothelial cells, and osteoclasts (Zhang *et al.*, 2014; Bastos *et al.*, 2017). This protein plays multiple roles in physiological and pathological processes, namely, it is involved in cellular growth, adhesion, and invasion in tumourigenesis and metastasis (Chen *et al.*, 2013; Bandopadhyay *et al.*, 2014; Briones-Orta *et al.*, 2017). Research has also demonstrated that *SPP1* activates the cancer stem population through its binding to CD44, which is a cellular adhesion molecule that is closely linked to cancer stem cells (Nallasamy *et al.*, 2021). This activation is induced by cancer-associated fibroblasts and is closely associated with the ECM protein, hyaluronan, which plays a role in the rigidity of the ECM and migratory capacity of tumour cells through the ECM (Nallasamy *et al.*, 2021).

To further unpack the role of SPP1 in PDAC, SWATH-MS was employed following *SPP-1* knockdown to ascertain which pathways are enriched during PDAC progression, and what effect co-administration of gemcitabine treatment would have.

4.3 Materials and Methods

4.3.1 Cell line preparation

MIA PaCa-2 cells were counted using the TC20^{TM} automated cell counter (1450102, Bio-Rad Laboratories, Hercules, CA, USA), and triplicate sets of 2.5 x 10^5 viable cells were subsequently seeded into 12-well plates (150628, ThermoFisher Scientific, Waltham, MA, USA). These cells were subjected to transfection with *SPPI* siRNA for 48 hours (section 3.2.3), as well as treatment with 10 µM gemcitabine (optimal concentration) for an additional 48 hours (section 3.2.4). Relevant controls were included. Adherent cells were rinsed with PBS and detached using trypsin-EDTA. Cells were centrifuged at 250 x g for five minutes. The resulting pellet underwent a series of four PBS washes and stored at -80 °C for later analysis. The Council for Scientific and Industrial Research (CSIR) centre was used for sample and data processing. Only proteins with a fold change of log2 and a p-value of ≤ 0.01 were considered differentially expressed.

4.3.2 Protein preparation for mass spectrometry

After pelleting the cells, they were resuspended in a 50 mM Tris-HCl pH 8.0 solution and 2% SDS. The Pierce Bicinchoninic assay (23225, ThermoFisher Scientific, Waltham, MA, USA) was used to measure protein concentration as per the manufacturer's instructions. To prepare the protein samples for analysis, each sample containing 10 µg of protein underwent a reduction step using 5 mM tris (2-carboxyethyl) phosphine, followed by alkylation at room temperature for 20 minutes with 10 mM 2-chloroacetamide. Subsequently, the samples were subjected to a purification process, removing detergents and salts, utilizing MagReSyn™ HILIC beads (MR-HLC005, ReSyn Biosciences Robindale, Randburg, South Africa) as previously described (Nweke *et al.,* 2020; Baichan *et al.,* 2023). On-bead protein digestion was carried out with a 1:20 ratio of protease to protein, employing sequencing-grade trypsin. The resulting peptides were then dried and preserved at -80°C until they were ready for LC-MS analysis.

4.3.3 LC-MS data acquisition

Samples, with about 1.5µg of peptides each, underwent analysis via a Dionex Ultimate 3000 RSLC system linked to a Sciex 5600 TripleTOF mass spectrometer. An Acclaim PepMap C18 trap column (75 µm × 2 cm; 2 min at 5 µl.min 1 using 2% ACN/0.2% formic (FA)) was used to inline de-salt injected peptides. Trapped peptides were gradient eluted and separated on a

Waters Acquity CSH C18 NanoEase column (75 µm × 25 cm, 1.7 µm particle size) at a flow rate of 0.3 µl.min 1 with a gradient of 6-40% B over 90 minutes (A: 0.1% FA; B: 80% ACN/0.1% FA). For SWATH, precursor scans were acquired from 400-1100 m/z with 50 milliseconds accumulation time, and fragment ions were acquired from 200-1800 m/z for 48 variable-width precursor windows with 0.5 Da overlap between windows and 20 milliseconds accumulation time per window.

4.3.4 LC-MS data analysis

SWATH data was processed using Spectronaut v17 software (Biognosys, Schlieren, Switzerland). For data processing, the default direct DIA identification and quantification settings were used. As a fixed modification, carbamidomethylation was added , and as variable modifications, N-terminal acetylation and methionine oxidation were added-Swiss-Prot Human sequences (downloaded on 09 June 2023 from www.uniprot.org). A q-value ≤ 0.01 cut-off was applied at the precursor and protein levels. Quantification was performed at the MS1 and MS2 levels. Label-free cross-run normalisation was employed using a global normalisation strategy. Candidate dysregulated proteins were filtered at a q-value ≤ 0.01 and absolute Log2 fold change (FC) ≥ 1.

4.3.5 Pathway and network analyses

To evaluate the dysregulated protein sets, functional enrichment analysis and network analysis using Cytoscape v3.8.2 were performed (Shannon *et al.*, 2003). The stringApp v2.0.1 (Doncheva *et al.*, 2019) within Cytoscape to examine the molecular functions, cellular compartments, and pathways (Reactome (Fabregat *et al.*, 2017) and KEGG (Kanehisa *et al.*, 2016) databases) associated with the dysregulated proteins. For the PCA analysis, a correlation matrix was employed to explain the maximal variance in samples, facilitating the delineation of the disease contexts. Significant loadings for the PCA were calculated using the equation: $1/\sqrt{n}$ (variables)−1. The values generated from this equation were utilised to determine the positive and negative significant loadings for the PCA analysis. Protein abundance data were analysed in Enrichr (Yeung *et al.*, 2008). The proteins were queried with filters including species: *Homo sapiens* as the reference species, a confidence threshold of 0.4 and zero additional interactors. Within the network, single non-interacting proteins were excluded.

4.4 Results

4.4.1 Identification of dysregulated proteins

A comprehensive proteomic analysis was conducted to further gain an understanding of *SPP1*'s role in PDAC and to provide deeper insights into the pathways affected by *SPP1*

downregulation. The protein profile of dysregulated proteins for the various treatments (gemcitabine treatment, *SPP1* knockdown, and combination treatment) compared to untreated MIA PaCa-2 cells are described (Table 4.1, 4.2 and 4.3).

In the gemcitabine-treated group, there were a total of 893 dysregulated proteins, of these 439 proteins were upregulated and 454 proteins were downregulated. In the *SPP1* knockdown-treated group, there were a total of 40 dysregulated proteins, 27 were upregulated and 13 were downregulated. The combination treatment group indicated that there were 188 proteins dysregulated compared to the untreated group. Of these, 110 proteins were upregulated and 78 proteins were downregulated.

Thereafter, unique and commonly dysregulated proteins across treatments were elucidated using Venny (v2.1.0). There were 741 proteins unique to gemcitabine treatment, 13 proteins unique to *SPP1* knockdown treatment, 34 proteins unique to combination treatment, and 13 proteins which were common across all 3 groups, as shown in Figure 4.1.

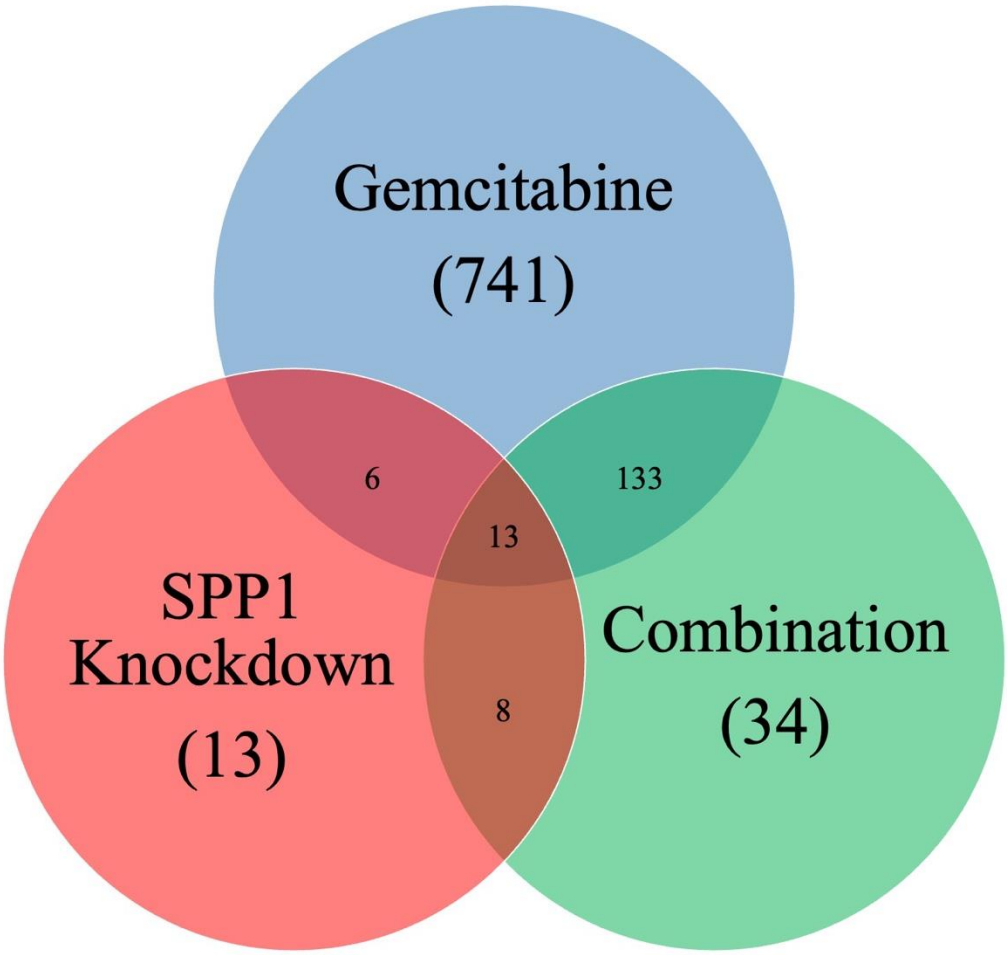


Figure 4. 1: Venn diagram depicting uniquely and commonly dysregulated proteins. There were 741, 13, and 34 proteins uniquely dysregulated in the gemcitabine, *SPP1* knockdown, and combination treatment groups, respectively. Thirteen proteins were commonly dysregulated across all 3 treatment groups.

Principal component analysis (PCA) was conducted to determine if the differentially expressed proteins in each treatment group could effectively distinguish between the groups. Method development data were acquired from four replicates (n = 4) for each workflow and this was consistent across the treatment groups, gemcitabine treatment, *SPP1* knockdown treatment, combination treatment, and untreated control. Figure 4.2 indicates the PCA analysis which shows that PC1 accounts for 63.92% variation and PC2 accounts for 10.05% variation. The gemcitabine treatment, *SPP1* knockdown treatment, and combination treatment are all well separated and distinguished along PC1. However, the *SPP1* knockdown and untreated sample groups, overlap showing that the protein profiles of these two groups are still very similar, notwithstanding that the untreated samples cluster tightly together while the *SPP1* knockdown shows more variance across PC2.

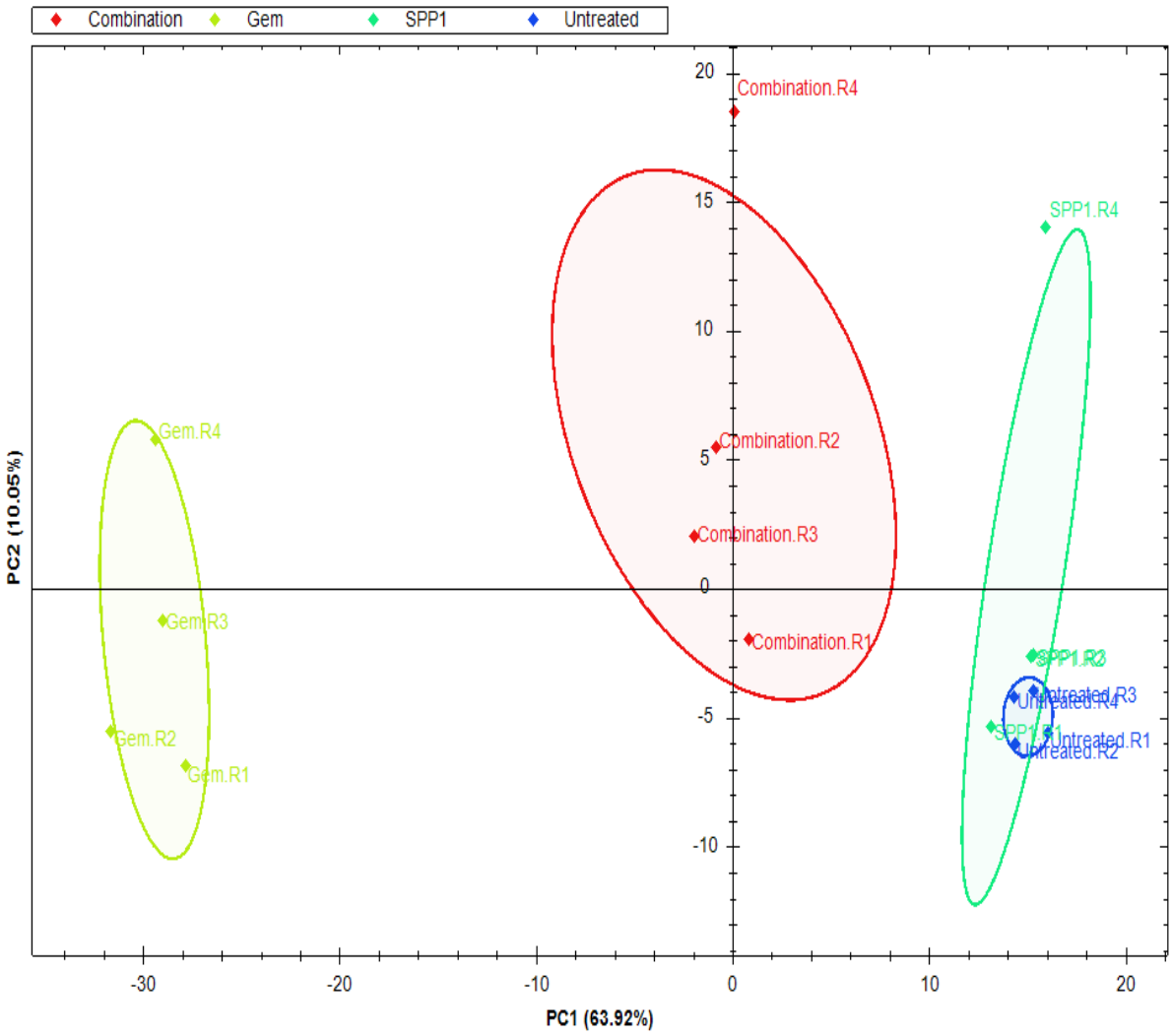


Figure 4. 2: Principal component analysis (PCA analysis) conducted on the different treatment groups. PCA analysis indicates that the treatment groups are separated and well differentiated from each other. Although the *SPP1* knockdown and untreated groups overlap, the untreated group clusters tightly with PC2 having a greater influence over the *SPP1* knockdown group.

4.4.2 Aberrant pathways with *SPP1* knockdown and combine treatment with gemcitabine

Pathway analysis on the differentially expressed proteins was performed to determine the specific processes the dysregulated proteins in gemcitabine treatment (Table 4.1), *SPP1* knockdown (Table 4.2), and combination treatment (Table 4.3) groups are involved in. Metabolism, including a member of the cytochrome p450 family, CYP51A1 is a top pathway enriched by the dysregulated proteins in the gemcitabine and combination treatment groups. Proteins involved in cell cycle were enriched in gemcitabine treatment only, including MCM5 which is involved in the transition from G0/G1/S phase

(https://www.genecards.org/Search/Keyword?queryString=MCM5); and proteins involved in apoptosis were downregulated particularly in cells undergoing gemcitabine treatment alone and when combined with *SPP1* knockdown. In the *SPP1* knockdown treatment group, transcription activation is one of the most enriched pathways by the differentially expressed proteins (Supplementary table 7), with an upregulation of *CDK1* a major player in cell cycle transition. Under a combination of both *SPP1* knockdown and gemcitabine treatment, not only were proteins involved in metabolism upregulated, but also proteins involved with collagen biosynthesis and extracellular matrix remodelling, including members of the *PLOD* family. Moreover, the downregulation of Toll-like receptor cascades and the upregulation of, for example, *STAT3* involved in particularly the adaptive immune response, suggests that MIA PaCa-2 cells were able to undertake compensatory mechanisms to ensure cell survival despite *SPP1* knockdown and gemcitabine treatment.

Table 4.1: Dysregulated pathways following gemcitabine treatment

Upregulated			Downregulated		
Pathway name	p-value	Proteins enriched	Pathway name	p-value	Proteins enriched
Metabolism	6.2×10^{-30}	CYP51A1,HSD17B10,OXCT1,UQCRC1,DLD,AAAS,TECR,SLC25A1,ACO2,MTHFD1	Metabolism of RNA	2.45×10^{-28}	PSMA4,PSMA3,PQBP1,PSMA2,CNOT2,RPS12,SNRPA,SNRPB2,LSM8,FUS
The citric acid (TCA) cycle and respiratory electron transport	4.76×10^{-28}	UQCRC1,DLD,ACO2,OGDH,NDUFS7,TRAP1,LRPPRC,ATP5B,NDUFS3,NNT	mRNA Splicing - Major Pathway	2.05×10^{-21}	PQBP1,SNRPA,SNRPB2,LSM8,FUS,SRSF1,WBP11,DNAJC8,NUDT21,SRRM2
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	1.54×10^{-16}	UQCRC1,NDUFS7,TRAP1,LRPPRC,ATP5B,NDUFS3,SDHA,NDUFA9,UQCRC2,ATP5O	Programmed Cell Death	8.59×10^{-17}	PSMA4,MAPK1,PSMA3,CDC37,PSMA2,YWHAH,PSMA6,PSMB1,MAPK3,YWHAE

Mitochondrial protein import	$2.60x10^{-15}$	ACO2,MTX2,TIMM10,ATP5,GRPEL1,VDAC1,SLC25A4,HSPA9,PAM16,CS	Apoptosis	$9.38x10^{-17}$	PSMA4,MAPK1,PSMA3,PSMA2,YWHAH,PSMA6,PSMB1,MAPK3,YWHAE,ADD1
Protein localization	$1.75x10^{-12}$	ACO2,MTX2,TIMM10,ATP5B,AGPS,GRPEL1,VDAC1,SLC25A4,HSPA9,PAM16	Cellular responses to external stimuli	$6.36x10^{-16}$	FKBP4,PSMA4,MAPK1,RBX1,PSMA3,GSR,PSMA2,RPS12,DYNC1LI2,PSMA6
Pyruvate metabolism and Citric Acid (TCA) cycle	$3.90x10^{-12}$	DLD,ACO2,OGDH,NNT,SDHA,VDAC1,DLAT,PDHB,ME2,MDH2	Regulation of expression of SLITs and ROBOs	$1.62x10^{-15}$	PSMA4,RBX1,PSMA3,PSMA2,RPS12,PSMA6,PSMB1,PSMA5,PSMB4,RPL38
Influenza Infection	$1.56x10^{-10}$	AAAS,RPL18A,NUP107,NUP155,NUP85,CANX,GRSF1,NUP205,RPS23,RPL15	Nervous system development	$1.99x10^{-14}$	PSMA4,MAPK1,RBX1,PSMA3,PSMA2,PFN1,ARPC3,RPS12,PSMA6,PSMB1
SRP-dependent cotranslational protein targeting to membrane	$9.64x10^{-10}$	RPL18A,RPN2,SEC61A1,SSR1,SPCS2,RPN1,RPS23,RPL15,SSR4,RPL3	Negative regulation of NOTCH4 signaling	$1.42x10^{-11}$	PSMA4,RBX1,PSMA3,PSMA2,SKP1,PSMA6,PSMB1,PSMA5,PSMB4,CUL1
Cristae formation	$9.64x10^{-10}$	MTX2,ATP5B,ATP5O,HSPA9,ATP5L,ATP5H,ATP5I,SAMM50,ATP5C1,MTX1	Membrane Trafficking	$2.01x10^{-11}$	PLIN3,ARPC3,TFG,YWHAH,DYNC1LI2,TBC1D1,CAPZA1,PACSIN2,AP2S1,YWHAE
Cell Cycle, Mitotic	$1.16x10^{-8}$	RFC2,AAAS,MCM5,NUP107,NUP155,NUP85,TUBGCP4,LMNB1,MCM4,CSNK2A2	FBXL7 down-regulates AURKA during mitotic entry and in early mitosis	$1.66x10^{-10}$	PSMA4,RBX1,PSMA3,PSMA2,SKP1,PSMA6,PSMB1,PSMA5,PSMB4,CUL1

Table 4.2: Dysregulated pathways following *SPP1* knockdown

Upregulated			Downregulated	
Pathway name	p-value	Proteins enriched	No functional enrichment	
Activation of gene expression by SREBF (SREBP)	9.70×10^{-4}	MVD,FASN,HMGCS1,LSS		
Cholesterol biosynthesis	5.4×10^{-3}	MVD,HMGCS1,LSS		
G1/S-Specific Transcription	5.6×10^{-3}	TK1,TYMS,CDK1		
Metabolism	2.25×10^{-2}	ACSS2,RRM1,MVD,TK1,FASN,TYMS,HMGCS1,FAR1,ACSL3,B4GALT1		
Mitotic G1 phase and G1/S transition	2.25×10^{-2}	TK1,TYMS,CDK1,TOP2A		

Table 4.3: Dysregulated pathways following a combination of *SPP1* knockdown andgemcitabine treatment

Upregulated			Downregulated		
Pathway name	p-value	Proteins enriched	Pathway name	p-value	Proteins enriched
Metabolism of proteins	3.22×10^{-7}	AAAS,MLEC,NUP155,MOGS,RPN2,SSR1,UGGT1,DERL1	Processing of Capped Intron-	2.03×10^{-9}	PQBP1,FYTTD1,WBP11,SARNP,GTF2F2,CHTOP,RBMX,SRSF11,YBX1,SRSF2

		,TMED2,C19orf10	Containing Pre-mRNA		
Asparagine N-linked glycosylation	$1.60x10^{-6}$	MLEC,MOGS,RPN2,UGGT1,DERL1,TMED2,RPN1,TMED10,CALR,MAGT1	mRNA Splicing - Major Pathway	$2.04x10^{-7}$	PQBP1,WBP11,GTF2F2,RBMX,SRSF11,YBX1,SRSF2,GTF2F1,BUD31,SRSF5
Collagen biosynthesis and modifying enzymes	$4.93x10^{-6}$	PLOD1,PLOD3,LEPRE1,COLGALT1,P4HA1,PLOD2,P4HB,P4HA2	Metabolism of RNA	$4.23x10^{-7}$	PQBP1,FYTTD1,WBP11,NOB1,SARNP,GTF2F2,CHTOP,RBMX,SRSF11,YBX1
Post-translational protein modification	$1.21\ x10^{-5}$	AAAS,MLEC,NUP155,MOGS,RPN2,UGGT1,DERL1,TMED2,VDAC1,RPN1	RNA Polymerase II Transcription	$1.60x10^{-4}$	FYTTD1,ATF2,JUNB,RBM14,SARNP,GTF2F2,GTF2E2,GATAD2B,CHTOP,SRSF11
Maturation of spike protein	$1.21x10^{-5}$	MOGS,RPN2,RPN1,MAGT1,STT3A,DDOST	TP53 Regulates Transcription of DNA Repair Genes	$1.5x10^{-3}$	ATF2,GTF2F2,JUN,GTF2F1,TCEA1
Extracellular matrix organization	$2.40x10^{-4}$	PLOD1,PLOD3,LEPRE1,COLGALT1,ITGAV,P4HA1,PLOD2,P4HB,FN1,HTRA1	Transcription of the HIV genome	$2.3x10^{-3}$	GTF2F2,GTF2E2,AK6,GTF2F1,TCEA1
Unwinding of DNA	$2.40x10^{-4}$	MCM5,MCM6,MCM7,MCM3	mRNA Splicing - Minor Pathway	$7.1x10^{-3}$	GTF2F2,YBX1,SRSF2,GTF2F1
DNA strand elongation	$2.70\ x10^{-4}$	MCM5,LIG1,MCM6,MCM7,MCM3	Toll-like Receptor Cascades	$3.06x10^{-2}$	ATF2,S100A9,JUN,LGMN,IKBKG
N-glycan trimming in the ER and Calnexin/Calreticulin cycle	$4.20x10^{-4}$	MLEC,MOGS,UGGT1,DERL1,CALR	Transcriptional Regulation by TP53	$4.18x10^{-3}$	ATF2,GTF2F2,GATAD2B,JUN,AK6,GTF2F1,TCEA1
Fatty acyl-CoA biosynthesis	$4.20x10^{-4}$	ACLY,HSD17B12,FASN,ACSL3,ACACA			

4.5 Discussion

Proteomics is a well-established and trustworthy methodology that involves the application of techniques designed for the identification and quantification of the complete protein composition present in cells and biological fluids. This approach has proven to be particularly valuable for the investigation of extensive protein profiles through mass spectrometry in a single analytical process. In this research, MIA PaCa-2 cells were subjected to proteomic analysis using SWATH-MS after undergoing various treatments, as previously mentioned. The analysis of these proteins and associated pathways shed light on the molecular mechanisms underscoring the phenotypes observed with *SPP1* knockdown and combination treatment with gemcitabine.

4.5.1 Treatment with gemcitabine induce pathways that may foster cell survival

Gemcitabine is considered the primary chemotherapy treatment for PDAC. However, its effectiveness is constrained by a low response rate and a limited capacity to extend progression-free survival (Stathis and Moore, 2010). The findings from this study highlight that MIA PaCa-2 cells undergo metabolic changes associated with enhanced cell survival under gemcitabine treatment.

The metabolic changes that occur in tumours are a well-established phenomenon that was initially identified by Otto Warburg (Warburg, 1956). Over the past 20 years, there has been a significant interest among researchers in the association between cell metabolism and tumourigenicity as well as tumour progression (Vander Heiden and DeBerardinis, 2017). Cellular metabolic alterations in cells are being targeted for therapeutic intervention, to balance metabolic demands with nutrient availability. Cancer cells, such as PDAC, require alterations in nutrient uptake and utilisation for growth and survival, as a result of the extreme hypoxia and limited nutrient access in the hypovascular, fibrotic tumour microenvironment. One major hallmark of cancer is deregulated metabolism whereby cancer cells prefer the less efficient glycolytic pathway as a source of energy (Hanahan, 2022). Importantly, PDAC is associated with certain genetic perturbations that have been linked to increased glycolytic pathway activity. For example, oncogenic KRAS promotes glucose uptake and directs the scavenging of serum lipids and proteins through macropinocytosis, a process that involves endocytosis (Commisso *et al.*, 2013).

Dysregulation of the Notch signalling pathway has been implicated in the development of tumour recurrence, metastasis, and chemoresistance in cancer patients (Espinoza and Miele,

2013). The Notch signalling pathway is critical for cell proliferation, differentiation, and homeostasis in PDAC. It is through the binding of specific ligands to various Notch receptors, including Notch-1, Notch-2, Notch-3, Notch-4, that Notch signalling cascades are activated (Espinoza and Miele, 2013). Notch serves as a significant mediator of chemoresistance in multiple types of cancer (Kajiyama *et al.*, 2007; Sayan *et al.*, 2009), and the downregulation of negative regulators of Notch signalling as identified in the present study indicates the induction of compensatory survival mechanisms in the face of gemcitabine treatment. The Notch and VEGF pathways are closely interconnected and play a crucial function in tumour angiogenesis triggered by hypoxia (Gu *et al.*, 2012). *In vivo* studies have revealed a 50% reduction in tumour growth following the co-administration of MRK-003 and gemcitabine, demonstrating a significant antitumour effect in PDAC xenografts. To build upon these findings, a Phase I clinical trial has commenced, to disrupt Notch signalling through the administration of the γ-secretase inhibitor MK-0752 in combination with gemcitabine for the treatment of PDAC patients (Cook *et al.*, 2018). Inhibiting the downstream targets of Notch in PDAC offers a promising therapeutic approach. Studies suggest that decreasing Notch receptor expression by blocking the Notch pathway in PDAC cells with gamma-secretase inhibitors (GSI) or MRK-003 impedes proliferation and growth (Mizuma *et al.*, 2012; Pramanik *et al.*, 2024). Primary drivers of PDAC are the *RAS* and TGFβ signalling pathways, which disrupt the G1/S cell cycle checkpoint machinery through *CDKN2A* and *TP53* deletions and, in some instances, impair DNA repair mechanisms through HRD or MMRD (Connor and Gallinger, 2022). In the present study, MIA PaCa-2 cells, upregulated proteins involved in the cell cycle suggested that those cells that did not succumb to the effects of gemcitibane were adapting to move through the cell cycle.

4.5.2 Pathways associated with *SPP1* knockdown

SREBPs signalling pathways, which were upregulated in the present study after *SPP1* knockdown, are essential transcription factors that regulate the expression of enzymes necessary for the synthesis of fatty acids, phospholipids, cholesterol, and triacylglycerol, which are vital for lipid metabolism (Eroglu *et al.*, 2023). Dysregulated lipid metabolism has been recognized as a defining characteristic of cancer cells and has been associated with various forms of the disease, including PDAC (Ettinger *et al.*, 2004; Li *et al.*, 2012), where altered lipid synthesis and accumulation contribute to tumour growth and progression. Consequently, SREBPs play a critical role in cellular lipid homeostasis (Parente *et al.*, 2023). High expression of SREBP1 has been shown to significantly influence the progression and metastasis of cancer

(Calvisi *et al.*, 2011). Several studies have indicated elevated de novo lipogenesis in PDAC cells (Menendez and Lupu, 2007; Walter *et al.*, 2009). Studies have demonstrated that the involvement of fatty acid metabolism is crucial for the tumourigenesis of PDAC (Swierczynski, Hebanowska and Sledzinski, 2014; Eroglu *et al.*, 2023). Aberrant activation of SREBPs is frequently observed in pancreatic tumours and has been linked to increased lipogenesis, providing cancer cells with the essential nutrients required for their survival and proliferation (Yin *et al.*, 2022). SREBP-mediated lipogenesis can affect other signalling pathways involved in cell cycle regulation, apoptosis resistance, angiogenesis, and metastasis (Zhao, Lin and Wang, 2022). Additionally, the knockdown of SREBP1 in a pancreatic cancer mouse model significantly reduces the viability of pancreatic cancer cells (C. Zhou *et al.*, 2019; Wang *et al.*, 2024).

Approximately about 78% of PDAC cases show mutations in cell cycle regulatory genes (Khan *et al.*, 2017). *TP53,* a vital regulator of the cell cycle, is one of the genes that has been identified to have mutations in 70% of PDAC cases, making it a significant contributor to the development of PDAC (Meireles Da Costa *et al.*, 2021). *CDK1* is another gene that plays a critical role in promoting the progression of PDAC cells through the cell cycle (Lim and Kaldis, 2013). The CDKs, including *CDK1*, are vital in cell-cycle regulation and transcriptional elongation, and their dysregulation has been implicated in the progression of various cancers, including PDAC (García-Reyes *et al.*, 2018). The G1/S specific transcription factors that were found to be upregulated despite *SPP1* knockdown, indicates different avenues by which MIA PaCa-2 cells can continue driving growth.

CDK1 regulates mitosis entry and progression, as well as collaborates with cyclins like D1, E, and A to manage the progression of the G1 phase and S phase transition (Novak *et al.*, 2007). In response to stress or DNA damage, *TP53* inhibits *CDK1* signalling, which prevents cancerous cell growth through apoptosis. However, overexpression of *CDK1* can promote cell replication with DNA abnormalities, contributing to cancer cell proliferation. It has been established that the activity of CDK1 is essential for the development of tumours (García-Reyes *et al.*, 2018). The expression of *CDK1* genes is significantly higher in tumour cells of PDAC patients, indicating advanced stages of PDAC and poorer survival outcomes (Dong *et al.*, 2019). The use of dinaciclib to inhibit *CDK1* is a promising strategy to overcome acquired resistance to pancreatic tumour immunity induced by IFNG (Huang *et al.*, 2021; Silva *et al.*, 2024). Further investigation is required to elucidate the precise role of *CDK1* in PDAC cells and to assess its potential as a therapeutic target.

4.5.3 Combination of *SPP1* knockdown and gemcitabine treatment induce pathways that may promote cell death

Proteomic analysis conducted following gemcitabine treatment resulted in the downregulation of negative regulation of NOTCH4 signalling, leading to increased activation of the pathway associated with tumour progression. Furthermore, *SPP1* knockdown resulted in the upregulation of cell cycle-related genes, including *TK1, TYMS, CDK1,* and *TOP2A,* indicating *CDK1*'s role in PDAC development by promoting cell cycle progression and inhibiting apoptosis. The combination of *SPP1* knockdown and gemcitabine treatment revealed an increase in the expression of proteins involved in collagen biosynthesis, a decrease in Toll-like receptor (TLR) cascades and a downregulation of transcriptional regulators of tumour suppressor protein, TP53. These findings highlight potential therapeutic targets in PDAC and emphasize the intricate interplay of signalling pathways in disease progression.

TLR appear to exhibit a dual function, as they can both promote and inhibit the progression of PDAC (Vaz and Andersson, 2014). TLR7 and TLR9 stimulation in either stromal or transformed epithelial cells has been reported to accelerate tumour progression (Ochi *et al.*, 2012; Zambirinis *et al.*, 2015). Studies have shown a significant increase in the expression of TLR2, TLR4, and TLR9 in PDAC (Grimmig *et al.*, 2016; Nurmi *et al.*, 2022; Topcu *et al.*, 2022). Our study found that the TLR signalling pathway's activity was downregulated, which may hinder PDAC cell growth by reducing inflammatory markers and promoting apoptosis, as shown by Ajay et al. (Ajay *et al.*, 2024). The combinational treatment of *SPP1* knockdown and gemcitabine treatment could limit PDAC tumour growth by decreasing TLR 7 and TLR 9, which are involved in tumour development.

Another crucial finding is the downregulation of transcriptional regulators of tumour suppressor protein, TP53. Amongst the key proteins enriching this pathway is GATAD2B. GATAD2B serves as an essential component of the Methyl-CpG binding proteins (MeCP1) complex, which regulates gene expression by selectively binding to, remodelling, and deacetylating methylated nucleosomes. Moreover, it promotes the relocation of MBD3 protein to specific nuclear domains (Brackertz *et al.*, 2002; Feng *et al.*, 2002; Fujitomo *et al.*, 2014). GATAD2B has been found to induce tumour growth and metastasis in lung cancer specifically those driven by *KRAS* (Grzeskowiak *et al.*, 2018). Studies have shown that approximately 90% of PDAC have *KRAS* mutations and are driven by them (Stephen *et al.*, 2014; Buscail, Bournet and Cordelier, 2020). A downregulation of GATAD2B may suggest an interplay of anti-tumour

mechanisms that may partly explain the phenotype observed during a combined treatment of *SPP1* knockdown and gemcitabine treatment.

Important to keep in mind are findings from the previous Chapter which highlighted that under treatment whereby approximately 24% of the cells were undergoing early apoptosis, and about 75% of the cells were still cycling. Possibly, with a longer duration in culture, suggesting the induction of later events in cell death; however, the results obtained indicate that surviving cells can downregulate proteins involved in cell death and upregulate pathways associated with metabolism and modulation of the tumour microenvironment for tumour progression. These results highlight cancer cells' adaptability to their environment (Xulu, Nweke and Augustine, 2023). This heterogeneity may lead to the emergence of different subtypes of cancer (intratumuoral heterogeneity) with distinct characteristics, making treatment challenging. Metabolism may potentially occur as cells attempt to enhance the efficacy of gemcitabine, while a smaller fraction may engage in processes facilitating adaptation. Combination of *SPP1* knockdown and gemcitabine treatment might be using compensatory mechanisms to overcome the effects of both the chemotherapy and *SPP1* knockdown.

The finding of upregulated pathways associated with increased collagen biosynthesis and extracellular matrix remodelling is of particular interest. PDAC is characterized by the presence of a dense stroma composed of rapidly proliferating myofibroblasts, also known as pancreatic stellate cells, as well as the accumulation of extracellular matrix components, including type I collagen, hyaluronic acid, and other extracellular matrix components (Ryan, Hong and Bardeesy, 2014). Collagen is the most abundant ECM protein, which is responsible for forming fibrils that support cell development and contribute to the mechanical strength of connective tissues (Arseni, Lombardi and Orioli, 2018). Collagen has been shown to increase tumour tissue stiffness, regulate immunity, and promote metastasis (Whatcott *et al.*, 2015; Yamauchi *et al.*, 2018). In our study, we discovered that genes belonging to the PLOD (1, 2 and 3) family exhibited significantly elevated expression levels in PDAC. This is consistent with the studies by Zhang et al. where PLOD family genes were primarily found to control collagen metabolism and extracellular matrix composition (Zhang *et al.*, 2022). Jiang et al. uncovered that PLOD1 influences the proliferation and invasion of osteosarcoma cells by modulating the Wnt/β-catenin signalling pathway (Jiang *et al.*, 2020). In PDAC, increased expression of PLOD2 in hypoxic conditions may enable the migration of cancer cells, thereby contributing to the progression of the tumour (Sada *et al.*, 2016). The upregulation of PLOD3 levels was found to substantially enhance the migratory and invasive capabilities of cancer cells in both glioma and

lung cancer (Baek *et al.*, 2018; Tsai *et al.*, 2018). While the precise function of PLODs in the development of PDAC is not fully understood, it infers that these proteins may be involved in enhancing tumour progression.

The enzyme P4HA2, which is vital for collagen synthesis, has been established as a dependable predictor of the progression of various malignancies (Wang *et al.*, 2019). Research conducted by Long et al. revealed that the four genes *P4HA2, ITGA5, MMP9* and *SPP1*, which are related to p-EMT, were overexpressed in HCC tissues compared to normal liver tissues. Additionally, the mRNA expression levels of all four genes were found to be associated with the prognosis of patients with HCC (Long *et al.*, 2018).

SPP1 has been implicated in facilitating the proliferation of liver cancer cells, but its influence on EMT in gastric cancer (GC) cells remains unexplored (Dong *et al.*, 2016). Previous research has indicated that *SPP1* stimulates EMT in liver cancer by enhancing the stability of vimentin. However, immunohistochemical (IHC) assessment revealed that vimentin was not expressed in GC cells. This suggests that *SPP1* does not regulate EMT through vimentin in GC cells. Studies by Liu at al. showed that the *SPP1-HER2* connection implied the involvement of *SPP1* in the *HER2*-mediated EMT process. However, the knockdown of *SPP1* did not impact *HER2* expression. Conversely, EMT-related genes: E-cadherin was elevated at the protein level, while N-cadherin and twist were reduced (Yalan Liu *et al.*, 2021). EMT is characterized by three primary types, each exhibiting distinct characteristics. Type I, which is a natural physiological process, serves a specific purpose in the body. Type II, on the other hand, is triggered by environmental toxins. Lastly, Type III promotes the spread of tumours by inducing angiogenesis, a process that stimulates the growth of new blood vessels (Stone *et al.*, 2016; Zhou *et al.*, 2020).

This study offers novel insights into the role and mechanisms of *SPP1* in PDAC cells by demonstrating that *SPP1* might influence the progression of ECM through the upregulation of collagen-related genes. Therefore, this study suggests that *SPP1* and collagen-related genes might be knocked down respectively in PDAC cell lines to explore their roles.

Conclusions

Proteomic analysis of MIA PaCa-2 cells revealed that PDAC cells can undertake compensatory mechanisms to ensure cell survival despite *SPP1* knockdown and gemcitabine treatment. The upregulation of pathways associated with cellular metabolism, dysregulation of Notch signalling and cell cycle transition highlights that cells that did not succumb to early apoptosis,

which were the vast majority as highlighted in the previous Chapter, adapted to the individual insult of *SPP1* knockdown or gemcitabine treatment. Of particular interest under combined therapy, was the downregulation of toll-like receptor pathway and the transcriptional regulators of TP53. Although there was an upregulation of proteins involved in extracellular matrix reorganisation and collagen biosynthesis. This finding cautions that despite the finding of functionally reduced migration, which may be explained by the downregulation of some key tumour-promoting pathways there are induced compensatory mechanisms known to enhance tumour heterogeneity and promote invasive capacity over a longer duration. This highlights the need for further studies in models such as mice investigating issues such as dosage and duration of treatment.

CHAPTER 5: OVERALL DISCUSSION

PDAC represent almost 90% of all pancreatic cancers (Skorupan *et al.*, 2023). A limited number of patients qualify for potentially curative surgery, with a considerable proportion of those undergoing surgical resection encountering recurrence within two years, with an anticipated recurrence rate of up to 97% (Groot *et al.*, 2018). Moreover, pancreatic cancer cells appear to be especially resistant to chemotherapy (Lowenfels and Maisonneuve, 2005).

The present study sought to determine differentially expressed genes in pancreatic tumours obtained from South African patients using pathway-specific RT^2 Profiler PCR arrays. One main limitation of this study was the small sample size of tissue samples; nevertheless, pathway analysis highlighted the significance of cytokine signalling pathways and their intersection with major signal transduction pathways in PDAC. Communication in the TME, and thus immune evasion and tumour progression, are dependent on cytokine signalling (Bhatia *et al.*, 2022). IL-4 and IL-13 activity, pathways of which were upregulated substantively in the present study, in PDAC are strongly associated with tumourigenesis and metastasis (Kornmann *et al.*, 1999; Gabitass *et al.*, 2011) In the present study the upregulation of genes associated with cell cycle (including cyclin d1, cyclin d2, PCNA and and BCL2L1) (Loo *et al.*, 2020), also highlighted PDACs aggressiveness by illustrating the intricate interplay between pushing cells through the cell cycle, avoiding the induction of apoptosis, and ensuring survival (Vahabi *et al.*, 2023).

These parameters were identified in the second part of the study, wherein, *SPP1*, which was found to be highly upregulated in PDAC patient samples compared to adjacent normal tissue, was knocked down in MIA PaCa-2, to understand its functional relevance.

SPP1 is a dual-functioning protein, serving both normal physiological functions and promoting the progression of multiple cancer types (Luo *et al.*, 2015). This is achieved through various mechanisms, such as enhancing cell migration, adhesion, and invasion, as well as promoting angiogenesis and inhibiting apoptosis. *SPP1*'s ability to enhance these processes is thought to

be due to its ability to induce EMT, autophagy, aberrant glucose metabolism, epigenetic modifications, and reduced drug absorption (Hao, Lane and Jiang, 2023; Matsubara *et al.*, 2023). In the present study, knockdown of *SPP1* combined with gemcitabine treatment resulted in significant inhibition of cell proliferation, migration, and induction of early apoptosis in PDAC cells. The results of this study suggest that *SPP1* knockdown can be used to enhance the effectiveness of chemotherapy drugs, particularly gemcitabine by triggering pathways involved in early apoptosis and reducing the migratory capacity of PDAC cells.

Gemcitabine is currently employed as the first-line chemotherapeutic agent for PDAC; however, PDAC cells become resistant to chemotherapy (Lowenfels and Maisonneuve, 2005) and also have a high rate of recurrence (Groot *et al.,* 2018), suggesting that intratumoural heterogeneity and evolutionary mechanisms for survival may be major players in PDAC (Xulu, Nweke and Augustine, 2023). Despite the proportion of cells undergoing early apoptosis and the decrease in migratory capacity viusalised at 48hours, further proteomic analysis of the effects of chemotherapy and *SPP1* knockdown revealed induced compensatory mechanisms for survival. This includes upregulation of both cell cycle/mitotic and metabolism pathways. The expression profiling data following *SPP1* knockdown indicated an up-regulation of genes related to cell cycle (*TK1, TYMS, CDK1, TOP2A*), and *CDK1* is thought to contribute to the development of PDAC by facilitating cell cycle progression and resisting apoptosis (Wijnen *et al.*, 2021). No genes were found to be downregulated following the downregulation of *SPP1*. Furthermore, gemcitabine treatment resulted in the downregulation of the negative regulation of NOTCH 4 signalling, which ultimately led to an increase in the activation of the NOTCH 4 pathway, which is correlated with tumour progression (Gao, Long and Wang, 2017). Moreover, additional research is necessary to comprehend the precise mechanisms through which the upregulation of the collagen biosynthesis pathway occurs upon the combination of *SPP1* knockdown and gemcitabine treatment. This upregulation may contribute to the promotion of migration and metastasis, both processes of which are associated with recurrence, and further investigation is required to fully elucidate these processes.

The current study indicated that the downregulation of *SPP1* through siRNA, in conjunction with gemcitabine treatment in MIA PaCa-2 PDAC cells, significantly inhibits various hallmark traits associated with PDAC. It can be suggested that the two treatments have a synergistic effect as shown in Figure 5.1, as a significant reduction in cell proliferation, an increase in apoptosis, and a decrease in migration capacity were observed over 48 hours. Additionally, the findings from the proteomic analysis conducted following combinatorial treatment indicated

the upregulation of several proteins involved in the collagen biosynthesis pathway and the downregulation of the Toll-like receptor cascades and transcriptional regulators of TP53. The collagen biosynthesis pathway plays a significant role in regulating the migration of cancer cells and increased expression of PLOD2 in hypoxic conditions may enable the migration of cancer cells, thereby contributing to the progression of the tumour (Zhang *et al.*, 2022). Collagen upregulation and extracellular matrix remodelling are important parameters of the tumour microenvironment that play a role in cell migration and therapeutic resistance (Öhlund *et al.*, 2017; Jena *et al.*, 2020).

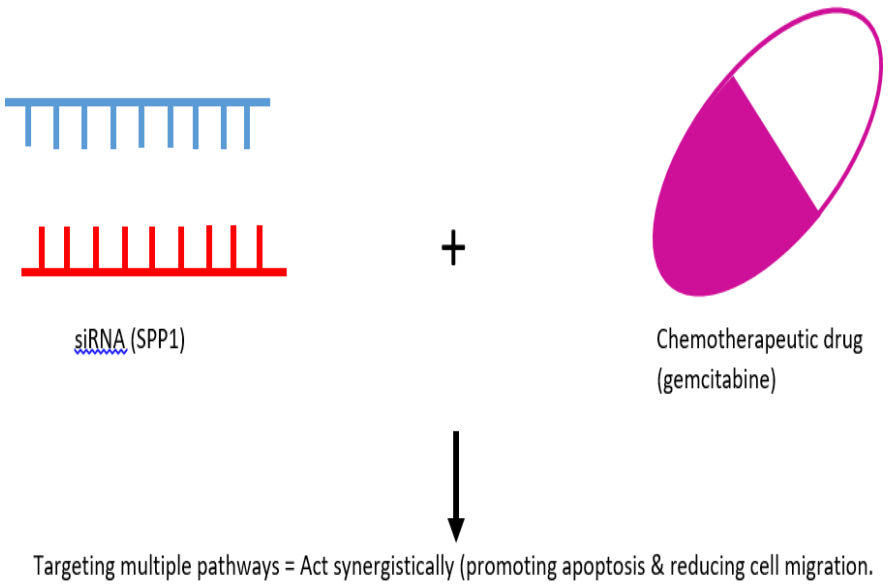


Figure 5.1: siRNA knockdown and gemcitabine treatment. Combining *SPP1* knockdown with conventional chemotherapy used for PDAC, gemcitabine, resulted in a synergistic effect, leading to an enhanced early apoptotic response.

The results of this study have thus unpacked major pathways and genes involved in PDAC; however, while identifying *SPP1* as a driver of PDAC, its knockdown while able to enhance response to chemotherapy *in vitro* requires further investigation *in vitro* and in *in vivo* animal models to ascertain whether its effects can limit PDAC progression overall, or provides an avenue for tumour cells to adapt their responses and initiate compensatory mechanisms for survival.

CHAPTER 6: STUDY LIMITATIONS

The primary limitation of this study was the small number of tissue samples, likely resulting from patients presenting at advanced disease stages. Consequently, further research with a larger cohort is necessary.

Lastly, due to budget constraints, tissue samples were pooled for the Pathway-Focused PCR assays.

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SUPPLEMENTARY TABLES

Supplementary table 1: RNA concentration of samples used in the validation of gene targets

Sample	Concentration (ng/µL)	500 ng/ µL volume
N1	378.7	1.32
N2	451.7	1.10
N3	520.9	0.95
N4	3551.3	0.14
N5	3758.7	0.13
N6	907.5	0.56
N7	2117	0.24
N8	3791.2	0.13
N9	204	2.45
N10	2388.8	0.21
N11	3822.9	0.16
N12	846	0.59
N13	751.8	0.67
N14	3352.4	0.15
N15	423.9	1.18
T1	393	1.27
T2	232	2.15
T3	1149.7	0.43
T4	1275.5	0.39
T5	1220.1	0.41
T6	132.4	3.78
T7	499	1
T9	228.8	2.19
T10	3789.4	0.18
T11	2431	0.21
T12	322.3	1.55
T13	3616.6	0.14
T14	554.6	0.90
T15	187.3	2.66

Notes: Sample ID- N: Corresponding normal tissue sample; T: Tumour samples.

Supplementary Table 2: Volumes and calculations of PCR Master Mix and Set up for PCR targets in normal and tissue samples.

Component	Reaction/ Volume (µl)	Reaction/ Volume Xn* (+1)
TaqMan Gene expression Master Mix (2X)	5	*5n*+1
Taqman Assay (20X, Primers)	0.5	*0.5n*+1
Reference gene	1	*1n*+1
Nuclease free water	3.5	*6.5n*+1
Total per reaction	10	*8n*+1

Notes: Real-Time PCR setup.

Supplementary Table 3: Volumes and calculations of PCR Master Mix and Set up for PCR targets in normal and tissue samples.

	Incubation	Polymerase activation	PCR (40 cycles)	PCR (40 cycles)
	Hold	Hold	Denature	Anneal/Extend
Temperature	50ºC	95ºC	95ºC	60ºC
Time	2 minutes	10 minutes	15 seconds	1 minute

Notes: PCR set up (cycling conditions).

Supplementary Table 4(a): List of genes and fold change (Growth factors upregulated).

Gene symbol	Description	Fold regulation
B2M	Beta-2-microglobulin	190.39
RPLP0	Ribosomal protein, large, P0	122.00
SPP1	Secreted phosphoprotein 1	73.73
JAG1	Jagged 1	60.65
ACTB	Actin, beta	41.30
TYMP	Thymidine phosphorylase	39.44
INHBA	Inhibin, beta A	33.11
IGF1	Insulin-like growth factor 1 (somatomedin C)	32.42
PTN	Pleiotrophin	28.32
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	16.74
PGF	Placental growth factor	11.95
CSF1	Colony stimulating factor 1 (macrophage)	10.30
FGF7	Fibroblast growth factor 7	9.42
GPI	Glucose-6-phosphate isomerase	8.31
TGFB1	Transforming growth factor, beta 1	8.15
PDGFC	Platelet derived growth factor C	7.96
FGF14	Fibroblast growth factor 14	6.71
BMP1	Bone morphogenetic protein 1	6.58
VEGFA	Vascular endothelial growth factor A	6.34
FGF1	Fibroblast growth factor 1	6.04
MSTN	Myostatin	5.70
FGF2	Fibroblast growth factor 2 (basic	5.67
GDF11	Growth differentiation factor 11	5.41
NTF3	Neurotrophin 3	5.33
IL18	Interleukin 18 (interferon-gamma-inducing factor	4.90
FGF9	Fibroblast growth factor 9	4.89
TNNT1	Troponin T type 1 (skeletal, slow	4.59
IL3	Interleukin 3 (colony-stimulating factor, multiple)	4.36
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	4.28
BMP5	Bone morphogenetic protein 5	4.12
BMP4	Bone morphogenetic protein 4	3.71
CECR1	Cat eye syndrome chromosome region, candidate 1	3.63
BMP7	Bone morphogenetic protein 7	3.62
ERAP1	Endoplasmic reticulum aminopeptidase 1	3.49
FGF13	Fibroblast growth factor 13	3.48
IGF2	Insulin-like growth factor 2 (somatomedin A)	3.40
NRG1	Neuregulin 1	3.22
HBEGF	Heparin-binding EGF-like growth factor	2.92
NODAL	Nodal homolog (mouse)	2.81
MDK	Midkine (neurite growth-promoting factor 2)	2.81
LTBP4	Latent transforming growth factor beta binding protein 4	2.81
IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic	2.81
FGF19	Fibroblast growth factor 19	2.81
BMP2	Bone morphogenetic protein 2	2.81
AMH	Anti-Mullerian hormone	2.81
BMP3	Bone morphogenetic protein 3	2.49
NRG3	Neuregulin 3	2.44
CSF3	Colony stimulating factor 3 (granulocyte)	2.35

Supplementary Table 4(b): List of genes and fold change (Growth factors downregulated).

Gene symbol	Description	Fold regulation
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	-7802.06
HGDC	Human Genomic DNA Contamination	-6.84
GDF10	Growth differentiation factor 10	-3.86
IL2	Interleukin 2	-3.29
THPO	Thrombopoietin	-3.10
TDGF1	Transforming growth factor, beta 1	-3.04
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-2.63
BMP10	Bone morphogenetic protein 10	-2.36
IL1B	Interleukin 1, beta	-2.32
NRG2	Neuregulin 2	-2.09

Supplementary Table 5(a): List of genes and fold change (Signal transduction factors upregulated).

Gene symbol	Description	Fold regulation
HMOX1	Heme oxygenase (decycling) 1	261.74
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related	162.02
ADM	Adrenomedullin	91.77
B2M	Beta-2-microglobulin	68.93
RPLP0	Ribosomal protein, large, P0	65.21
ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67)	45.73
ACSL4	Acyl-CoA synthetase long-chain family member 4	26.06
FTH1	Ferritin, heavy polypeptide 1	25.32
BCL2	B-cell CLL/lymphoma 2	19.13
CCL5	Chemokine (C-C motif) ligand 5	18.77
ACTB	Actin, beta	17.98
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	16.61
HES1	Hairy and enhancer of split 1, (Drosophila)	14.94
JAG1	Jagged 1	14.57
SQSTM1	Sequestosome 1	14.53
CCND1	Cyclin D1	14.31
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	12.91
CA9	Carbonic anhydrase IX	12.89
EGFR	Epidermal growth factor receptor	12.59
HERPUD1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	12.43
HEY1	Hairy/enhancer-of-split related with YRPW motif 1	11.93
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	11.00
TXN	Thioredoxin	10.43
NQO1	NAD(P)H dehydrogenase, quinone 1	10.20
ICAM1	Intercellular adhesion molecule 1	9.90
CCND2	Cyclin D2	8.83
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	8.08
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1	8.06
BAX	BCL2-associated X protein	7.96
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	7.38
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	7.06
GADD45B	Growth arrest and DNA-damage-inducible, beta	6.67
GCLC	Glutamate-cysteine ligase, catalytic subuni	6.08
PPARD	Peroxisome proliferator-activated receptor delta	5.87
ACSL5	Acyl-CoA synthetase long-chain family member 5	5.80
VEGFA	Vascular endothelial growth factor A	5.54
FOSL1	FOS-like antigen 1	5.40
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	5.32
ARNT	Aryl hydrocarbon receptor nuclear translocator	5.10
BCL2A1	BCL2-related protein A1	5.07
EMP1	Epithelial membrane protein 1	4.98
HEYL	Hairy/enhancer-of-split related with YRPW motif-like	4.92
LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	4.25
STAT1	Signal transducer and activator of transcription 1, 91kDa	4.11
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila	4.08
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	3.94
LRG1	Leucine-rich alpha-2-glycoprotein 1	3.91
TXNRD1	Thioredoxin reductase 1	3.44
PCNA	Proliferating cell nuclear antigen	2.81
BCL2L1	BCL2-like 1	2.78
CPT2	Carnitine palmitoyltransferase 2	2.72
WISP1	WNT1 inducible signaling pathway protein 1	2.71
LDHA	Lactate dehydrogenase A	2.70
IRF1	Interferon regulatory factor 1	2.68
SORBS1	Sorbin and SH3 domain containing 1	2.54
GADD45A	Growth arrest and DNA-damage-inducible, alpha	2.10

Supplementary table 5(b): List of genes and fold change (Signal transduction factors downregulated).

Gene symbol	Description	Fold regulation
HEY2	Hairy/enhancer-of-split related with YRPW motif 2	-5.66
FABP1	Fatty acid binding protein 1, liver	-4.50
EPO	Erythropoietin	-3.71
TNF	Tumor necrosis factor	-3.65
HGDC	Human Genomic DNA Contamination	-3.57
GATA3	GATA binding protein 3	-3.49
OLR1	Oxidized low density lipoprotein (lectin-like) recepto	-3.17
IFNG	Interferon, gamma	-2.96
WNT6	Wingless-type MMTV integration site family, membe	-2.70
NOTCH1	Notch 1	-2.09

Supplementary table 6: Dysregulated protein list in Gemcitabine treatment cells compared to untreated cells

Protein Group	Protein Name	Average Log_2 Ratio	p-value	q-value
Q92743	HTRA1	6,36075094	0,001983194	0,001295509
P04183	KITH	4,993683485	5,08E-08	3,75E-07
Q14807	KIF22	4,724610813	0,002410518	0,001520709
Q9Y3S1	WNK2	4,641889483	0,006610611	0,003575573
Q53GQ0	DHB12	3,72755852	0,001662164	0,001114653
Q9HCL2	GPAT1	3,704508082	0,00046688	0,000390628
P02768	ALBU	3,641947395	2,20E-05	3,62E-05
P11388	TOP2A	3,511741934	1,50E-05	2,71E-05
P05109	S10A8	3,492923744	0,000121449	0,000134112
P06702	S10A9	3,242595926	0,00287781	0,001765379
Q8WVX9	FACR1	3,237677925	1,73E-05	3,00E-05
Q14643	ITPR1	3,210988628	1,74E-05	3,02E-05
P68871	HBB	3,205508911	7,38E-05	9,10E-05
Q9BTV4	TMM43	2,982659383	9,64E-06	1,91E-05
Q16799	RTN1	2,965335993	4,69E-05	6,46E-05
Q96C36	P5CR2	2,910110587	2,10E-05	3,49E-05
Q9HD42	CHM1A	2,86205827	0,002103574	0,001361097
Q969G5	CAVN3	2,814139946	0,012221675	0,006013747
Q8N4H5	TOM5	2,778469482	0,001167674	0,000833645
O75027	ABCB7	2,76540812	6,44E-05	8,21E-05
O75844	FACE1	2,759042326	0,000238232	0,000230017
O60306	AQR	2,754341743	4,15E-05	5,91E-05
Q9NRG9	AAAS	2,722692449	4,47E-07	1,86E-06
Q8N766	EMC1	2,704603254	3,79E-09	6,01E-08
Q96IX5	ATPMK	2,672531565	0,000481985	0,000401539
Q16647	PTGIS	2,671225009	0,000600718	0,000482274
P48723	HSP13	2,668769132	0,000377294	0,000330699
Q7Z7H5	TMED4	2,638968994	4,97E-06	1,17E-05
P39656	OST48	2,633903839	7,51E-09	9,73E-08
P51571	SSRD	2,633020862	3,03E-07	1,38E-06

Supplementary table 7: Dysregulated protein list in SPP1 knockdown treated cells compared to untreated cells

Protein Group	Protein Name	Average Log_2 Ratio	p-value	q-value
Q92743	HTRA1	2,024162353	0,000556759	0,000452671
P09486	SPRC	1,764590583	2,63E-08	2,43E-07
O95573	ACSL3	1,69381142	1,15E-05	2,22E-05
P60520	GBRL2	1,561508453	4,23E-05	6,01E-05
Q8IVF2	AHNK2	1,538202295	1,51E-06	4,70E-06
Q9Y6Y0	NS1BP	1,528844793	0,000370061	0,000325615
P23921	RIR1	1,516365611	1,82E-05	3,12E-05
P06493	CDK1	1,505854963	3,91E-07	1,68E-06
Q9NR19	ACSA	1,497225897	4,30E-06	1,04E-05
P11388	TOP2A	1,486241943	1,69E-05	2,95E-05
P48449	LSS	1,40371772	0,000136154	0,00014686
Q8WVX9	FACR1	1,340778598	1,62E-08	1,67E-07
P53602	MVD1	1,31977847	0,000208073	0,000206758
P15291	B4GT1	1,274292259	0,000171598	0,000176876
Q6ZRY4	RBPS2	1,22237874	2,38E-06	6,80E-06
Q9UBP9	GULP1	1,198399689	0,000449621	0,000379136
Q92896	GSLG1	1,197434339	1,68E-06	5,10E-06
P40763	STAT3	1,17149528	2,80E-05	4,38E-05
Q01581	HMCS1	1,116856904	5,28E-08	3,85E-07
O95479	G6PE	1,102566544	1,80E-07	9,57E-07
P02751	FINC	1,093568904	8,66E-07	3,08E-06
Q8WZ42	TITIN	1,05965208	0,020437116	0,009303667
P41212	ETV6	1,056967876	7,61E-05	9,31E-05
P49327	FAS	1,054834111	3,98E-05	5,73E-05
P04183	KITH	1,020011342	2,22E-12	3,03E-10
P04818	TYSY	1,009068452	3,98E-11	2,62E-09
Q9H1A3	METL9	1,000571196	2,21E-05	3,63E-05
Q99541	PLIN2	-1,038527615	1,35E-05	2,49E-05
Q5TC12	ATPF1	-1,049675738	0,001213023	0,000861209
P27105	STOM	-1,174828889	2,06E-08	2,04E-07
P68871	HBB	-1,184873902	0,001300475	0,000910021
Q07817	B2CL1	-1,252029237	0,005384914	0,003024667
O75976	CBPD	-1,411734705	3,28E-08	2,85E-07
P17275	JUNB	-1,416891471	9,01E-08	5,69E-07
Q9H0Q0	CYRIA	-1,435622725	0,000373005	0,000327567
P02794	FRIH	-1,452809181	1,09E-06	3,72E-06
P17301	ITA2	-1,457350715	0,003634749	0,002150963
P02538	K2C6A	-1,696169557	0,006907277	0,003713433
P19823	ITIH2	-1,701850852	0,022144541	0,009983114
P02787	TRFE	-2,466018866	1,51E-06	4,70E-06

Supplementary table 8: Dysregulated protein list for the combination of gemcitabine and SPP1 knockdown treatment compared to untreated cells.

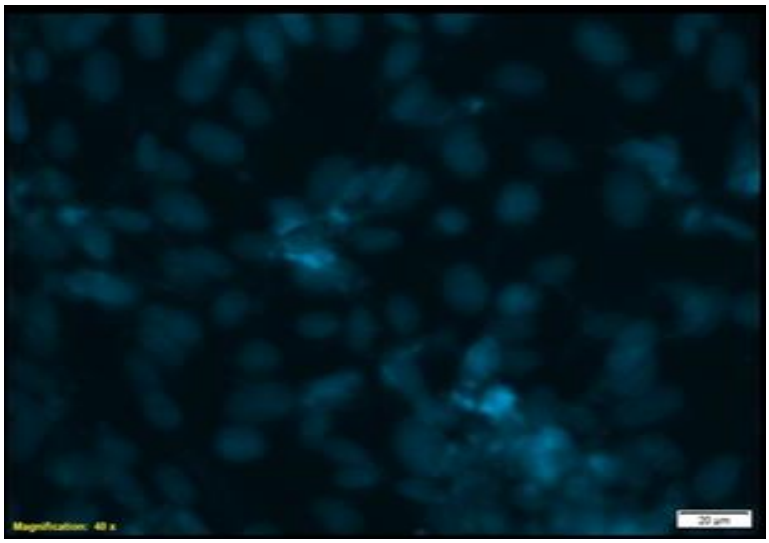
Protein Groups	Protein Name	Average Log_2 Ratio	p-value	q-value
Q92743	HTRA	4,660691077	0,001028165	0,0007514
P04183	KITH	3,703863075	4,62E-09	6,74E-08
P02768	ALBU	2,990881771	8,88E-07	3,14E-06
Q9Y3S1	WNK2	2,867061728	2,43E-05	3,90E-05
P02751	FINC	2,407851242	0,000277797	0,0002586
P11388	TOP2	2,207524978	2,31E-07	1,13E-06
Q16799	RTN1	2,207366162	7,04E-06	1,52E-05
P48449	LSS_	2,165981151	0,000331015	0,0002994
Q8N4H5	TOM5	2,053320967	0,003483892	0,0020731
Q8WVX9	FACR	1,934024425	2,92E-06	7,92E-06
Q9NR19	ACSA	1,924470323	8,72E-06	1,78E-05

Q96HE7	ERO1	1,919120775	0,001301734	0,0009106
O95573	ACSL	1,909870546	7,39E-05	9,10E-05
P68871	HBB_	1,893064462	0,002138989	0,0013788
P55290	CAD1	1,868186745	7,04E-07	2,64E-06
P23141	EST1	1,855482505	0,000762334	0,0005884
O00469	PLOD	1,84853244	0,000209513	0,0002078
Q92896	GSLG	1,798989661	5,02E-08	3,73E-07
O95479	G6PE	1,796612313	2,04E-07	1,04E-06
Q9UBM7	DHCR	1,777061587	5,69E-05	7,47E-05
O15460	P4HA	1,743364179	3,22E-06	8,52E-06
Q96DZ1	ERLE	1,741975541	3,18E-05	4,82E-05
P69905	HBA_	1,723945184	5,17E-05	6,94E-05
Q8NE86	MCU_	1,71775822	0,001063125	0,0007725
Q02809	PLOD	1,664489608	2,37E-10	8,51E-09
Q8IXB1	DJC1	1,622043234	1,86E-07	9,74E-07
Q14643	ITPR	1,597050533	1,22E-05	2,32E-05
Q9HDC9	APMA	1,587223444	1,75E-07	9,41E-07
Q8IVF2	AHNK	1,536528197	0,000903698	0,0006764
Q8NBJ5	GT25	1,514673395	1,93E-05	3,24E-05
P07237	PDIA	1,445956932	1,43E-05	2,61E-05
Q14165	MLEC	1,444277308	8,93E-05	0,0001051
Q969G5	CAVN	1,436494289	0,000601377	0,0004826
P33992	MCM5	1,419895259	5,20E-06	1,21E-05
P27797	CALR	1,379524531	5,51E-05	7,31E-05
Q9BUE6	ISCA	1,358436247	7,54E-05	9,24E-05
P39656	OST4	1,354906272	4,17E-08	3,41E-07
Q13423	NNTM	1,354351386	2,38E-06	6,80E-06
Q9Y2I1	NISC	1,34835604	0,000103383	0,0001186
O15260	SURF	1,344499343	0,000131363	0,0001432
Q9Y6Y0	NS1B	1,330302959	0,003334182	0,0019962
P51571	SSRD	1,326206559	2,49E-05	3,98E-05
Q53GQ0	DHB1	1,326113647	5,89E-05	7,66E-05
P40763	STAT	1,313304964	0,00020658	0,0002055
O60568	PLOD	1,309314677	3,33E-05	4,98E-05
Q96AY3	FKB1	1,309058502	6,78E-06	1,48E-05
Q8IY17	PLPL	1,275367517	9,01E-06	1,82E-05
Q92544	TM9S	1,270314479	0,000112934	0,0001266
Q9NYU2	UGGG	1,268966105	9,09E-08	5,70E-07
P18858	DNLI	1,26859082	0,001124914	0,0008101
P11166	GTR1	1,268341407	0,000581805	0,0004697
P31040	SDHA	1,262275106	3,30E-08	2,86E-07
Q9H1A3	METL	1,261690629	0,000148296	0,0001575
P49327	FAS_	1,259758236	4,88E-06	1,16E-05
P46977	STT3	1,252879022	1,23E-06	4,05E-06
Q15363	TMED	1,248891488	0,000896849	0,000672
P25205	MCM3	1,248423168	5,28E-05	7,06E-05
Q6ZXV5	TMTC	1,233991808	3,90E-05	5,63E-05
Q13217	DNJC	1,227511691	0,000733815	0,0005696
Q9UGP8	SEC6	1,226802306	1,27E-05	2,38E-05
P43307	SSRA	1,213556348	4,32E-07	1,82E-06
P13807	GYS1	1,211387086	1,82E-07	9,60E-07
Q12769	NU16	1,190592289	9,32E-05	0,000109
Q9BUN8	DERL	1,184606248	0,000225917	0,0002201
P04844	RPN2	1,184019706	4,13E-05	5,89E-05
P06493	CDK1	1,174091454	4,10E-09	6,20E-08
O75027	ABCB	1,165639859	0,000299492	0,000275
Q96RP9	EFGM	1,159147576	1,30E-08	1,42E-07
P06396	GELS	1,158801943	0,000588781	0,000474
Q9NRG9	AAAS	1,150007382	5,59E-05	7,39E-05
Q9Y223	GLCN	1,147398095	6,13E-05	7,89E-05
P45880	VDAC	1,141169833	6,29E-09	8,52E-08
Q8N2K0	ABD1	1,139441723	7,32E-05	9,05E-05
Q9Y2H1	ST38	1,138001545	6,65E-05	8,39E-05
Q9HCL2	GPAT	1,136722502	0,003567581	0,002118
Q969H8	MYDG	1,132131325	0,002353707	0,0014914
Q9H0U3	MAGT	1,12953473	8,81E-08	5,58E-07
P46976	GLYG	1,119003918	0,000154487	0,0001628
P54709	AT1B	1,111711458	0,000654924	0,0005193
Q9BTV4	TMM4	1,105905142	3,43E-13	8,13E-11

Q13643	FHL3	1,104523446	0,014911503	0,0071363
P06756	ITAV	1,09763448	0,000722546	0,0005637
Q14566	MCM6	1,096186735	3,56E-06	9,02E-06
P49755	TMED	1,094917874	0,000104692	0,0001195
P21796	VDAC	1,094217848	1,42E-09	2,94E-08
Q13724	MOGS	1,0894588	0,004999803	0,0028317
Q7Z7H5	TMED	1,088025931	4,65E-08	3,63E-07
Q9NZM1	MYOF	1,08775237	2,19E-07	1,09E-06
P14625	ENPL	1,085283131	2,29E-07	1,13E-06
P33993	MCM7	1,078705416	2,58E-06	7,25E-06
Q9NQL2	RRAG	1,078423384	0,00162028	0,0010937
P15291	B4GT	1,075857572	0,000443145	0,0003744
O43292	GPAA	1,068430859	1,20E-05	2,29E-05
P05386	RLA1	1,065390947	0,001663686	0,0011153
Q9Y4L1	HYOU	1,059387458	1,03E-06	3,54E-06
Q12789	TF3C	1,055756849	0,000111847	0,0001258
O94919	ENDD	1,05254701	5,09E-05	6,86E-05
Q9HD42	CHM1	1,050561798	0,00377191	0,0022186
Q9UBP9	GULP	1,048520135	0,008461383	0,0043858
Q9NRP0	OSTC	1,045789697	1,45E-06	4,59E-06
Q9NZJ7	MTCH	1,030526136	1,42E-05	2,61E-05
Q13085	ACAC	1,028924914	0,00048823	0,0004062
Q9H857	NT5D	1,026857094	2,04E-05	3,40E-05
P04843	RPN1	1,023356291	5,46E-06	1,26E-05
P13674	P4HA	1,020206155	5,57E-06	1,28E-05
Q9UEY8	ADDG	1,019262881	6,16E-08	4,34E-07
O75694	NU15	1,018733952	8,08E-05	9,73E-05
P53396	ACLY	1,011317447	3,21E-07	1,44E-06
Q9Y512	SAM5	1,006288539	0,000205853	0,000205
Q32P28	P3H1	1,001063368	1,69E-07	9,21E-07
O95372	LYPA	-1,000721469	8,10E-05	9,75E-05
P05412	JUN_	-1,012277744	2,39E-05	3,86E-05
Q96I24	FUBP	-1,018053139	0,000502477	0,0004162
O43399	TPD5	-1,018625207	6,94E-05	8,67E-05
Q9ULX3	NOB1	-1,019822396	0,000186527	0,0001898
P30566	PUR8	-1,021359191	4,94E-05	6,71E-05
P02533	K1C1	-1,023782188	0,00794745	0,0041654
P49591	SYSC	-1,026871206	1,53E-06	4,75E-06
P62861	RS30	-1,029506486	0,000389127	0,0003385
Q8IVM0	CCD5	-1,034549813	0,000155715	0,0001637
Q9UKG1	DP13	-1,035510756	0,005760538	0,0031996
Q06203	PUR1	-1,047853347	6,80E-05	8,54E-05
P46937	YAP1	-1,051026833	0,000570795	0,0004621
Q9Y2W2	WBP1	-1,054160614	3,62E-06	9,12E-06
P53999	TCP4	-1,070858088	0,000394594	0,0003419
Q9UN86	G3BP	-1,073033372	7,47E-05	9,18E-05
P38159	RBMX	-1,079016208	3,41E-06	8,79E-06
P35269	T2FA	-1,082843535	0,000766262	0,0005908
P29084	T2EB	-1,085989496	0,019584797	0,0089836
Q9BWF3	RBM4	-1,086475844	5,06E-05	6,84E-05
P13984	T2FB	-1,09304074	8,62E-05	0,0001025
P06702	S10A	-1,099373868	0,000336116	0,0003031
P23588	IF4B	-1,104751178	0,000202964	0,0002033
P15336	ATF2	-1,133439842	0,001143787	0,0008197
Q7Z739	YTHD	-1,134854394	0,000320761	0,0002915
Q15287	RNPS	-1,139410186	8,63E-07	3,07E-06
Q6NYC1	JMJD	-1,146263349	0,000507101	0,0004193
Q8WXI9	P66B	-1,146640084	6,76E-07	2,55E-06
Q9NR56	MBNL	-1,151209158	7,10E-06	1,53E-05
Q16594	TAF9	-1,151375214	0,001104593	0,0007981
Q05519	SRS1	-1,162192907	0,000312961	0,0002858
O75475	PSIP	-1,164580955	1,72E-06	5,21E-06
O60869	EDF1	-1,169623464	7,90E-06	1,64E-05
P26583	HMGB	-1,171058764	5,22E-06	1,21E-05
P78330	SERB	-1,176804482	4,70E-05	6,46E-05
Q9H089	LSG1	-1,178633047	0,000858679	0,0006482
P27144	KAD4	-1,187796632	1,97E-08	1,97E-07
O60885	BRD4	-1,197283296	5,03E-06	1,18E-05
Q9UHB6	LIMA	-1,200295995	0,000104324	0,0001193

Q15056	IF4H	-1,200718353	1,34E-08	1,46E-07
P82979	SARN	-1,210347473	0,000117353	0,0001304
Q7Z4V5	HDGR	-1,213721291	0,010247621	0,0051604
Q9NPA8	ENY2	-1,218120794	7,82E-05	9,48E-05
P07339	CATD	-1,234348629	6,21E-07	2,38E-06
O60828	PQBP	-1,286841584	0,000482082	0,0004015
P41223	BUD3	-1,331027003	0,001366414	0,0009497
Q96PU8	QKI_	-1,36169063	2,40E-08	2,26E-07
Q92522	H1X_	-1,365126913	7,26E-07	2,70E-06
Q9H098	F107	-1,372508362	2,88E-07	1,33E-06
Q13283	G3BP	-1,388791311	4,66E-07	1,91E-06
P23193	TCEA	-1,390199443	2,70E-05	4,24E-05
Q13243	SRSF	-1,397985992	3,74E-05	5,44E-05
Q9Y3Y2	CHTO	-1,401604375	3,35E-07	1,50E-06
O60716	CTND	-1,407280606	1,94E-06	5,75E-06
Q96RT1	ERBI	-1,412008728	1,45E-05	2,63E-05
Q96PK6	RBM1	-1,450771945	1,32E-10	5,88E-09
Q99848	EBP2	-1,490582207	9,90E-08	6,08E-07
Q9Y6K9	NEMO	-1,492878355	4,60E-07	1,89E-06
Q92541	RTF1	-1,498838619	5,76E-07	2,27E-06
Q01130	SRSF	-1,505088372	4,82E-06	1,14E-05
Q14011	CIRB	-1,53422986	7,44E-06	1,58E-05
Q7Z417	NUFP	-1,547410096	3,27E-06	8,60E-06
Q9H875	PKRI	-1,609712519	2,05E-07	1,04E-06
P48634	PRC2	-1,615450011	3,32E-09	5,42E-08
Q9Y520	PRC2	-1,616771554	3,90E-06	9,64E-06
Q96QD9	UIF_	-1,653559083	2,35E-08	2,23E-07
Q96LD4	TRI4	-1,690740802	9,37E-07	3,28E-06
Q9UI15	TAGL	-1,708648446	7,47E-06	1,59E-05
P62256	UBE2	-1,71180883	0,000237276	0,0002293
Q9NZN4	EHD2	-1,829740676	2,43E-11	1,78E-09
O43805	SSNA	-1,942749578	2,64E-06	7,39E-06
P67809	YBOX	-1,944218904	5,50E-06	1,27E-05
P41567	EIF1	-2,125004136	1,63E-07	9,00E-07
Q99538	LGMN	-2,129171795	4,50E-05	6,25E-05
Q14978	NOLC	-2,155484516	1,16E-06	3,86E-06
P02538	K2C6	-2,199878099	0,00589893	0,0032632
Q5JSZ5	PRC2	-2,415856972	9,28E-09	1,14E-07
P17275	JUNB	-3,189742424	4,99E-10	1,38E-08

SUPPLEMENTARY FIGURES



Supplementary fig. 1: DAPI staining of nuclei for non-specific mycoplasma detection

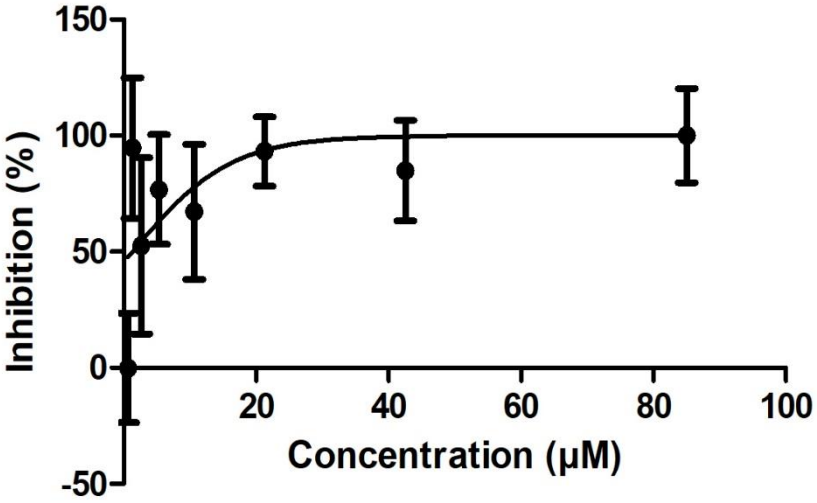
A

Layout	01	02	03	04	05	06	07	08	09	10	11	12
	AMH	BDNF	BMP1	BMP10	BMP2	BMP3	BMP4	BMP5	BMP6	BMP7	BMP8B	CECR1
A	2.81	-1.14	6.58	-2.36	2.81	2.49	3.71	4.12	-1.56	3.62	-1.26	3.63
	C	B	B	B	C	B	B	B	B	B	B	B
	CLC	CSF1	CSF2	CSF3	CSPG5	CXCL1	DKK1	ERAP1	EREG	FGF1	FGF11	FGF13
B	-1.06	10.30	-7802.06	2.35	-1.61	-2.63	1.52	3.49	-1.29	6.04	1.14	3.48
	B	B	A	B	B	B	B	B	B	B	B	B
	FGF14	FGF17	FGF19	FGF2	FGF22	FGF23	FGF5	FGF6	FGF7	FGF9	FIGF	GDF10
C	6.71	-1.98	2.81	5.67	1.90	-1.56	1.16	1.32	9.42	4.89	-1.12	-3.86
	B	B	C	B	B	B	B	B	B	B	B	B
	GDF11	GDNF	GPI	HBEGF	IGF1	IGF2	IL10	IL11	IL12B	IL18	IL1A	IL1B
D	5.41	-1.25	8.31	2.92	32.42	3.40	-1.02	1.72	2.81	4.90	1.37	-2.32
	B	B	B	B	A	B	B	B	C	B	B	B
	IL2	IL3	IL4	INHA	INHBA	INHBB	JAG1	JAG2	LEFTY1	LEFTY2	LIF	LTBP4
E	-3.29	4.36	-1.63	1.46	33.11	-1.61	60.65	1.07	-1.00	1.24	4.28	2.81
	B	B	B	B	A	B	A	B	B	B	B	C
	MDK	MSTN	NDP	NGF	NODAL	NRG1	NRG2	NRG3	NRTN	NTF3	OSGIN1	PDGFC
F	2.81	5.70	1.38	1.51	2.81	3.22	-2.09	2.44	1.70	5.33	1.32	7.96
	C	B	B	B	C	B	B	B	B	B	B	B
	PGF	PSPN	PTN	SLCO1A2	SPP1	TDGF1	TGFB1	THPO	TNNT1	TYMP	VEGFA	VEGFC
G	11.95	1.73	28.32	1.40	73.73	-3.04	8.15	-3.10	4.59	39.44	6.34	1.45
	B	B	A	B	A	B	A	B	B	B	B	B

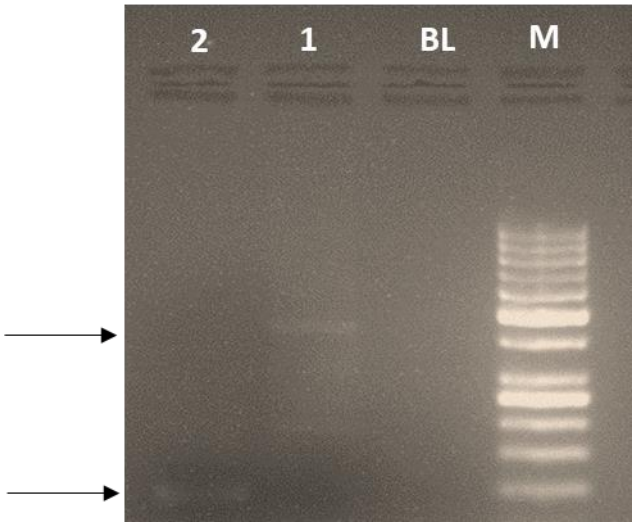
B

Layout	01	02	03	04	05	06	07	08	09	10	11	12
	ACSL3	ACSL4	ACSL5	ADM	ARNT	ATF4	AXIN2	BAX	BBC3	BCL2	BCL2A1	BCL2L1
A	1.77	26.06	5.80	91.77	5.10	45.73	1.53	7.96	-1.80	19.13	5.07	2.78
	C	B	B	A	B	A	B	B	B	B	B	B
	BIRC3	BMP2	BMP4	BTG2	CA9	CCL5	CCND1	CCND2	CDKN1A	CDKN1B	CEBPD	CPT2
B	-1.82	-1.69	-1.54	1.81	12.89	18.77	14.31	8.83	8.06	7.38	1.25	2.72
	B	B	B	B	B	B	B	A	A	B	B	B
	CSF1	DAB2	EGFR	EMP1	EPO	FABP1	FAS	FCER2	FOSL1	FTH1	GADD45A	GADD45B
C	-1.52	4.08	12.59	4.98	-3.71	-4.50	1.41	1.01	5.40	25.32	2.10	6.67
	B	B	B	B	B	B	B	B	B		B	B
	GATA3	GCLC	GCLM	GSR	HERPUD1	HES1		HEY1	HEY2	HEYL	HMOX1	ICAM1
D	-3.49	6.08	1.09	-1.47	12.43	14.94	N/A	11.93	-5.66	4.92	261.74	9.90
	B	B	B	B	A	B		B	B	B	A	B
	ID1	IFNG	IFRD1	IRF1	JAG1	LDHA	LFNG	LRG1	MCL1	MMP7	MYC	NOTCH1
E	11.00	-2.96	1.59	2.68	14.57	2.70	4.25	3.91	162.02	16.61	12.91	-2.09
	B	B	B	B	A		B	B	A	A	A	B
	NQO1	OLR1	PCNA	PPARD	PTCH1	RB1	SERPINE1	SLC27A4	SLC2A1	SOCS3	SORBS1	SQSTM1
F	10.20	-3.17	2.81	5.87	-1.07	1.66	8.08	-1.96	7.06	1.65	2.54	14.53
	B	B	B	B	B	B	A	B	B	B	B	A
	STAT1	TNF	TNFSF10	TXN	TXNRD1	VEGFA	WISP1	WNT1	WNT2B	WNT3A	WNT5A	WNT6
G	4.11	-3.65	3.94	10.43	3.44	5.54	2.71	-1.24	-1.04	-1.49	-1.69	-2.70
	B	B	B	A	B	B	B	B	B	B	B	B

Supplementary fig. 2: Plate layout of the 96 genes. (A) Growth factor array (B) Signal transduction array



Supplementary fig.3: IC50. Cytotoxic effect of gemcitabine on MIA PaCa-2 cells. Gemcitabine required at least 10 µM to induce cytotoxicity. With an IC50 value of 10.01±3.02 µM in MIA PaCa-2 cells.



Supplementary fig.4: Agarose gel electrophoresis results demonstrate successful amplification of the *SPP1* gene in MIA PaCa-2 cells.

APPENDIX A: TURNITIN REPORT

Xelwa Ntombikayise H Feb 2024_FINAL_TA_EN-3.docx

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

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From: Ekene Nweke
To: 1583242@students.wits.ac.za
Subject: Turnitin report- Ms Xelwa
Date: Thursday, 07 March 2024 12:00:00
Attachments: Pages from Xelwa Ntombikayise H Feb 2024_FINAL_TA_EN-3.docx.pdf

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Dear Ms Xelwa,

Herewith your MMed plagiarism report is attached. Your overall similarity index is 18% with a maximum single match of 8 % which is from your already published PhD paper https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2021.683788/full. All other matches are ≤ 1%. Therefore, your similarity index is **ACCEPTABLE** within our Department.

For PG submissions - Please only submit the attached page in the attached report, as well as this email stating that your plagiarism report is **ACCEPTABLE** within the Department of Surgery.

APPENDIX B: ETHICS CLEARANCE CERTIFICATE and accompanying documents

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms N Mhlambi

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190735

NAME: (Principal Investigator)	Ms N Mhlambi
DEPARTMENT:	School of Clinical Medicine Department of Surgery Medical School University
PROJECT TITLE:	Gene expression patterns of signalling pathways in pancreatic cancer: towards inhibiting metastases
DATE CONSIDERED:	2019/07/26
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Professor G Candy; Drs E Nweke and J Devar
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/01/27

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **July** and will therefore reports and re-certification will be due early in the month of **July** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES

WITS UNIVERSITY

STUDY INFORMATION DOCUMENT

Study title: **GENE EXPRESSION PATTERNS OF SIGNALLING PATHWAYS IN PANCREATIC DUCTAL ADENOCARCINOMA (PDAC) : TOWARDS INHIBITING METASTASES.**

Greetings Sir/Madam:

Introduction:
Good day, we, Prof. Geoffrey Candy, Dr. Ekene Nwake and Ms Ntombikayise Mhlambi, are doing research on Pancreatic cancer. Research is a process used in seeking new knowledge. In this study we want to learn the role of signalling pathways involved in a tumour progression, which will help in understanding the disease. Tissue biopsy will be taken during the Whipple procedure when the patient will be unconscious.

This is a research study and is not part of your normal and routine care which you will receive while in hospital. The study hopes to find out why the cancer starts, what makes it different, and hopefully find ways to best kill it by using drugs or other treatment therapies.

Invitation to Participate: In order for us to study the gene expression patterns of signalling pathways in PDAC cancer, we are asking you to take part in this research study.

What is involved in the study: You have been diagnosed with Pancreatic adenocarcinoma cancer and in this study your Tissue biopsy will be taken during the Whipple procedure when the patient will be unconscious. Various techniques will be used to analyse the gene expression of signalling pathways from the tissue samples.

Risks of being involved in the study: Tissue biopsy will be taken during the Whipple procedure when the patient will be unconscious and this could cause some discomfort and pains. Furthermore, this surgical procedure will be done by the Surgeons in the surgery division unit 1, which are trained to do so safely.

Benefits of being in the study: This is a research study. We do not know at this time what we will find, if anything. Research studies like this can take years to complete and we need samples from a number of patients to make sure we are seeing the same differences in all the patients. So at this time you will not benefit from taking part in this study. However, if you do take part, we may find better ways to treat or diagnose other PDAC patients in the future. However, should there be a discovery which may benefit you we would contact your treating doctor who would contact you through your clinic.

Participation is voluntary: If you decide not participate in the study, this is your right and this will not influence the way you are treated while in hospital. If you decline to take part, we simply will not take the samples indicated. If you change your mind later, we would destroy the samples we have taken. Again there will be no penalty or loss of benefits or care that you receive. Either way

you will need to contact Prof. Geoffrey Candy to ensure that this happens – the contact details are below. Finally, your participation in this study will not cost you anything.

Reimbursements: Participation in the study is entirely voluntary and you would not be paid for taking part in the study. None of the scientists or doctors are being paid if you decide to participate in the study.

Confidentiality: As with any medical records, every effort will be made to keep all your personal information confidential. Your name will not be disclosed to any external third party. In order to keep your identity confidential, a specialised identity code will be assigned to you; only the investigators of this study will have access to the files which links your name to the specialised identity code. However, absolute confidentiality cannot be guaranteed and personal information may be disclosed if required by law. Organisations such as the Research Ethics Committee of the University of the Witwatersrand may inspect and/or copy your research records for quality assurance and data analysis where this is required (this includes groups such as the Medicines Control Council.
For this study we are only interested in the pancreatic ductal adenocarcinoma cancer and diagnosis, not in names and personal information. It is our intention to publish the data, showing blood samples which may be of interest for all the patients in the group – not that of individuals participating in the study.

Contact details of researcher/s: If you have further queries or questions, Prof. Geoffrey Candy can be contacted on 011-717 2574 or 083-226 2436

Outputs: As previously mentioned, it is our intention to publish the data, showing blood samples which may be of interest for all the patients in the group – not that of individuals participating in the study and these articles would be shown to you if interested.

Contact details of HREC administrator and chair:
This study has been approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg ("Committee"). A principal function of this Committee is to safeguard the rights and dignity of all human subjects who agree to participate in a research project and the integrity of the research.
If you have any concern over the way the study is being conducted, please contact the Chairperson of this Committee who is Professor Clement Penny, who may be contacted on telephone number 011 717 2301, or by e-mail on Clement.Penny@wits.ac.za. The telephone numbers for the Committee secretariat are 011 717 2700/1234 and the e-mail addresses are Zanele.Ndlovu@wits.ac.za and Rhulani.Mukansi@wits.ac.za

Thank you for reading this Study Information Sheet.

Date: January 2020

The text in the Consent Sheets below is intended to be neither exhaustive, nor applicable in every circumstance. Researchers are free to customize them according to the particulars of their study

WITS UNIVERSITY

PARTICIPANT CONSENT SHEET

GENE EXPRESSION PATTERNS IN SIGNALING PATHWAYS OF PDAC: TOWARDS INHIBITING METASTASES

1. I have been given a Participant Information Sheet which explains the nature and processes involved in this study, which is attached hereto;
2. I was given time to read it, or had it read to me, in the language I best understand;
3. I was given time to ask any questions I wanted to and found any answers given to me to be reasonable and satisfactory;
4. I believe I fully understand why the study is being conducted and what the intended outcomes will be;
5. I understand that there will be no immediate benefit to me, should I agree to participate, nor will I receive any payment; conversely, participation will not cost me anything but my time;
6. I understand that, even if I initially consent to take part in the study, I may subsequently withdraw at any time and would not be required to give any reasons; if that happened, any data collected about me for the purposes of the study would immediately be destroyed, unless I give consent for it to be retained
7. I have been given a range of contact details, listed below. If I require further information or become concerned about any aspect of this study I am free to speak to any of these contacts.

Contact details:

Ms. Ntombikayise Mhlambi, Principal Investigator, telephone no. 0786829002, or by e-mail at 1583242@students.wits.ac.za.

Prof. Geoffrey Candy/Dr. Ekene Nweke, Supervisor, on telephone no.011 717-2574/0735384461, or by e-mail at Geoffrey.Candy@wits.ac.za/Ekene.Nweke@wits.ac.za

Professor CB Penny, Chairperson of the Human Research Ethics Committee (Medical) at the University of Witwatersrand, on telephone no.011 717 2301, or by e-mail at Clement.Penny@wits.ac.za.
Ms. Z Ndlovu or Mr Rhulani Mkansi, Committee Secretariat, telephone nos.: 011 717 2700 or 1234, or by e-mail at: Zanele.Ndlovu@wits.ac.za or Rhulani.Mkansi@wits.ac.za

All consenting Participants should be given a copy of this Form to keep, for their records, after signature

The text in the Consent Sheets below is intended to be neither exhaustive, nor applicable in every circumstance. Researchers are free to customize them according to the particulars of their study

Name of Participant: _____
Date: _____
Place: _____
Signature or mark _____

Witnessed by:

Name of Witness: _____
Signature: _____
Date: _____

20180329

Iain0007/GenericConsent.docx

All consenting Participants should be given a copy of this Form to keep, for their records, after signature

The text in the Consent Sheets below is intended to be neither exhaustive, nor applicable in every circumstance. Researchers are free to customize them according to the particulars of their study

CONSENT FORM FOR AUDIO RECORDINGOF STUDY PARTICIPATION

Project Title

I hereby consent to audiorecording of the interview[1], or focus group discussion[1], or classroom interaction[1]

.

I understand that:

- The recording will be stored in a secure location (a locked cupboard or password protected computer) with restricted access to the researcherand the research supervisor.
- The recording will be transcribed and any information that could identify me will be removed,
- The recordings will be erased within either (a) two (2) years of the publication of the research findings, or (b) six (6) years, if no publications arise from this research
- Anyone wishing to access this information in the future will first have to obtain the approval of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg
- Direct quotes from my interview, without any information that could identify me, may be cited in the research report or other write-ups of research.

Name of Participant: _____
Date: _____
Place: _____
Signature or mark _____

Witnessed by:

Name of Witness: _____
Signature: _____
Date: _____

[1] Delete as appropriate

20180329

Iain0007/GenericConsent.docx

All consenting Participants should be given a copy of this Form to keep, for their records, after signature

The text in the Consent Sheets below is intended to be neither exhaustive, nor applicable in every circumstance. Researchers are free to customize them according to the particulars of their study

WITS UNIVERSITY

CONSENT FORM FOR VISUAL RECORDINGOF STUDY PARTICIPATION

Project Title

I hereby consent to visual recording of the interview,[1] or focus group discussion,[1] or classroom interaction[1]

.

I understand that:

- The recording will be stored in a secure location (a locked cupboard or password protected computer) with restricted access to the researcher and the research supervisor.
- The recording will be transcribed and any information that could identify me will be removed
- My face and voice will not be identifiable in any visual recording
- The recordings will normally be erased within either (a) two (2) years of the publication of the research findings, or (b) six (6) years, if no publications arise from this research, or:
- The film, with all identifying information directly linked to me removed, will be stored permanently and may be used for future research
- Anyone wishing to access this information in the future will first have to obtain the approval of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg
- Direct quotes from my interview, without any information that could identify me, may be cited in the research report or other write-ups of research.

Name of Participant: _____
Date: _____
Place: _____
Signature or mark _____

Witnessed by:

Name of Witness: _____
Signature: _____
Date: _____

[1] Delete as appropriate

20180329

Iain0007/GenericConsent.docx

All consenting Participants should be given a copy of this Form to keep, for their records, after signature

The text in the Consent Sheets below is intended to be neither exhaustive, nor applicable in every circumstance. Researchers are free to customize them according to the particulars of their study

PARTICIPANT ASSENT SHEET FOR MINORS*

Project Title

1. I have been given a Participant Information Sheet for Minors, which explains what this study is about;
2. The study was explained to me and I understand what will happen if I take part;
3. I was given time to ask any questions I wanted to and was happy with the answers I was given;
4. I understand that I will not benefit from the study, should I agree to take part. I also understand that I will not be paid to take part in the study; taking part will not cost me anything either;
5. I have been given a range of contact details, repeated below, should I require further information at a later stage, or have any cause for concern over anything which is done to me during the study; and
6. I understand that even if I agree to take part in the study, I can change my mind later and stop being a part of the study
7. My parent(s) or guardian(s) know that I have been invited to take part in the study. They agree that I may do so, but the decision to take part is also mine.

Contact details:

XXX, Principal Investigator, telephone no. XXX, or by e-mail at XXX,

XXX, Supervisor, on telephone no. XXX, or by e-mail at XXX

Professor CB Penny, Chairperson of the Human Research Ethics Committee (Medical) at the University of Witwatersrand, on telephone no.011 717 2301, or by e-mail at Clement.Penny@wits.ac.za.
Ms. Z Ndlovu or Mr Rhulani Mkansi, Committee Secretariat, telephone nos.: 011 717 2700 or 1234, or by e-mail at: Zanele.Ndlovu@wits.ac.za or Rhulani.Mkansi@wits.ac.za

Name of Participant: _____
Date: _____
Place: _____
Signature or mark _____

Name of Parent or Guardian: _____
Date: _____
Place: _____
Signature or mark _____

Witnessed by:

Name of Witness: _____
Signature: _____
Date: _____

*Defined as being persons under the age of 18

20180329

All consenting Participants should be given a copy of this Form to keep, for their records, after signature