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HEALTH SCIENCES

Identification of (Novel) Immune Targets with Potential Roles in the Progression of Pancreatic Ductal Adenocarcinoma (PDAC)

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A thesis submitted to the Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy.

JOHANNESBURG, 2024

DECLARATION

I, Zanele Nsingwane, declare that this thesis titled “Identification of (Novel) Immune Targets with Potential Roles in the Progression of Pancreatic Ductal Adenocarcinoma (PDAC)” is my own unaided work. It is being submitted for a degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

22nd day of April 2024

Date

DEDICATION

This PhD is dedicated to every black child from a disadvantaged background. Despite seemingly unattainable goals, persist, seek God's guidance—for “vain is the help of man”—and witness the remarkable achievements you'll reach.

“Do what you can, with what you have, where you are.”

Theodore Roosevelt.

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ABSTRACT

Background: Pancreatic ductal adenocarcinoma (PDAC) is a lethal cancer with a growing incidence and mortality despite novel therapeutic strategies. Its aggressiveness and difficulty to treat suggest the need for a better understanding of associated molecular mechanisms which could be targeted for treatment. The complement signalling pathway may play diverse roles in PDAC by eliciting an immune response, inducing inflammatory responses, and may elevate pathways linked to chemoresistance. However, their role in the progression of PDAC is not fully understood. This study aimed to identify potential immune response-related targets in a group of patients. **Methods:** In this study, 30 tissue samples (tumours and corresponding normal tissues) were obtained from 15 PDAC patients, 34 plasma samples were obtained from 25 PDAC patients, 6 patients with chronic pancreatitis, and 3 healthy control participants. Targeted pathway-specific PCR analysis was conducted to determine the gene expression profiles of immune-response-related genes. The circulating levels of complement proteins C3 and C5 were further investigated. Pharmacological inhibition of the complement pathway in MIA PaCa-2 pancreatic cancer cell lines was performed and the effect on cells was assessed by cell proliferation, cell migration, and cell cycle assays. Finally, SWATH-mass spectrometry was performed to identify potential molecular mechanisms during inhibition. **Results:** The results identified C3 to be overly expressed in early PDAC compared to later stages in plasma ($p=0.047$). Pharmacological inhibition of the complement pathway led to increased cell growth ($p<0.0001$), proliferation ($p=0.001$) and migration ($p=0.002$) *in vitro*. Proteomic analysis implicated several proteins such as the mitochondrial and histone proteins, that could play a role in inducing this phenotype. **Conclusion:** Both Complement C3 and C5 are elevated in PDAC samples compared to healthy ones. Furthermore, the inhibition of the complement pathway was shown *in vitro* to result in a more aggressive phenotype by stimulating cellular growth, proliferation, and migration, indicating the involvement of complement C3 and C5 in tumour progression. This study helps to further delineate the role of the complement pathway in PDAC progression.

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LIST OF ABBREVIATIONS AND SYMBOLS

Symbol & Abbreviations	Full meaning
α	Alpha
β	Beta
$^{\circ}$	Degree
μL	Microliter
Δ	Delta
©	Copyright
®	Registered Trademark
™	Trademark
APC	Antigen Presenting Cells
B2M	Beta-2-Microglobulin
CAR	Chimeric Antigen Receptor
cDNA	Complementary Deoxyribonucleic Acid
CEA	Carcinoembryonic Antigen
C3	Complement component 3
C5	Complement component 5
CCR4	Chemokine Receptor 4
CRP	C Reactive Protein
CRPs	Complement regulatory proteins
CTLA	Cytotoxic T lymphocyte-associated antigen
DCs	Dendritic Cells

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ERCP	Endoscopic retrograde cholangiopancreatography
FBS	Fetal Bovine serum
FC	Fold Change
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GENT	Gene Expression Patterns across Normal and Tumour tissues
HEK 293	Human Embryonic Kidney 293 cell
ED ₅₀	Median Effective Dose
IL	Interleukin
INR	International Normalized Ratio
LMP	Latent Membrane Protein
MAC	Membrane Attack Complex
MAPK	Mitogen-Activated Protein Kinase
MBL	Mannose Binding Lectin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MDSCs	Myeloid-Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MPV	Mean Platelet Volume

mRNA	Messenger Ribonucleic Acid
MRPL	Mitochondrial Ribosomal Protein L19
NKCs	Natural Killer Cells
NS	Not Significant
PCR	Polymerase Chain Reaction
PD	Programmed cell death protein 1
PDL1	Programmed cell death Ligand 1
PDAC	Pancreatic Ductal Adenocarcinoma
Pen-Strep	Penicillin Streptomycin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPLP0	Ribosomal Protein Lateral stalk subunit P0
SPC	Streptavidin Peroxidase Conjugate
TAMs	Tumour-associated Macrophages
TBE	Tris Borate EDTA
TGF- β	Transforming Growth Factor Beta
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
Tregs	T regulatory cells
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

This chapter gives a concise overview of PDAC and its global impact. The processes by which PDAC develops and progresses are also outlined. Furthermore, the immunopathogenesis of pancreatic ductal adenocarcinoma is elucidated. Additionally, the role of the immune system and immune cells in combating PDAC, and current therapeutic approaches in the field are discussed.

1.1 Anatomy and function of the pancreas

The pancreas is a glandular organ in the abdomen, positioned just behind the lower part of the stomach measuring about 15cm in length. It serves as an accessory organ in digestion, although it is not part of the digestive tract. The pancreas comprises five anatomical parts: the head, uncinate process, neck, body, and tail (Figure 1.1). It plays a role in the endocrine and exocrine systems, functioning as a gland. Its endocrine function involves synthesizing and releasing hormones like insulin and glucagon into the bloodstream, which regulate the metabolism of glucose, lipids, protein, and blood sugar (Karpińska & Czauderna, 2022). Conversely, its exocrine role entails the production and secretion of digestive enzymes into the duodenum of the small intestine. In most cases, the pancreas is connected to the common bile duct through the pancreatic duct, also known as the Wirsung duct, although in approximately one-third of patients, these ducts may open separately into the duodenum (Lee et al., 2012).

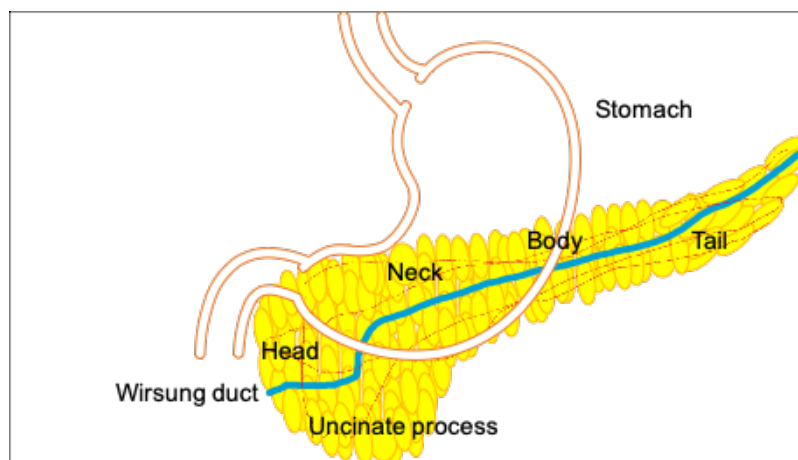


Figure 1.1: The anatomy of the pancreas. The pancreas is divided into five anatomical parts: the head, uncinate process, neck, body and tail.

1.1.1 Diseases of the pancreas

The pancreas is susceptible to various medical conditions, collectively referred to as pancreatic diseases. These encompass a spectrum of ailments, including pancreatitis, pancreatic cysts, and pancreatic cancer. Pancreatitis involves inflammation of the pancreas, which can manifest as either an acute or chronic form. Acute pancreatitis is marked by abrupt and intense inflammation, frequently triggered by factors like gallstones or excessive alcohol consumption (Klochkov et al., 2023). Conversely, chronic pancreatitis denotes a persistent inflammation of the pancreas, potentially resulting in lasting harm and compromised organ function. Chronic pancreatitis is a known risk factor of pancreatic cancer development, often exhibiting precancerous changes similar to those observed in pancreatic cancer patients (Gandhi et al., 2022; Le Cosquer et al., 2023; Seki et al., 2023). These changes which encompass the presence of pancreatic intraepithelial neoplasia, suggest the potential connection between chronic pancreatitis and pancreatic cancer onset (Gutierrez and Lennon, 2019).

Pancreatic cancer is a highly aggressive and fatal malignancy that originates in the pancreatic tissue. Pancreatic cancer can be classified into different types, including exocrine pancreatic cancer and neuroendocrine tumours (Table 1.1). Pancreatic adenocarcinoma, is the most common type accounting for the vast majority of pancreatic cancer cases, with more than 90% of all diagnoses falling into this category (Gordon-Dseagu et al., 2018). This type of cancer originates from the cells lining the pancreatic ducts. Neuroendocrine tumours (NETs) constitute a minority of pancreatic cancer cases, accounting for a smaller fraction, which is less than 2% (Sulciner and Clancy, 2023). These tumours develop from the hormone-producing cells in the pancreas and exhibit unique features and behaviours distinct from those of exocrine pancreatic cancer. Chronic inflammatory diseases, such as chronic pancreatitis, have the potential to progress towards neoplasia, as evidenced by the presence of premalignant changes in patients with chronic pancreatitis (Kandikattu et al., 2020). Interestingly, specific subtypes of chronic pancreatitis, such as hereditary pancreatitis or tropical form, have been identified to carry a notably increased risk of pancreatic cancer (Vlavcheski et al., 2022). The development of pancreatic cancer is multifactorial, with various risk factors playing a role. One of these factors is the increased cell turnover associated with chronic pancreatitis, which can lead to mutations and progress to cancer (Thomas and Radhakrishnan, 2019).

Table 1.1: Different malignant pancreatic tumours.

Pancreatic cancer type	Characteristic/origin
Pancreatic ductal adenocarcinoma	The most common type of pancreatic cancer, arising from the cells lining the pancreatic ducts.
Neuroendocrine tumours (NETs)	Originate from the hormone-producing cells of the pancreas.
Acinar cell carcinoma	Originate from the pancreatic acinar cells, these tumours are rare but can be aggressive.
Pancreatic cystic neoplasms	Include mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs), which have the potential to become malignant.
Pancreatic sarcomas	Rare tumours that originate from the pancreas connective tissue.
Solid pseudopapillary neoplasms (SPNs)	These tumours are often benign, but can exhibit malignant behaviour in some cases.

1.1.2 Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma (PDAC) stands as the predominant form of pancreatic cancer, representing over 90% of all cases. (Kleeff et al., 2016). It is prevalently lethal, fast-growing and aggressive (Siegel et al., 2022). PDAC is the fourth (4th) leading cause of cancer-related deaths, especially in developed countries such as the United States of America (Rahib et al., 2014), European countries (Pancreatic Cancer Europe, 2020) and the fifth leading cause of cancer-related deaths in Australia (Rawla et al., 2019). With the escalation in occurrence and fatality rates, PDAC is projected to become the world's second leading cause of cancer-related deaths by 2030 (Quante et al., 2016). PDAC has a poor overall survival of 5% below five years; in Europe, it is as low as 3%, showing how severe the disease is. Early diagnostic and better therapeutic options need to be identified speedily.

The development of PDAC involves a series of processes. Initially, pancreatic acinar cells undergo transdifferentiation, adopting a ductal phenotype as neoplastic development begins (Hanahan, 2022). Early-stage abnormalities called pancreatic intraepithelial neoplasms (PanIN) and intraductal papillary mucinous neoplasm (IPMN) play a crucial role in the development of PDAC. Approximately, 85-90% of PDAC arise in the backdrop of PanINs and the rest in

IPMNs (Yamakawa et al., 2021). PanIN is believed to be the prominent precursor lesion for PDAC, often found in proximity to resected tumours. However, its exact significance in predicting post-resection prognosis remains unclear (Hassid et al., 2014). In the process of genetic progression of PDAC, mutations classified as early, intermediate, and late occur and are key events in PDAC initiation and progression. In the earlier process, telomeres are shortened, and activation of *RAS* mutation occurs within PanIN-1 lesions (low grade PanIN) (Kanda et al., 2012). During the intermediate event, PanIN-2 (low grade PanIN) accumulates inactivating mutations and epigenetic suppression of *CDKN2A*. Ultimately, in the advanced stage, inactivating mutations in *TP53* and *SMAD4* arise within PanIN-3 (high grade PanIN). The specific occurrences driving the progression of pancreatic cancer remain undisclosed (Figure 1.2).

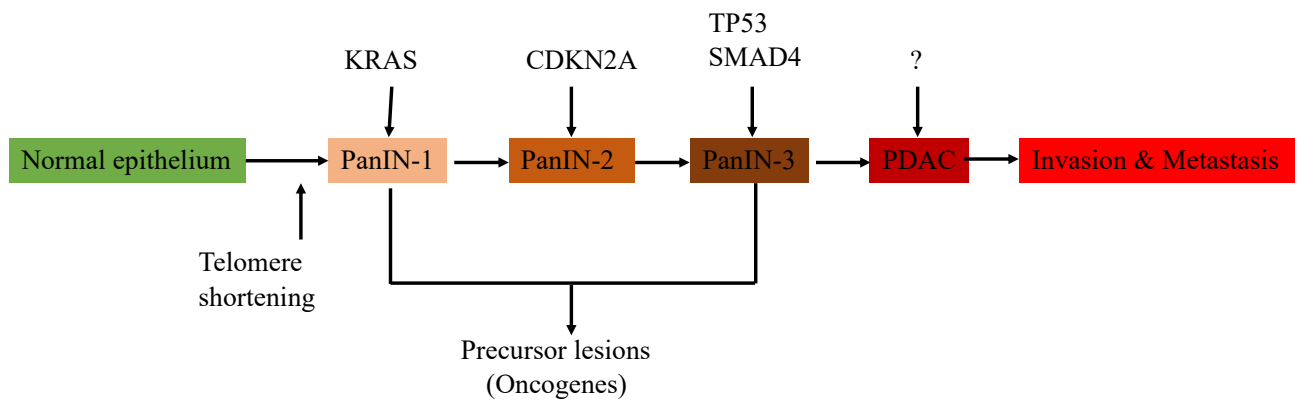


Figure 1.2: Genetic progression of Pancreatic Ductal Adenocarcinoma (PDAC). Genetic events involving mutations accumulate during the initiation and progression of PDAC.

1.1.3 Risk factors

PDAC risk factors are conditions that may elevate an individual's likelihood of developing PDAC. Approximately, 10% of PDAC cases arise in backdrop of germline mutations or due to a strong family history. Most of these risk factors tend to be associated with inflammation, and individuals exposed to these factors may be at a higher risk of developing the disease. For example, living an unhealthy lifestyle that involves heavy smoking and alcohol consumption may lead to inflammation of the pancreas, also known as pancreatitis (Żorniak et al., 2020). When this inflammation persists over a long period, it may develop into chronic pancreatitis, which could elevate one's risk of PDAC, especially when combined with other environmental and genetic factors (Momi et al., 2012). Additionally, several other variables, such as age, gender, and ethnicity, could also present as risk factors for the disease. For instance, black African males are more predisposed to PDAC, and their risk increases with age. Generally,

most cases occur after the age of 45, with approximately 90% of diagnoses being made in people over 55 years of age (Gordon-Dseagu et al., 2018).

1.1.4 Diagnosis

PDAC does not show signs and symptoms in its early stages. Clinicopathological characteristics gathered from clinical assessment encompass patient-reported signs and symptoms, their medical history, findings from physical examinations, and outcomes of laboratory tests (Kanji and Gallinger, 2013). Approximately, 25-40% of patients present with new onset diabetes or worsening hyperglycemia. Specific indications like unexplained weight loss, abdominal pain, jaundice, and disruptions in digestion are also characteristic of PDAC (Takikawa et al., 2022). Additionally, risk factors, like a family history of PDAC or particular genetic disorders, can further aid diagnosis (Molina-Montes et al., 2018).

Jaundice is one of the common symptoms of PDAC; it appears due to a blocked common bile duct by a tumour (Huggett and Pereira, 2011; Shen et al., 2018). Bile accumulates in the system and can be identified by yellow skin and eyes (Chen et al., 2018). PDAC patients also excrete dark brown urine that almost looks rusty, indicating a build-up of bilirubin. A greasy and clay-looking stool can also be a sign of PDAC caused by the pancreas' inability to break down fats and insufficient bilirubin (due to blockage), which usually gives the stool its brown colour (Tonini and Zanni, 2021). Radiological imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS), provide detailed information on the anatomical location, size, and involvement of adjacent structures by the tumour (Canto et al., 2006). These imaging studies can help determine the stage of the disease, including local tumour extension, lymph node involvement, and the presence of distant metastasis (Kanji and Gallinger, 2013; Mizrahi et al., 2020). A more definitive diagnosis of PDAC is usually confirmed by histopathological examination of a tissue sample obtained via biopsy or surgical resection (King et al., 2021). The pathologist evaluates various features, including tumour type (e.g., adenocarcinoma, neuroendocrine tumour), grade (degree of differentiation), and stage (extent of local invasion and distant spread).

1.1.5 Pancreatic ductal adenocarcinoma staging

The National Comprehensive Cancer Network classifies PDAC into four stages, namely resectable, borderline resectable, locally advanced, and metastatic (Tempero et al., 2021). PDAC is asymptomatic at its early stages, but symptoms begin to appear as it progresses, although these are often non-specific and transient, leading to misdiagnosis. Most patients are diagnosed late with locally advanced and metastatic disease at first presentation, causing the mortality rate of PDAC to escalate. The median overall survival for locally advanced patients

ranges from 12 to 14 months, while metastatic candidates often live for approximately eight to eleven months after diagnosis (Conroy et al., 2011; Vogel et al., 2016).

In stage I, the tumour can be surgically removed. In this case, the tumour is located in the superficial layers of the pancreatic duct and has not spread beyond the pancreas and the portal vein. It is important to note that some patients with more advanced stage disease may also be resected. In stage II, borderline resectable tumours share a similar phenotype but typically involve invasion into the portal vein (Grossberg et al., 2020). In stage III, the tumour is considered locally advanced, making surgical removal challenging, although it remains non-metastatic. At this stage, the tumour has extended beyond the pancreas and has spread to nearby lymph nodes, the portal vein, and the superior mesenteric artery. However, it has not invaded other major blood vessels, nerves, or distant sites. In stage IV, the tumour has advanced to a metastatic state and has spread from its original site to surrounding areas and organs. Common sites of metastasis include the liver: the most frequently affected organ, and the lungs (Houg and Bijlsma, 2018; Z. Liu et al., 2023). It is important to note that the metastases are not limited to these organs alone. By understanding the distinct characteristics of each stage, we gain valuable insights into the progression and severity.

1.1.6 Therapeutic strategies for PDAC

Therapeutic options for diagnosed PDAC patients are very few. They comprise of surgery, chemotherapy, and radiotherapy. The combination of surgery and chemotherapy is the only proven treatment for providing long-term survival in PDAC patients. However, this approach is effective only for patients whose tumours can be surgically resected, and unfortunately, over 80% of patients are diagnosed with tumours that cannot be surgically removed (Lee et al., 2019). Hence, almost all PDAC patients undergo chemotherapy. Gemcitabine is one of the most commonly used chemotherapy drugs for PDAC. It was first approved by the FDA in 1996 (Moore, 1996) even though it showed minimal efficacy in clinical trial subjects, at the time, there wasn't a better option. Gemcitabine showed increased efficacy in patient survival when used in combination studies with albumin-bound (Nab) Paclitaxel (Von Hoff et al., 2013). Another commonly used chemotherapy drug for PDAC is FOLFIRINOX, a multi-drug that combines 5-fluorouracil, Leucovorin, Irinotecan and Oxaliplatin. According to a study conducted by Principe and colleagues (Principe et al., 2021) treatment with 5-fluorouracil produced improved results, in patients with metastatic PDAC, in comparison to Gemcitabine. The median survival increased from 15.5 months to 25.3 months among those who underwent surgery (Underwood et al., 2023). However, it is important to note that using 5-fluorouracil can also lead to serious adverse effects in patients, as highlighted by (Conroy et al., 2018).

Chemotherapy serves various purposes in cancer treatment. In some cases, it is administered with the intent to convert an unresectable tumour into a resectable one, known as conversion therapy. Additionally, chemotherapy may be given before surgery to shrink the tumour, termed neo-adjuvant therapy, or after surgery to prevent recurrence, referred to as adjuvant therapy. Conversely, when the goal of treatment is symptom management and improving quality of life rather than curing the disease, chemotherapy is used palliatively. Due to its potential toxicity to normal cells and symptoms related to that, some patients choose to pursue best supportive care instead of palliative chemotherapy (Bowcock et al., 2004). Currently, none of these therapeutic options are near effective, mostly due to the disease's complex nature and heterogeneity.

Over recent years immunotherapy has shown promise as an effective cancer therapeutic strategy. Immunotherapy primes the host's immune system to recognise and kill cancer cells. Single checkpoint immunotherapy has shown successful outcomes in melanoma and other cancers, although it doesn't yield the same results in PDAC (Kabacaoglu et al., 2018). Nonetheless, there is hope in combination treatment, including immune checkpoint inhibitors. Identifying biomarkers and other options for early diagnosis of PDAC could potentially help increase the effectiveness of treatments, reduce the mortality rate and increase the survival rate.

1.2 Immune system in cancer

The immune system comprises a network of specialised cells, tissues and organs that work together to defend the body from external and internal threats such as pathogens and damaged cells. It is activated in response to infection by pathogens or transformed cells (Balkwill and Mantovani, 2012). During tumourigenesis, tumour-specific antigens are identified by the immune system through a complex process of immune surveillance (Kallinger et al., 2023). The immune system continuously monitors the body for the presence of abnormal or foreign substances, including cancer cells. Any cell that expresses abnormal surface proteins or antigens will be recognized as non-self. This manifestation allows the immune system to function like an intrinsic tumour suppressor system (Inman, 2014). During tumorigenesis, normal functioning cells exhibit an uncontrolled proliferation phenotype, becoming cancerous and displaying abnormal tumour-associated antigens on their surface. The immune system identifies these antigens and eliminates tumour cells by eliciting an immune response against affected cells. However, cancer cells have established ways to evade immune detection. Avoiding immune recognition, elimination and immune evasions are among the identified hallmarks of cancer (Hanahan, 2022). Mechanisms used by tumour cells to avoid recognition include secreting factors that suppress the immune system, such as the inflammatory cytokine

IL-10, and TGF- β . Tumours elevate the expression of IL-10 and TGF- β as part of their immune evasion strategy, creating an immunosuppressive tumour microenvironment. This upregulation is often associated with the promotion of tumour growth, progression, and immune escape (de Streel and Lucas, 2021; Zhao et al., 2021).

Cancer and the immune system share a connection that unfolds in distinct phases: elimination, equilibrium, and escape, collectively termed immunoediting. This process dictates whether the immune system restrains or fosters cancer progression (O'Donnell et al., 2019). In the elimination phase, the immune system identifies and eliminates cancer cells. Helper T cells activate dendritic cells, triggering CD8⁺ T cells to eradicate both pre-cancerous and cancerous cells. Additionally, mature B cells release antibodies that counteract and label foreign pathogens and cancer cells for obliteration. The equilibrium phase ensues when cancer cells or tumours persist following the initial elimination attempt. During this period, the immune system may still effectively prevent tumour expansion. This equilibrium phase is believed to be the most prolonged stage of immunoediting. It can persist for years, with the immune system continually eradicating cancer cells while also encountering and giving rise to resistant cancer variants (Bhatia & Kumar, 2011). The escape phase, in which a tumour becomes clinically detectable, marks a compromised ability of the immune system to eliminate and suppress cancer cells. The tumour disrupts the regulation of the immune response by diminishing the availability of antigen-presenting cells and expressing immune checkpoint inhibitors like PD-L1, CTLA-4, and LAG-3 (discussed in section 1.4). Additionally, the tumour cells express frequent mutations and acquire properties that confer resistance and survival advantages to tumour cells (McCoach & Bivona, 2018).

Immunoediting is a process that describes the interaction between the immune system and cancer and emphasises that the immune system can either eliminate, control or facilitate the escape of cancer cells (O'Donnell et al., 2019). It has significantly influenced the development of cancer immunotherapy strategies, such as immune checkpoint inhibitors and adoptive T-cell therapies, which aim to reactivate the immune system's ability to recognize and target cancer cells, especially those that have escaped immune surveillance (Liu et al., 2023; Vesely and Schreiber, 2013).

1.2.1 Immune cells and their roles in PDAC

The immune system is separated into two lines of defence: the innate and the adaptive immune response. The innate immune system is the first line of defence that protects the host from invading pathogens (Marshall et al., 2018). Its activation triggers a response to remove dead cells and begin wound healing. In contrast, an adaptive immune response is elicited when

pathogens overwhelm the innate immune defence and will activate a long-lasting immune response against specific infectious agents (Inman, 2014; Sepand et al., 2022). The immune system relies on a diverse range of immune cell types to combat foreign invaders and protect the body against infections. The immune system requires cell types from both the innate and adaptive immune system including macrophages, regulatory T cells (Tregs), dendritic cells, natural killer cells, cytotoxic CD8⁺ T cells and CD4 helper T cells, to function effectively against cancer. The specific functions of these cells concerning PDAC are described below.

1.2.1.1 Macrophages

Macrophages, specialised cells responsible for recognising, phagocytosis, and destroying pathogens, play a crucial role in the immune system. In the innate immune response, macrophages function to clear dead or dying cells. In the adaptive immune response, they differentiate from M1 (classically activated) to M2 (alternatively activated) cells (*in vitro* macrophage phenotype). Classically activated macrophages, promote inflammation, while alternatively activated macrophages, have anti-inflammatory properties. Alternatively activated macrophages contribute to angiogenesis, tumour metastasis, and suppressing the adaptive immune response (Liu et al., 2021; Yunna et al., 2020). Macrophages are very diverse and play significant roles in cancer development. Tumour-Associated Macrophages (TAMs) can be polarized into classically activated or alternatively activated types, with alternatively activated TAMs often promoting tumour growth. TAMs share characteristics with alternatively activated TAMs and play roles in immunosuppression (Yang et al., 2021). Monocyte-Derived Macrophages (MDMs) from the bloodstream can also influence the tumour immune response. Their polarization can be influenced by signals from the tumour microenvironment. They often play a role in shaping the immune response within the tumour and can contribute to pro- and anti-tumour activities (Boutillier and Elswa, 2021). Perivascular macrophages are involved in angiogenesis, while Metastasis-Associated Macrophages (MAMs) facilitate cancer cell spread. Macrophages can either support or hinder cancer progression, making them important targets for cancer therapy research. TAMs are the most extensively studied macrophages in the context of cancer. They are a key component of the tumour microenvironment and can either promote or inhibit cancer development depending on their functional polarization (Feng et al., 2022). MDSCs are immature myeloid cells responsible for suppressing both the innate and adaptive immune response. Monocytic myeloid-derived suppressor cells (M-MDSCs) are derived from monocytes in the bone marrow and play a significant role in immunosuppression, particularly in cancer. They are influenced by various factors in the tumour microenvironment, which promote their expansion and activation, enabling them to suppress the activity of immune cells,

such as T cells and natural killer cells (Joshi and Sharabi, 2022). MDSCs are present at higher levels in the circulation of PDAC patients (Trovato et al., 2019).

1.2.1.2 Regulatory T cells (Tregs)

Regulatory T cells are CD4⁺ CD25⁺ FOXP3⁺ cells present in significant quantities within the tumour microenvironment, where they are recruited and bind multiple chemokines (Noyes et al., 2022; Zhang et al., 2020). Regulatory T cells express various molecules, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), secrete IL-10 and TGF- β to suppress excessive immune response (Huppert et al., 2022). This suppression echoes the critical role of regulatory T cells in preventing autoimmune diseases by ensuring that host cells do not trigger an immune response (Verma et al., 2019). Tregs are the most abundant immune cells in the tumour microenvironment and circulation in PDAC. The high levels of Tregs in the tumour site are associated with disease progression (Cheng et al., 2016; Gao et al., 2022). Unexpectedly, efforts to reduce Tregs in PDAC patients showed no significant improvement in immunosuppression and instead worsened tumour progression (Li et al., 2020). Decreased levels of Tregs in circulation post-tumour resection are associated with improved survival (Gao et al., 2022; Liu et al., 2017).

1.2.1.3 Dendritic Cells (DCs)

Dendritic cells are professional antigen-presenting cells among the main regulators of the anti-tumour immune response (Sideras et al., 2014). They are responsible for antigen presentation to CD4⁺ and CD8⁺ T cells by capture, internalisation, processing, and presentation. In PDAC patients, DCs are significantly reduced and less functional; this is one of the mechanisms in which PDAC evades the immune response, compromises DCs maturation and survival, affects antigen presentation and contributes to immune tolerance (Hegde et al., 2020). The presence of DCs in tumour tissue and circulation can enhance CD8⁺ T cell function and type 1 helper T cell (Th1) activity to better combat cancer and are associated with prolonged survival of patients (Hegde et al., 2020).

1.2.1.4 Natural Killer (NK) cells

Natural killer cells are a type of large granular lymphocytes primarily involved in the innate immune response. They also play a secondary role in the adaptive immune response through communication with cells of the adaptive immune system. In response to infection or cancer, NK cells are activated and express activation receptors. These identify cancer cells as well as infectious pathogens and bind to the target cell's ligands resulting in a signalling cascade that activates NK cells. Activated NK cells use their effector functions to induce cytotoxicity by releasing cytotoxic granules and a host of cytokines. These include interleukins; IL-2, IL-5, IL-

10, IL-13, and anti-tumour molecules like tumour necrosis factor-alpha (TNF- α), GM-CSF and IFN- γ (Paul and Lal, 2017). PDAC cells have developed mechanisms to evade or suppress the immune response by reducing NK cell levels in the tumour site, often associated with poor prognosis (Malchiodi & Weiner, 2021). Correspondingly, increased NK cells post-tumour resection in PDAC patients positively correlates with improved survival (Peng et al., 2021).

1.2.1.5 Cytotoxic CD8⁺ lymphocytes

Cytotoxic CD8⁺ lymphocytes, also known as tumour-specific CD8⁺ T cells (T-killer cells/cytolytic T cells), are immune effector cells responsible for cell-mediated immune response. They attack and kill cancer cells by producing cytolytic granules such as perforin and granzymes and express the Fas ligand that bind the death receptor (Fas) and triggers a series of intracellular events that ultimately lead to apoptosis, or programmed cell death. The function of perforins involves punching holes into the plasma membrane of target cells, aiding the release of pro-apoptotic granzymes from the host cell into the target cell through the holes created (Santos-Zas et al., 2021). CD8⁺ cells play a significant role in anti-tumour immune response although their function in the microenvironment is defective due to several immunosuppressive mechanisms. Activation of CD8⁺ cells occurs following an immunological synapse between an antigen-presenting cell and antigen presented by MHC 1 molecule to CD8⁺ cells. PDAC cells downregulate the expression of MHC 1 molecules resulting in the restriction of CD8⁺ T cell activation; and also use the loss of Fas expression on PDAC tumours as an immune evasion mechanism (Goulart et al., 2021). PDAC patients show a dramatically reduced CD8⁺ count as a sign of a compromised immune system, especially as PDAC progresses; however, CD8⁺ T cell tumour infiltration may be seen following tumour resection, and this is associated with improved patient outcome (Goulart et al., 2021).

1.2.1.6 CD4⁺ helper T cells

CD4⁺ helper T cells, also known as CD4⁺ Th cells, are essential cells of the adaptive immune system. They are critical players in anti-cancer immunity, and it is estimated that approximately 80% of tumour infiltrating lymphocytes (TILs) are CD4⁺ and CD8⁺ T cells, with the rest being regulatory T cells (Muller et al., 2022). CD4⁺ Th cells differentiate into two subsets of cells, Th1 and Th2. The Th1 response is cell-mediated and includes the release of IL-2 and IFN- γ ; it is also involved in tumour killing (X. Zhao et al., 2019). PDAC tends to promote the Th2 response, which intersects with humoral immunity, potentially leading to immunosuppression, and demonstrates a tumour-promoting phenotype associated with poor prognosis (Liu et al., 2019). The proliferation and migration of CD4⁺ T cells in PDAC was reported to be obstructed, and this is one way in which PDAC can evade immune detection (Fogar et al., 2011).

Furthermore, their ability to transdifferentiate from Th2 to Th1 and *vice versa* qualifies them as suitable candidates to be targeted for therapeutic purposes (Martinez-Sanchez et al., 2018).

Table 1.2: Immune response cells and their role in PDAC progression.

Immune cells	Presence in PDAC	Interpretation of Presence	References
Tregs	Abundant in tumour site and circulation	In tumour site - advanced presentation of disease In circulation - less than one year of resection= improved survival	(Gao et al., 2022)
MDSCs	Abundant in circulation	Independent poor prognostic factor; poor overall survival	(Trovato et al., 2019)
TAMs	Abundant in tumour site	Disease progression	(Yang et al., 2021)
Cytotoxic CD8 ⁺ T cells	Decreased in circulation and tissue	Disease progression	(Goulart et al., 2021)
CD4 ⁺ helper T cells	Increased in tissue	Poor prognosis	(Goulart et al., 2021)
DCs	Decreased in circulation	Unable to stimulate T-lymphocyte proliferation	(Hegde et al., 2020)
NKs	Decreased in tumour site	Poor prognosis and represents suppressed immune response	(Malchiodi and Weiner, 2021)

1.3 Immunotherapy as a promising strategy for PDAC treatment and management

Despite conventional therapy, the prognosis for PDAC remains grim, necessitating the swift development of innovative approaches (Chang et al., 2016). Immunotherapy presents a range of benefits by harnessing the body's immune system to yield improved results. When compared to chemotherapy, radiation, and surgery, immunotherapy stands out as the most promising treatment avenue for PDAC. Diverse immunotherapies deployed thus far in treating PDAC encompass adoptive cell therapy, checkpoint inhibitors, monoclonal antibodies, and therapeutic cancer vaccines.

1.3.1 Adoptive cell therapy

Adoptive cell therapy, for example chimeric antigen receptor CAR-T, represents a form of passive treatment involving extracting and modifying a patient's T cells within a laboratory setting (Rohaani et al., 2019). Subsequently, these modified cells are reintroduced into the patient's body to enable them to combat cancer cells. While this therapy holds the promise of effectively addressing cancer, it is not exempt from certain drawbacks. Notably, the cost

associated with this approach is expected to be substantial, thereby potentially limiting accessibility for a broader range of patients (Kandra et al., 2022). Additionally, the personalised nature of this treatment precludes its application for multiple patients, as each individual possesses a distinct immune system. The inherent uniqueness of immune responses, coupled with the considerable variability of tumours among individuals, renders the notion of universally applicable cancer treatments challenging, if not unfeasible (Cruz-Ramos and García-Foncillas, 2019).

1.3.2 Antibody-based therapy

Specific antibody fragments are proteins synthesized in laboratories to target either antigens or other surface markers present on cancer cells. Their structure is reminiscent of the antibodies naturally generated by the human body to combat infections. Some monoclonal antibodies (mAbs) promote tumour destruction through immune-mediated processes like antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), as seen with drugs such as rituximab and trastuzumab (Golay and Taylor, 2020). Whereas other mAbs target growth factor receptors that are highly expressed in tumour cells. They hinder tumour growth by preventing the binding of growth factors to their receptors (e.g., anti-EGFR mAbs cetuximab and panitumumab), inhibiting receptor dimerization (e.g., anti-HER-2 mAb pertuzumab), impeding angiogenesis (e.g., anti-VEGF blocking mAb bevacizumab), promoting apoptosis (e.g., anti-CD20 mAb rituximab), and delivering potent radioisotopes for therapy (e.g., ibritumomab tiuxetan) (Arias-Pinilla and Modjtahedi, 2021). This type of therapy involves a patient receiving an infusion of engineered monoclonal antibodies (mAb) labelled with a radioactive atom using the antibody to locate the tumour and the radioactive component to detect and visualize, and to eradicate the cancer cells.

In the context of PDAC, specific immunotherapeutic strategies that use monoclonal antibodies have shown promising results in preclinical settings. These antibodies are designed to target molecules such as CEA (Lwin et al., 2022), mucin 1/4/5 (Wang et al., 2020), mesothelin (Hassan et al., 2020), PD-1 (Torphy et al., 2018), and CTLA-4 (Le et al., 2013). It is important to note that PD-1 and CTLA-4 have failed in PDAC as monotherapy and dual immunotherapy although showing promising results in other cancers. For example, ipilimumab targets and inhibits CTLA-4, thereby enhancing the anti-tumour immune response (not in PDAC). The utilization of mAbs in tumour diagnostics and visualization holds great promise and the identification of new biomarkers will further enhance the precision of tumour imaging (Sorbara et al., 2022).

1.3.3 Therapeutic cancer vaccines

Therapeutic cancer vaccines, also called anticancer vaccines, encompass a segment of promising immunotherapeutic methodologies under scrutiny to treat PDAC. In contrast to conventional vaccines that bolster the immune system and establish resistance to onset of diseases, cancer vaccines are employed specifically for treatment (Singh et al., 2021). These anticancer vaccines operate as adjuncts to activate and amplify pre-existing immune responses, encompassing humoral and cellular immunity, to surmount immune suppression within tumours (Le et al., 2022; Liu et al., 2022). Personalized RNA neoantigen vaccines are designed to teach the patient's immune system to recognize and attack the specific mutations present in their own tumour. Since PDAC is often associated with a low mutational burden, personalized vaccines that target neoantigens become particularly important in harnessing the immune response against the cancer. This approach holds promise for improving pancreatic therapeutics (Rojas et al., 2023). It is important to note that most therapeutic cancer vaccines have worked in minimal residual disease (post-surgical stage) but did not succeed in advanced disease settings in PDAC.

Within this vaccination paradigm, tumour antigens are administered to induce tumour-specific immune reactions. These antigens are dispensed in various formats, including whole tumour entities, proteins, peptides, DNA, RNA, and even antigen-loaded dendritic cells. An illustrative instance is the pancreatic GVAX vaccine, which has demonstrated limited success in trials involving metastatic PDAC patients (Laheru et al., 2008). Another notable example is the peptide-based PDAC vaccine Targovax (TG01), designed to target RAS mutations. This vaccine reached phase I/II clinical trials and exhibited notable post-resection enhancements compared to alternative treatments in Europe (Biocom, 2019). There is an indication that combining TG01 with chemotherapy could augment their collective efficacy.

1.3.4 Neoantigens

Neoantigens denote antigens stemming from genes specifically mutated within tumours, primarily emerging due to abnormal splicing events (Park and Chung, 2019). These neoantigens hold promise as an avenue for immunotherapy, as they can be recognised by the immune system as foreign entities, consequently triggering an immune response. Most PDAC cells express neoantigens, potentially provoking a robust immune reaction. However, this response is often hindered by mechanisms such as immune evasion, where the tumour microenvironment curtails the presentation of neoantigens to tumours (Bailey et al., 2016).

Among the potential neoantigens linked to PDAC, those emanating from KRAS codon 12 mutations, including G12V and G12D (Wang et al., 2016). In mouse models, distinct T-cell

receptors have been identified that recognise and bind to these mutation-derived neoantigens, underlining their significance as potential therapeutic targets (Sim and Sun, 2022; Verdon and Jenkins, 2021).

1.3.5 Targeting the microenvironment

The challenge in effectively implementing immunotherapy for PDAC arises from the intricate tumour microenvironment. This environment is characterised by a dense fibrous stroma, which obstructs drug delivery, culminating in drug resistance and unfavourable treatment outcomes. Addressing the cancer stroma can potentially revolutionise the treatment of this devastating disease. One approach involves targeting cancer-associated fibroblasts (CAFs) to mitigate desmoplasia caused by stromal cells. Drugs formerly used for stroma targeting were paclitaxel, pirfenidone, CD40 agonists, and PEGylated human hyaluronidase (PEGPH20). These agents hinder CAF proliferation, inducing a quiescent phenotype. However, a phase II clinical trial employing hedgehog pathway inhibitors to target proliferating CAFs and trials targeting PEGPH20 did not succeed (Amakye et al., 2013; Murakami et al., 2019). Furthermore, the dense and fibrotic stromal tissue surrounding PDAC tumours presents a significant challenge by promoting tumour progression and therapy resistance by stromal cells further complicating treatment strategies targeting them. Alternative methods to target CAFs warrant exploration and development.

Another promising strategy is targeting immunosuppressive cells. In melanoma patients, tumour-infiltrating Tregs exhibit heightened expression of chemokine receptor type 4 (CCR4). Tregs can be depleted by targeting this chemokine. Notably, the CCR4 antibody has demonstrated the removal of effector-type Tregs both *in vitro* and *in vivo* (Sugiyama et al., 2013). Furthermore, CD40-activated macrophages have been shown to infiltrate tumours and contribute to stroma reduction (Ene-Obong et al., 2013). Immunosuppressive cells like MDSCs dendritic cells, which hinder T-cell activation, could be viable targets. Targeting these cells might stimulate an appropriate immune response against PDAC, thereby enhancing the effectiveness of immunotherapeutic drugs.

1.4 Some immune checkpoints in PDAC

Immune checkpoints operate as regulators of immune activation, overseen by an array of co-stimulatory and inhibitory signals that dictate the functional behaviours of T-lymphocytes and regulate their activation process. Analogous to ‘brakes’ or ‘gatekeepers’, these checkpoints maintain control over the immune system, ensuring it refrains from attacking its own host tissues (He & Xu, 2020). Their significance in cancer immunotherapy has garnered considerable attention due to their potential for manipulation to prompt an appropriate immune

response against tumours. Immune checkpoint inhibitors, often pharmaceutical agents, are tailored to release these ‘brakes’, empowering the immune system to engage and eradicate cancer cells (Himmel et al., 2020). Extensive research has been devoted to understanding numerous immune checkpoints, and their role in the context of PDAC, along with their performance in ongoing clinical trials, will be briefly discussed.

1.4.1 PD-1/ PD-L1

The PD-1 receptor alongside its counterpart PD-L1, is found on the surface of T cells and serves as a mechanism for suppressing the immune response. These components contribute to regulating immune responses by dampening immune system functions and fostering self-tolerance by suppressing inflammatory activity within T-lymphocytes (Syn et al., 2017). Notably, PD-1 tends to inhibit the immune system from targeting and eliminating cancer cells. In the context of PDAC, PD-1 is often overexpressed, which correlates with tumour infiltration and a poorer prognosis (Geng et al., 2008; Nomi et al., 2007). Intriguingly, a decrease in PD-1 expression has been shown to hinder the proliferation of pancreatic tumours by increasing T cell activity and enhanced immune surveillance (Song et al., 2014).

Across various malignancies, PD1 has emerged as a pivotal immunotherapeutic target. Some early achievements were attained by using PD1 pathway blockade as a monotherapy. In the case of PDAC, clinical trials evaluated PD1 inhibitors like durvalumab and atezolizumab as standalone treatments for patients with PDAC, but regrettably, no clinical successes were observed (Mucileanu et al., 2021). However, limited efficacy was demonstrated by nivolumab and other PD-L1 inhibitors in PDAC patients (Karamitopoulou et al., 2021). Conversely, combining PD1/PD-L1 inhibitors with FOLFIRINOX chemotherapy showcased promising outcomes, prolonging patient survival compared to chemotherapy alone (L. Zhou et al., 2022). This underscores the prospect of combination therapies offering hope for PDAC, even in cases where monotherapies have fallen short.

1.4.2 CTLA-4

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), also recognised as CD152, functions as a protein receptor in the immune system, serving as an immune checkpoint that curtails immune responses. CTLA-4 is present in regulatory T-lymphocytes and is often overexpressed on the activation of Tregs following CD28 and T cell receptor (TCR) signalling (Rowshanravan et al., 2018; Syn et al., 2017). The CTLA-4 pathway influences T-lymphocyte infiltration into PDAC (Bengsch et al., 2017).

Despite its inability to effectively treat PDAC, CTLA-4 blockade therapy does influence how T cells respond to PDAC by modifying their immunobiology. This alteration can potentially positively impact T-cell immunotherapy in the future (Bengsch et al., 2017; Li et al., 2021).

1.5 Inflammation and the immune response

Inflammation serves as the immune system's defence strategy against infections and injuries. When the body encounters a harmful trigger, such as a virus, bacterium, damaged cells, or toxic substances, it activates the immune system. This prompts the immune system to signal leukocytes, which then work to safeguard the host from the intruder (Chen et al., 2017). Eliminating foreign invaders is a direct outcome of the immune response (Chen et al., 2017).

The sequence of inflammation manifests through four distinctive signs. Initially, the affected area becomes red due to blood vessel dilation, followed by a subsequent warmth due to an accumulation of blood flow. This is accompanied by swelling due to the accumulation of fluids outside the blood vessels and pain originating from tissue deformation or chemical mediators of inflammation. While not all types of inflammation are externally visible, as in the case of internal organs, inflammation can manifest as visceral pain.

Two primary categories of inflammation exist: acute and chronic. The distinguishing factor between them lies in the duration of the inflammation. These can also be referred to as short-term and long-term inflammation. Acute inflammation may persist for hours to days, while chronic inflammation can extend from months to even years (Furman et al., 2019). The presence of inflammation in the pancreas may result in acute and chronic pancreatitis which often results in the development of PDAC. Acute pancreatitis typically presents suddenly and is often reversible with prompt medical intervention, while chronic pancreatitis is characterized by ongoing inflammation and irreversible damage to the pancreas (Benjamin and Lappin, 2023).

Other components of inflammation may include cytokines, chemokines, acute-phase proteins and complement activation among others. The complement components C3a and C5a are anaphylatoxins with potent pro-inflammatory properties. They bind mast cells to release histamine for vascular permeability and promote the recruitment and activation of immune cells, including neutrophils and macrophages, to the site of infection or injury. These inflammatory immune response mediators exploit their vasoactive and chemotactic attributes to heighten inflammation (Carvelli et al., 2020) which may cause cancer. Complement factors C3a and C5a may participate in pathways that can exert both tumour-promoting and tumour-suppressing effects (Kolev et al., 2022; Revel et al., 2020; Xiao et al., 2022). These pathways might encourage the growth, survival, and mobility of cancer cells (Rolfe et al., 2020) or,

conversely, elicit an immune response to thwart aberrant cell proliferation. This dualistic nature is manifested as an anti- or pro-tumour response, which either eliminates abnormal cell growth or stimulates the expansion of tumour cells.

1.6 Justification

Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest human malignancies, accounting for about 8% of all cancer-related mortalities worldwide (Siegel et al., 2022). It is asymptomatic, especially in the early stages, and aggressive, leading to difficulty in diagnosis and treatment. A PDAC diagnosis involves a combination of physical and laboratory assessments, scans, and potentially endoscopic retrograde cholangiopancreatography (ERCP). Unfortunately, this cancer progresses fast, and when these tests are carried out, it is often too late, highlighting the importance of identifying new molecular markers specific to PDAC. The incidence and mortality of PDAC is increasing in South Africa and worldwide. Recent epidemiology studies have estimated that, in the next seven years (2030), PDAC is anticipated to be the second leading cause of cancer-related deaths (Kenner et al., 2016).

In a normal functioning immune system, immune cells can identify and eliminate cancer cells; however, they have discovered new mechanisms to evade immune recognition and elimination. Interestingly, in the initial publication of the hallmarks of cancer by Hanahan and Weinberg (Hanahan and Weinberg, 2000), the attributes that cancer cells must acquire for progression did not include immune evasion. It wasn't until a decade later that they recognised the significant role immune escape plays in facilitating cancer progression and metastatic dissemination (Hanahan, 2022; Hanahan & Weinberg, 2011).

Recently, there have been advances in the treatment of PDAC using immunotherapy. However, there is still a lack of effectiveness. This emphasises the need to discover novel potential immune targets. Most studies on PDAC and other cancers are focused on patients of European descent, and almost none investigate black African patients. Furthermore, some of the findings on cancer biomarkers may not necessarily apply to black African patients, given how they are highly predisposed to PDAC and have a distinct genomic makeup. Therefore, it is crucial to conduct comprehensive studies involving black African individuals and have their data represented alongside the existing body of knowledge. This study uniquely uses specimens from a local South African cohort to assess the immune response at different stages of PDAC, further enabling the identification of targets involved in tumour progression.

1.7 Aim of the study

This study aims to identify and functionally verify novel immune-related potential targets in PDAC progression.

1.8 Objectives

The objectives of this study are as follows:

1. To investigate the expression profile of immune-related genes in pancreatic tissues using RT² Profiler™ PCR arrays human innate and adaptive immune responses to identify dysregulated targets.
2. To verify gene expression of potential target (s) in a larger resectable PDAC sample cohort using real-time PCR.
3. To investigate the expression profile of potential protein target (s) in the circulation of patients in different stages of PDAC using Enzyme Linked Immunosorbent Assay.
4. To determine the inhibition effect of identified targets on PDAC (MIA PaCa-2) cell line using cell-based assays (cell proliferation XTT assay, cell cycle, cell migration).
5. To analyse the proteomic profile of MIA PaCa-2 cell lines following complement inhibition using SWATH-mass spectroscopy.

This thesis is structured into six chapters, with the initial chapter providing an introduction and comprehensive review of the literature concerning pancreatic ductal adenocarcinoma. The second chapter elaborates on the expression profile of immune response-related genes in PDAC tumours. The third chapter focuses on the circulating levels of C3 and C5 in the plasma of PDAC patients. The fourth chapter investigates the effect of pharmacological inhibition of the complement pathway in MIA PaCa-2 cells using cell-based assays. Chapter five analyses the proteomic profile of the complement pathway inhibition on PDAC cells, and the last chapter is the overall discussion and conclusion.

CHAPTER 2

2. EXPRESSION OF IMMUNE RESPONSE-RELATED GENES IN TUMOURS

2.1 Tumour microenvironment and immune response in PDAC

The tumour microenvironment (TME) is an ecosystem that surrounds a tumour and provides for its needs, such as nutrition and protection. It comprises immune cells, extracellular matrix (ECM), blood vessels, cancer-associated fibroblasts (CAFs), and stromal cells. Cancers such as PDAC depend mainly on the microenvironment to promote uncontrolled cell proliferation, evade growth suppressors, activate invasion and metastasis, resist apoptosis, activate angiogenesis, maintain proliferative signalling, evade immune destruction, and promote genome instability and mutations. These are cancer characteristics, and they are responsible for forming a complex microenvironment that promotes cancer progression and makes treatment ineffective with a poor prognosis (Wang et al., 2017).

The pancreatic tumour microenvironment is very heterogeneous and characterised by a dense desmoplastic stroma and an accumulation of hyaluronan (HA). The dense desmoplastic stroma surrounding the tumour was first thought to inhibit tumour development; however, the opposite is true. PDAC uses this stroma for nourishing the tumour and promote disease progression (Zhang et al., 2022). Targeting components of the tumour microenvironment, such as degrading hyaluronan by using a PEGylated human hyaluronidase (PEGPH20), may increase drug delivery (Murakami et al., 2019). Targeting other pathways involved in the TME has been proposed as a promising strategy for treating PDAC (Duan et al., 2022).

The PDAC tumour microenvironment has substantial immunosuppression and cancer-associated fibroblasts (CAFs), which provide cancer cells nutrients in cases of glucose depletion and induce dense desmoplasia, which promotes cancer cell migration, immune evasion and block vascularisation (Gorchs and Kaipe, 2021). The PDAC tumour microenvironment contains highly immunosuppressive components such as cancer cells, stroma and immune cells that have implications for the immune response and treatment (Chang et al., 2016; Ene-Obong et al., 2013; Feig et al., 2013; Homma et al., 2014; Pei et al., 2014; Shevchenko et al., 2013). CAFs comprise most PDAC stroma acquired from pancreatic stellate cells, distinct progenitor cells, epithelial, endothelial and mesenchymal cells. They can be activated by platelet-derived growth factor (PDGF), tumour necrosis factor- α (TNF- α), transforming growth factor β (TGF- β), sonic hedgehog, and cytokines IL-1, IL-6 and IL-10 (von Ahrens et al., 2017).

Cancer cells recruit Tregs, MDSCs and TAMs, which induce angiogenesis for the tumour's blood supply and metastasis. Pancreatic stellate cells (PSCs) make up the tumour stromal cells and contribute to the progression of PDAC. They secrete ECM (responsible for clinical manifestations when ECM-related proteins are overexpressed), matrix metalloproteinase (MMP), pro-angiogenic factors, cytokines and growth factors to maintain the microenvironment. Some types of cancers rearrange their stromal microenvironment (the supportive connective tissue) into structures similar to the lymphoid tissue of the immune system. This rearrangement is crucial in recruiting immune cells like Tregs and MDSCs to promote immune tolerance and tumour progression (Bremnes et al., 2011; Danenberg et al., 2022; Xulu et al., 2023).

The tumour microenvironment employs various mechanisms that help create a blood supply for tumour growth (angiogenesis), suppress the immune response, and induce cancer survival, invasion, and metastasis (Wang et al., 2023). Angiogenesis is induced post-activation of VEGF, Ang-1 and Ang-2 by the tumour. It down-regulates MHC 1 on CD8⁺ T cells while stimulating TAMs, MDSCs, and Tregs, which inhibit the antitumour immune response of CD8⁺ T cells. CD4⁺ T cells usually activate CD8⁺ T cells, but, in this case, they are inhibited by MDSCs. CAFs are activated by several cytokines and growth factors such as TNF- α , PDGF, IL-1, IL-6, IL-10 and TGF- β . Activation of CAFs increases desmoplasia. They stimulate the ECM, which increases cancer cell survival and invasion. CAFs are also involved in stimulating endothelial mesenchymal transition (EMT) and metastasis. Furthermore, the TGF- β also inhibits CD8⁺ T cell activation while enhancing cancer growth.

Drug targets, such as pirfenidone and PEGPH20, slow the proliferation of CAFs and break down stromal hyaluronic acid, aiding chemotherapy delivery. Immunosuppressive cells like MDSCs inhibit T cell activation; when targeted, they elicit an appropriate immune response against tumours. Tumour-infiltrating Tregs eradicate effector Tregs, while CD40-activated (Figure 2.1) (Nsingwane et al., 2020).

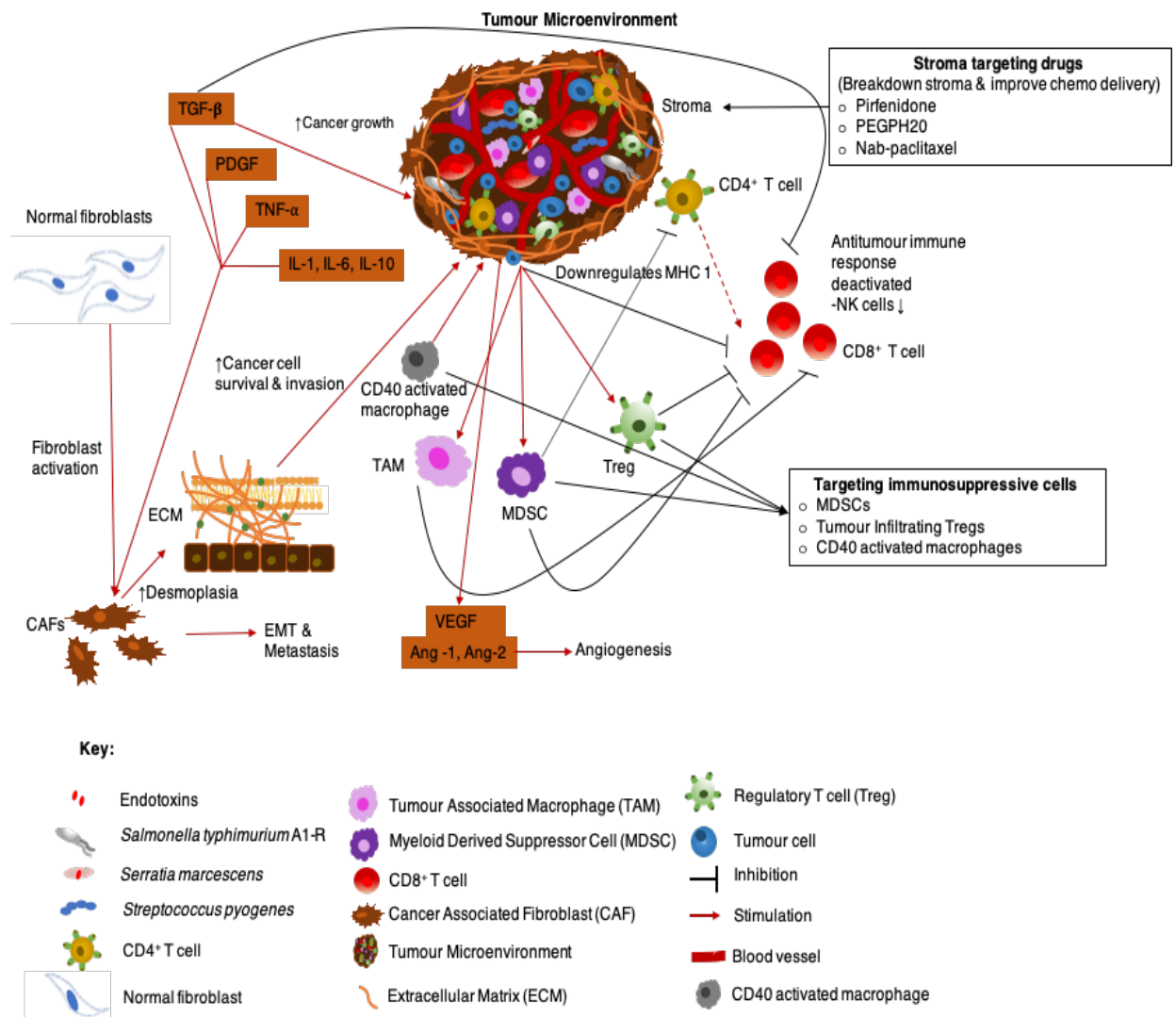


Figure 2.1: Pancreatic tumour microenvironment with potential therapeutic strategies involving stromal targeting drugs and immunosuppressive cells. The pancreatic tumour microenvironment plays a major role in PDAC progression. VEGF, Ang-1, Ang-2, MHC-1, TAMs, MDSCs, regulatory T cells, TNF-α, PDGF, IL-1, IL-6 and IL-10, TGF-β, CAFs, EMT, PEGPH20). (Nsingwane et al., 2020).

Given the complexity of the pancreatic tumour microenvironment, it is essential to thoroughly study and understand the functional role of genes and their respective pathways to target them. A comprehensive understanding of the above-mentioned might shed more light on how specific gene markers promote disease progression and how we can target them to achieve better patient outcomes.

2.2 MATERIALS AND METHODS

All equipment, reagents, kits, software and materials/consumables used in this study are in the supplementary (Tables S7, S8, S9, S10 and S11 respectively). Patients with PDAC who were undergoing the Whipple procedure were recruited, and from each patient, approximately 2mm of tumour and normal tissue were obtained after ethical clearance and patient consent. Total RNA was extracted, quantified, and cDNA synthesis performed, followed by the utilization of a pathway-focused PCR array to examine the expression of immune-related genes. Due to the pooling of tissues, qPCR was employed to confirm the observed gene expression (Figure 2.2).

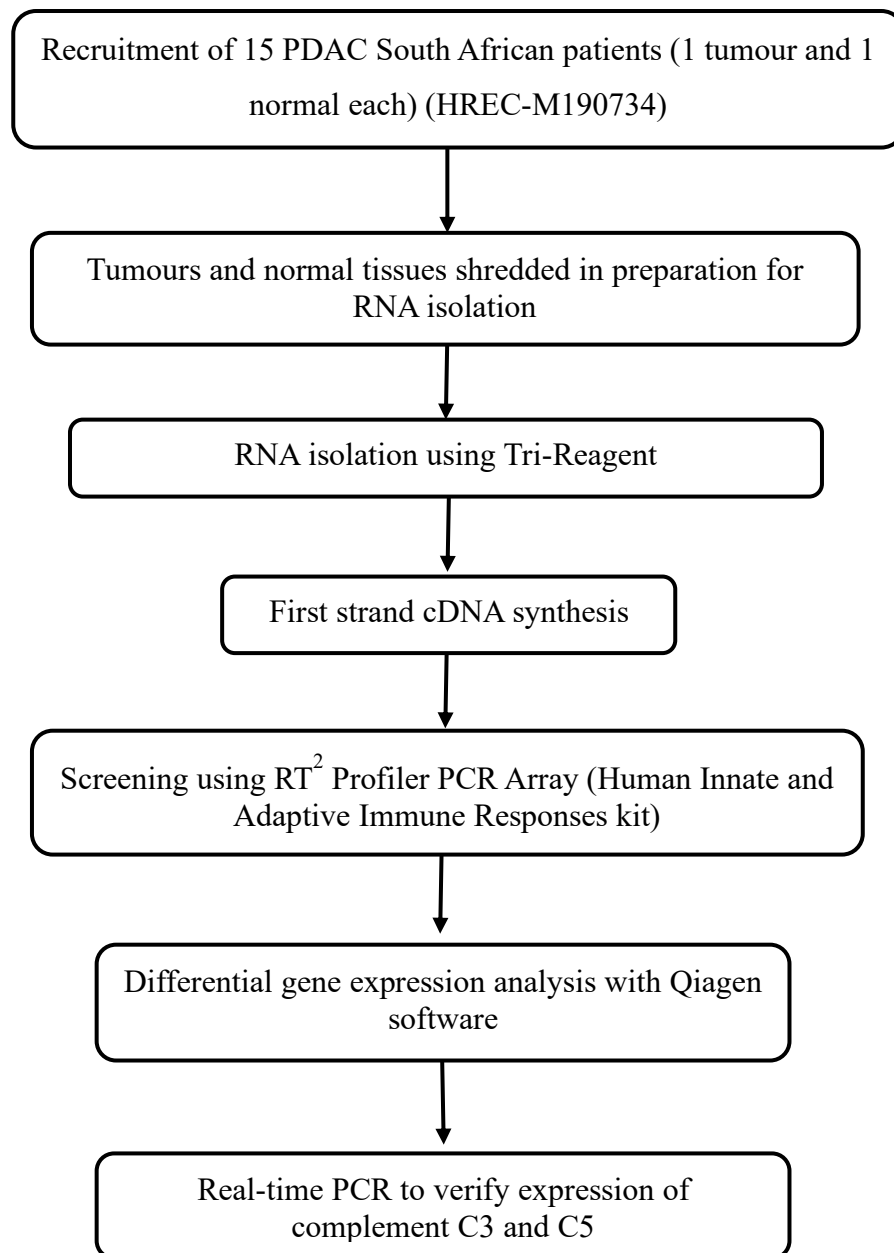


Figure 2.2: Flow diagram of the screening method for identifying potential genes.

2.2.1 Patient selection and recruitment

Patients were recruited from the Hepatopancreatobiliary (HPB) unit of Chris Hani Baragwanath Academic Hospital (CHBAH) in Johannesburg between January 2017 and March 2020. Recruited patients were self-identified black Africans. The inclusion criteria encompassed individuals aged 18 years and above, of African ancestry who were histologically diagnosed with PDAC. Patients presenting with acute inflammation, sepsis, organ failure and those undergoing immunotherapy and chemotherapy were excluded from the study. This research was conducted in accordance with the Declaration of Helsinki. All patients were clinically and histopathologically diagnosed with PDAC following the screening of the abdomen using a contrast-enhanced triple-phased CT scan. Histopathological diagnosis was either by endoscope or surgery. Endoscopic pathological diagnosis was by brush cytopathology of structures at endoscopic retrograde-cholangiopancreatography (ERCP), or biopsy of pancreas mass by endoscopic ultrasonography (EUS). All participants signed an informed consent form and ethical clearance for the use of human specimens was obtained from the University of the Witwatersrand Human Research Ethics Committee (HREC) (M190734).

Tissues: Surgery samples (a tumour and corresponding normal tissue) from fifteen patients (Nweke et al., 2020) scheduled for a Whipple procedure (surgical resection of cancerous tumours located on the head of the pancreas) were obtained and immediately immersed in RNAlater™ stabilisation solution (Invitrogen™, Massachusetts, United States) and stored at -80°C until required. Patients didn't receive neoadjuvant therapy. Analysis was conducted within six months.

2.2.2 Total RNA extraction from tissues

Total RNA was extracted using the TRIzol method (Chomczynski, 1993). Tissues (15 – 25mg) were shredded using sterilised surgical blades and transferred into 15mL Falcon tubes containing 700µL of TRIzol reagent (cat no. T9424, Sigma Aldrich, St. Louis, Missouri, United States) and then homogenised using a TissueRuptor (cat no. 9002755, Qiagen, Hilden, Germany). Samples were allowed to stand for 5 minutes at room temperature to ensure nucleoprotein complexes were fully dissociated. Chloroform (150µL) (cat no. 02487, Sigma Aldrich) was added and shaken vigorously for 15 seconds until the mixture turned milky and left to stand for 8 minutes before being centrifuged at 12 000xg for 10 minutes at 4 °C. The aqueous phase was transferred into sterile Eppendorf tubes, and 350µL of isopropanol (cat no. 9516, Sigma Aldrich, St. Louis, Missouri, United States) was added to the sample, mixed by resuspension and allowed to stand for 7 minutes. The mixture was centrifuged at 12 000xg for 8 minutes at 4 °C. The supernatant was discarded, and the pellet was washed with 700µL of

75% cold ethanol (cat no. 34870, Sigma Aldrich, St. Louis, Missouri, United States). The sample was vortexed for 5 seconds and centrifuged at 7500xg for 5 minutes at 4°C. The supernatant was discarded, and the pellet was air-dried for 10 minutes and placed into a heating block (55°C) for further drying. A total of 30µL nuclease-free water (cat no. 129317, Qiagen, Hilden Germany) was added to the pellet, mixed repeatedly with a pipette, placed in the heating block for 5 minutes at 55°C, and immediately placed on ice. Total RNA concentration and quality were assessed using the Nanodrop® ND 1000 (Thermo Fisher, Waltham, Massachusetts, United States) with RNA averaging 1955 ng/µl and 1162 ng/µl for normal and tumour tissues respectively. The A260/A280 and A260/230 of the samples were all above 2.0.

2.2.3 cDNA synthesis for pathway-focused PCR array analysis

The RT² first strand kit (cat no. 330401, Qiagen, Hilden, Germany) was used for cDNA synthesis per the manufacturer's instructions from 2µg of total RNA. Each of the 15 normal and tumour tissues were pooled into two different PCR tubes labelled normal and tumour, followed by RNA quantification using the Nanodrop®. The RT² first strand reagents were thawed and briefly centrifuged for 15 seconds to bring contents to the bottom of the tube, followed by genomic DNA elimination. For each group, genomic DNA elimination mix was prepared by adding 0.8µl RNA, 2µl buffer GE and 7.2µl RNase-free water in a PCR tube (normal), 0.5µl RNA, 2µl buffer GE and 7.5µl RNase-free water (tumour). Contents were mixed by resuspension, centrifuged briefly, incubated at 42° C for 5 minutes and placed immediately on ice for 1 minute. Reverse transcription mix was prepared as follows: 5X buffer BC3 (8µl), control P2 (µl), RE3 Rev transcriptase mix (4µl) and RNase-free water (6µl) were added in a PCR tube and mixed. Reverse transcription mix (10µl) was added to each tube containing 10µl of the genomic DNA elimination mix, resuspended and incubated at 42° C for 15 minutes and 95° C for 5 minutes to stop the reaction. RNase-free water (91µl) was added to each sample, resuspended and stored at -20° C.

2.2.4 Pathway-focused PCR array analysis

The RT² SYBR green Mastermix (cat no. 330500, Qiagen, Hilden Germany) and the RT² Profiler PCR Human Innate and Adaptive immune responses (Cat no. PAHS-052ZA 330231, Qiagen) panel were used to perform a pathway-focused gene expression analysis of immune response genes. RT² SYBR green mastermix (1350µl) was centrifuged to bring contents to the bottom of the tube. Then, total RNA sample (102µl) and 1248µl RNase-free water were added to a 5ml Falcon tube, resuspended and poured into a loading reservoir. A multi-channel pipette was used to transfer 25µl of the mixture from the loading reservoir to each well of the RT²

Profiler PCR array, Human Innate and Adaptive immune responses kit. The plate was sealed with optical thin-wall 8-cap strips and centrifuged at 700 rpm for 1 minute at room temperature (25° C) to remove any bubbles and then processed on a QuantStudio1 real-time thermocycler (cat no. A26332. 366.00, Thermo Fisher Scientific). The Ct values were exported, and fold changes were analysed using the QIAGEN RT² Profiler PCR analysis software tool (<https://www.qiagen.com/us/product-categories/instruments-andautomation/analytics-software> accessed 12th September 2019).

2.2.5 cDNA synthesis and verification of gene targets using real-time PCR

Verification of gene targets on the tissue samples was achieved by performing cDNA synthesis followed by real-time PCR according to the MIQE guidelines (Bustin et al., 2009). The Invitrogen™ SuperScript™_VILO™ cDNA synthesis kit (Cat no 11754-050, ThermoFisher Scientific) was used, followed by real-time PCR. The method for cDNA synthesis involved adding 4µl of the 5X VILO™ reaction mix, 2µl 10X SuperScript™ enzyme mix, 2.5µg RNA in each tube placed on ice and adding DEPC-treated water to 20 µl, gently mixed and incubated at 25°C for 10 minutes. Samples were further incubated at 42° C for 1 hour. The reaction was then terminated at 85° C for 5 minutes. Validated predesigned TaqMan Gene Expression assays (Thermo Fisher Scientific) were used for the 3 genes; *C3* (Assay ID- Hs00163811_m1), *C5* (Assay ID- Hs01004342_m1) and *MRPL19* (Assay ID- Hs00608519_m1). The method for real-time PCR involved adding 5µl of TaqMan® Gene Expression Master Mix (2X), 0.5µl TaqMan® Gene Expression Assay 20X) for target gene and reference gene, 3µl cDNA template, Human Male Raji cDNA template (controls), and 1µl nuclease-free water to a PCR plate, briefly vortexed, centrifuged and run according to the manufacturer's instructions (Thermo Fisher Scientific). Fold changes were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.2.6 Statistical and bioinformatics analyses

To compare the differences in fold change between the tumour and normal group, following normality, a one-way ANOVA was used to compare the tumour and normal samples. The differences were regarded as significant at $p < 0.05$.

2.3 RESULTS

2.3.1 Dysregulated immune response genes in pancreatic tumours

The gene expression profile of immune response genes was investigated in pancreatic tumours compared to corresponding normal tissues. Altogether, 13 and 62 genes (Table S1) were

upregulated and downregulated, respectively as illustrated by the red and green squares on the heatmap (Figure 2.3a). The complement *C3* was the most upregulated gene (fold change = 562.22) and the chemokine (C-C motif) receptor 4 (*CCR4*) was the most downregulated gene (fold change = -83.19). Furthermore, among the top 5 upregulated targets, 3 were major histocompatibility complex molecules (major histocompatibility complex class 1 A, major histocompatibility complex class 1 E, and the Beta-2-microglobulin), and their expression levels were 20 -100 times lower compared to that of complement *C3* (Table 2.1).

Due to the fact that complement *C3* highest fold change and its involvement in many processes linked to tumour progression such as inflammation and immune response, it was selected as a gene of interest in this study. Real-time PCR was then used to verify the expression of the complement *C3* in the sample groups as samples were pooled previously for the RT² profiler PCR array due to costs. The real-time PCR verified the increase of the complement *C3* in pancreatic tumours (125.3 ± 393.79) compared to the normal tissues (61.9 ± 162.09), although the increase was not significant ($p=0.72$).

The expression of another crucial downstream component of the complement pathway, *C5*, which was not included in the PCR array, was also evaluated. A significant increase in the expression of complement *C5* in pancreatic tumours (59.8 ± 166.42) compared to normal tissues (5.4 ± 2.58) was observed ($p<0.0001$) (Figure 2.3b).

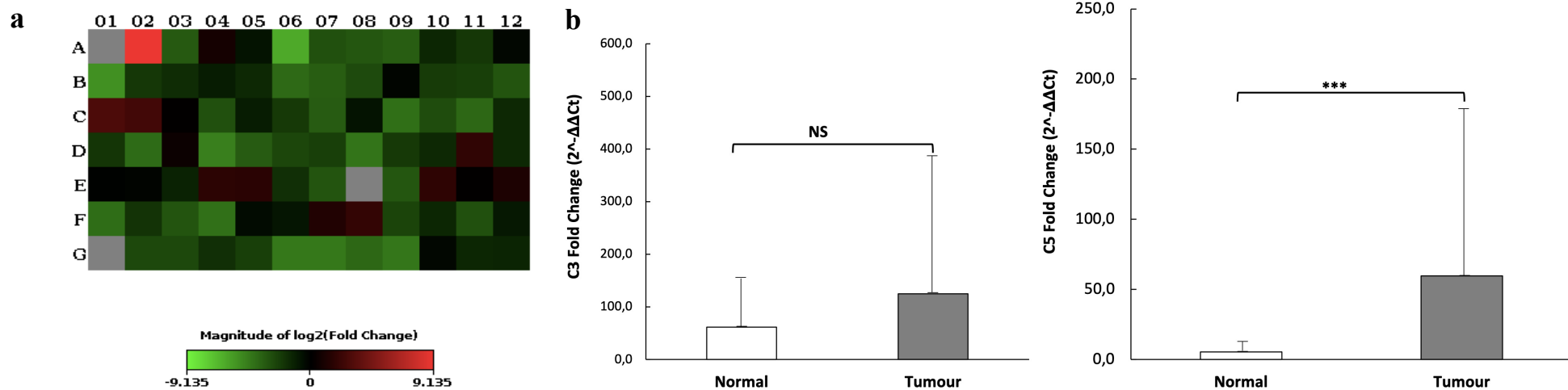


Figure 2.3: Gene expression profile of immune response genes in PDAC tumours. (a) Heatmap showing differentially expressed genes in the form of fold regulation. Red represents upregulated genes with positive values, and green represents downregulated targets with negative values. (b) Real-time PCR verifying the gene expression profile of complement *C3* and *C5* in resectable PDAC tumours. *C3* and *C5* are upregulated in pancreatic tumours and downregulated in normal tissues. Values are indicated as mean $\pm$ SD, The experiment was conducted twice n=2. NS indicate non-significant, ***p<0.0001.

Table 2.1: Top 5 upregulated and downregulated immune targets with their respective fold changes

Upregulated Targets	Fold change	Downregulated Targets	Fold change
Complement C3	562.22	Chemokine (C-C motif) receptor 4	-83.19
Beta-2-microglobulin	27.30	CD40 ligand	-44.48
Major Histocompatibility Complex Class 1 A	7.84	Interleukin 2	-28.49
Ribosomal protein large P0	6.64	Toll-Like Receptor 7	-22.75
Major Histocompatibility Complex Class 1 E	5.98	Toll-Like Receptor 6	-22.48

2.3.2 Gene network and functional pathway analysis

The RT² profiler PCR array used comprised multiple genes whose roles were analysed using the Qiagen software. The differentially expressed genes were grouped according to their function, e.g., whether they function in the innate or the adaptive immune response based on their classification in the array panel. It was observed that most of the genes investigated function in the innate immune response. The adaptive immune response genes investigated mainly function as cytokines and Th2 markers (Figure 2.4).

Upon inspecting the top 5 up- and downregulated genes, and excluding those that function as housekeeping/reference genes, eight dysregulated genes were analysed. Furthermore, it was observed that these genes predominantly function in the innate immune response, although they have some roles in the adaptive immune response. The complement *C3* and the chemokine receptor 4 were the top up- and downregulated genes respectively. They were shown to function in multiple pathways, either innate or adaptive based on the gene pathway and interaction analysis. The complement *C3* was shown to function in the innate immune, humoral and inflammatory response, whereas the chemokine receptor 4 functions exclusively in the adaptive immune response and as a marker of Th2 cells and Tregs within the adaptive pathway (Figure 2.4).

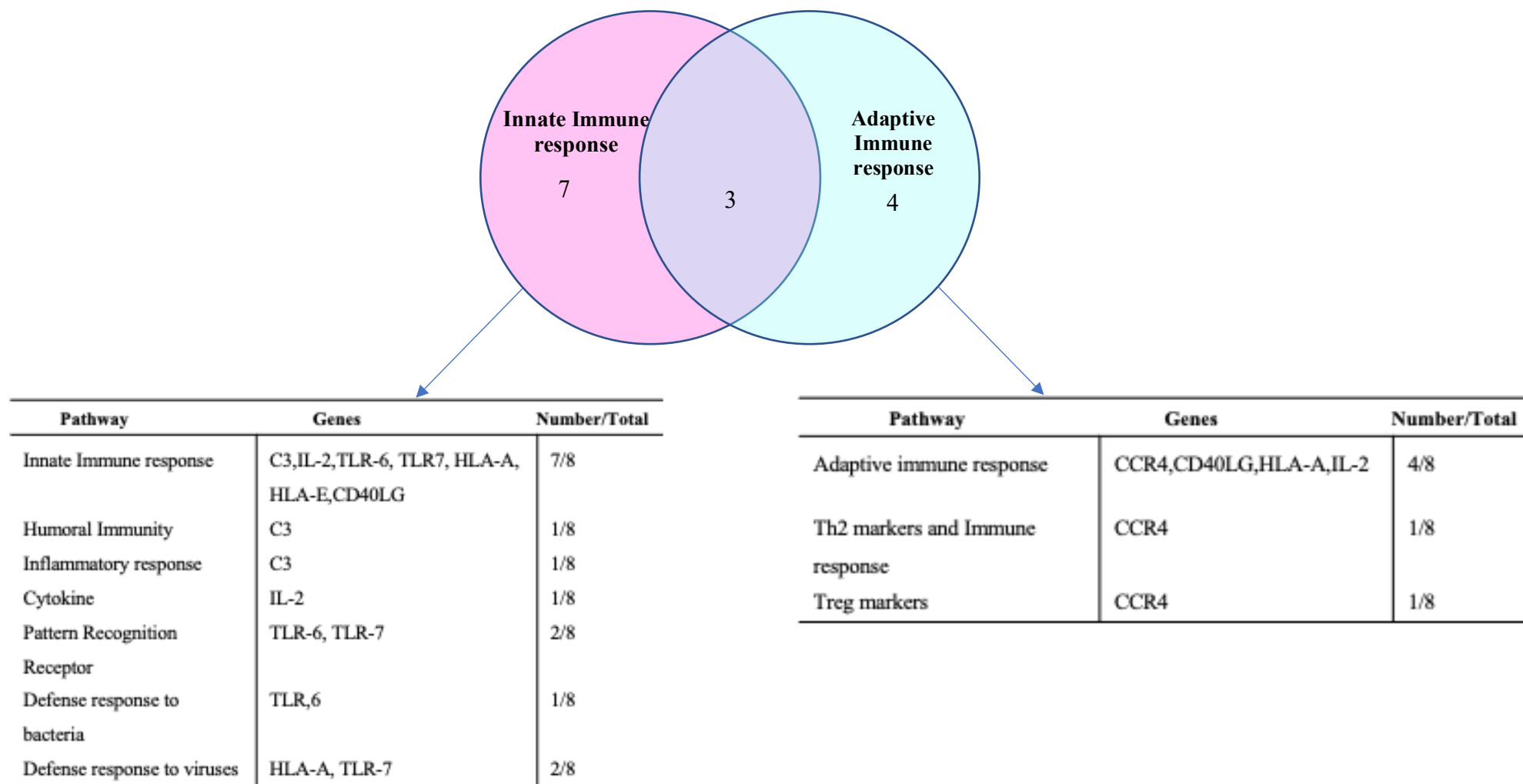


Figure 2.4: Gene expression profiles of immune response genes in PDAC tumours. The Venn diagram shows that most genes function predominantly in the innate immune response but also have some role in the adaptive immune response.

2.4 DISCUSSION

PDAC has a complex molecular landscape and a heterogeneous tumour microenvironment, rendering treatments ineffective (Narayanan et al., 2021). The lack of early diagnostic and prognostic biomarkers and ineffective therapeutic options contribute to the factors that result in poor outcomes. Patients diagnosed with PDAC do not survive past five years regardless of surgery or best standard of care chemotherapy, and radiation (Siegel et al., 2022). Furthermore, the lack of an appropriate immune response, in addition to cancer cell immune evasion, makes early diagnosis impossible and manufacturing effective anti-cancer therapeutics unfeasible. Nonetheless, immunotherapy seems like a strategy with more potential to treat PDAC. Investigating and understanding key targets that regulate the immune response and their underlying mechanisms is very important to developing innovative treatments.

In this study, 15 consenting South African PDAC patients scheduled for a Whipple procedure (surgical resection of cancerous tumours located on the head of the pancreas) were recruited. A resectable tumour sample and a corresponding 'normal' tissue were obtained from each patient, adding up to 30 tissue specimens. Total RNA was isolated, and pathway-focused PCR arrays were used to identify differentially expressed genes. The results showed an overexpression of the complement component *C3*, which was further verified with real-time PCR.

The RT² Profiler PCR array determined the level of expression of multiple genes relevant to the innate and adaptive immune responses. A total of 13 genes were overexpressed, and 61 were downregulated. From the 13 upregulated targets, it was no surprise that *STAT1* and *STAT3* were among them. These genes are commonly known to be involved in the JAK/STAT pathway, where they induce the gene expression of the vascular endothelial growth factor, which induces angiogenesis in pancreatic cancer. *STAT3* has also been shown to play a critical role in KRAS-induced pancreatic tumorigenesis (Corcoran et al., 2011). Other genes, such as the *IFR3*, *MYD88*, *LYZ*, *ACTB* and *NFKB1A*, were observed to be overexpressed. These have various roles in cancer development and progression and have been thoroughly studied previously, and their fold change was below four. The top five up and downregulated targets have interesting roles in cancer.

2.4.1 Expression of MHC I and related genes in PDAC

The major histocompatibility complex (MHC) class I antigen presentation pathway is essential in alerting the immune system about threats and infected cells. Most cancers target the loss of MHC class I antigen presentation as a mechanism to evade immune recognition (Dhatchinamoorthy et al., 2021). Three out of five identified upregulated immune genes were related to the MHC and an MHC subunit, β 2-microglobulin (*B2M*), and the ribosomal protein,

large, P0 (*RPLP0*). However, the over-expression of these genes was still 20-100 fold less than that of the complement *C3*. The expression of MHC is often altered on epithelial cells of several solid tumours. Previous literature has shown a strong correlation between MHC expression, disease severity and activity of intra-tumoural inflammation in PDAC (Gaida et al., 2012). This suggests that the expression observed on the tested resectable tissues may change as PDAC progresses to exhibit the behaviour of all cancers which downregulate the MHC I (Dhatchinamoorthy et al., 2021; Huang et al., 2020; Taylor and Balko, 2022), depending on the level of inflammation. The overexpression of the MHC 1 and its related genes in this study can be explained by a study done by Garrido and colleagues (Garrido et al., 2016), where mice with fibrosarcoma displayed substantial heterogeneity in the primary tumours. This study showed that primary tumours display diverse expression patterns of MHC-1 genes and molecules, suggesting that, depending on the level of inflammation, the expression of the MHC I may change as the cancer progresses.

β 2-microglobulin (*B2M*) has an immunological role as an *HLA class I* molecule and a prognostic marker for renal diseases. It plays an essential role in regulating precursor-like protein 2 and the migration of PDAC cells. The *B2M* gene is constantly expressed instead of downregulated in several tumour types (Liu et al., 2015; Sliker et al., 2019a; Zhang et al., 2021) and PDAC cell lines, including PANC-1 and MIA PaCa-2, corroborating its expression in this study. The role of *B2M* in PDAC was investigated by knocking down its expression by siRNA transfection, where it reduced the rate of migration of PANC-1 cells but increased migration in MIA PaCa-2 cells (Sliker et al., 2019b). Sliker and colleagues proved that, although B2M is a member of the MHC I family of molecules, it is perpetually expressed in human PDAC cell lines and not lost or downregulated like other MHC -1 genes in all cancers (Sliker et al., 2019b).

The human ribosomal protein, large, P0 (*RPLP0*), is encoded by the *RPLP0* gene in humans and forms part of the ribosomes that catalyse protein synthesis. Many ribosomal proteins (RPs) are overexpressed in PDAC and have been implicated in numerous pathological processes, especially tumourigenesis and cell transformation (El-Khoury & Nasr, 2021). The effects of PRs are associated with KRAS, which has a well-recognised role in PDAC, which initiates the transformation of normal pancreatic cells into cancerous cells and driving tumour progression. They are suspected to play a role in the initiation and progression of PDAC, where most of them (*RPSA*, *RPS15A*, *RPL26*, *RPL29* and *RPL34*) have been thoroughly studied and could be used as targets and biomarkers for PDAC. *RPLP0* is usually used as a reference gene in most studies due to its role in maintaining basic cellular functions as well as its expression in all human cells in normal pathophysiological circumstances (Ahn et al., 2021; Fan et al., 2020;

Ren et al., 2021). The expression of *RPLP0* was observed to be overexpressed in pancreatic tumours in this study corroborating with published literature (Artero-Castro et al., 2011; El-Khoury and Nasr, 2021). *RPLP0* is a prognostic marker for renal cancer (Uhlén et al., 2015). It has been studied in several cancers and its overexpression is observed to increase in gynecological cancers (Artero-Castro et al., 2011), increase apoptosis and decrease cell viability and proliferation in cervical cancer (El-Khoury & Nasr, 2021). Furthermore, a reduced expression of *RPLP0* was shown to suppress cell growth and cell cycle progression through CDK2 and increase cell apoptosis in gastric cancer cells (Teller et al., 2015).

2.4.2 *CCR4*, *CD40* ligand, *IL-2* and *TLR 6* and *7* are downregulated in PDAC

The top five downregulated genes comprised the chemokine receptor 4 (*CCR4*), cluster of differentiation 40 ligand (*CD40LG*), Interleukin-2 (*IL-2*) and the toll-like receptor 6 and 7 (*TLR-6* and *TLR-7*). These genes have important roles in the innate immune response with the *CCR4*, which is commonly known for its roles in the adaptive immune response (Khanam et al., 2023), whereas the *CD40LG* and *IL-2* function in both the innate and adaptive immune response. The *CCR4* is a marker expressed by Th2 and Tregs. It has been shown to have a role in PDAC progression where it is associated with elevated EMT, which invigorates an immunosuppressive tumour microenvironment and promotes resistance to immunotherapies (Sharma et al., 2022). *CCR4* is differentially expressed in different kinds of cancers such as lung squamous carcinoma, and uterine carcinoma (Song et al., 2021), and it is associated with controlling tumour trafficking of Tregs and recruitment of Th2 subsets, which promotes a highly immunosuppressive tumour microenvironment (Uhlén et al., 2015). Additionally, *CD40LG* and *IL-2* also have roles in the adaptive immune response. The downregulation of these genes could hint that the adaptive immune response might be active but not high functioning at this stage.

On the contrary, toll-like receptors 6 and 7 function in the defence against bacteria and viruses. *TLR-7* has been shown to increase tumour growth and proliferation in PDAC when overexpressed (Słotwiński and Słotwińska, 2017). The downregulation observed could suggest that the innate response is compromised which may enable PDAC cells to evade detection and subsequently proliferate (Orlacchio and Mazzone, 2021); (Urban-Wojciuk et al., 2019). Lastly, TLRs function to trigger inflammation in response to infection or tissue damage (Orlacchio and Mazzone, 2021). The downregulation of TLRs could indicate reduced inflammation in the PDAC microenvironment by recruiting immune cells to the tumour site for surveillance and elimination.

2.4.3 Complement *C3* and *C5* are overexpressed in pancreatic tumours

Complement *C3* and *C5* were overexpressed in PDAC tumours compared to normal tissues. Although verification studies showed that *C3* was overexpressed in tumours and not in normal tissues, its overexpression was not significant likely due to the small sample size. However, the complement *C5* was found to be significantly elevated in tumours. The complement *C3* is a central protein responsible for activating the complement system (a major component of the innate immune system). Activation of complement results in cell opsonisation, killing pathogens through the membrane attack complex (MAC) and inducing inflammation responsible for cancer initiation and progression. Complement signalling is known to impede anti-tumour immunity (Kolev et al., 2022). PDAC cells hijack normal cellular processes to foster tumour growth and proliferation. One possible mechanism is increasing the levels of *C3* and *C5* leading to the production of *C3a* and *C5a* and the subsequent recruitment of immune cells to the tumour site by chemotaxis (Zhou et al., 2022). This immune response can aid tumour growth by secreting cytokines like $\text{TNF-}\alpha$ and IL-6 which promote angiogenesis and subsequently supply the growing tumour with nutrients and oxygen required for survival (Geindreau et al., 2022).

Furthermore, the production of *C3a* and *C5a* can attract immunosuppressors such as $\text{TGF-}\beta$ and VEGF and suppressive myeloid cells to the tumour microenvironment and inhibit the anti-tumour functions of CD4 and CD8 cells (Thurman et al., 2020). However, the expression of these complement components may vary based on the type of cancer and their expression may not follow a consistent pattern across all cancers. For instance, *C3* is overexpressed in almost all cancer types but *C5* is downregulated in ovarian serous cystadenocarcinoma, uveal melanoma, Bladder carcinoma, rectum adenocarcinoma, and colon adenocarcinoma (Cerami et al., 2012; Roumenina et al., 2019). The behaviour of complement factors can vary widely even within the same type of cancer and this is dependent on the tumour microenvironment, tumour type and tumour stage (Corrales et al., 2012; Kurihara et al., 2010; Thurman et al., 2020; Zhang et al., 2019a). This implies that PDAC may increase *C3* and *C5* to support tumour growth and facilitate the development of a suppressive microenvironment, as observed in this study.

2.5 CONCLUSION

This study showed the expression of complement *C3* and *C5* to be elevated in tumours compared to corresponding “normal” tissues. The level of expression of both complement *C3* and *C5* hint that the complement system has a unique role in PDAC, either in the development or progression of the disease and needs to be further investigated. As this study utilized a small sample size, a larger patient cohort needs to be investigated to validate these findings.

Furthermore, this study utilized whole surgical tumour which makes it difficult to assess the expression of these targets within the heterogenous tumour microenvironment. Future studies would need to utilize appropriate technologies, such as spatial transcriptomics or single-cell sequencing, enabling the characterization of gene expression patterns at the cellular level within the tumour microenvironment context to address this challenge.

CHAPTER 3

3. CIRCULATING LEVELS OF COMPLEMENT C3 AND C5 IN PLASMA

3.1 The Complement system

The complement system is an enzyme cascade that forms part of the innate immune system with defensive mechanisms that protect against invading pathogens. It enhances the innate immune response, killing infectious pathogens and eliminating damaged cells. Studies have shown an additional role of complement in adaptive immunity, specifically the humoral antibody response (Bennett et al., 2017). The complement system plays a significant role in immunosurveillance and homeostasis (Ricklin et al., 2010). It comprises more than 50 plasma, serosal and serum proteins produced by the liver and found in the circulating blood (Kolev and Kemper, 2017). These proteins play a role in host defence. Their primary responsibility in the immune system is to clear invading infectious pathogens by lysis, opsonisation, and activation of inflammation (Sandhu and Quan, 2017). Inflammation is a protective immune response that involves host cells, blood vessels, proteins and other mediators and aims to eliminate foreign invaders and damaged cells. It can be classified into acute and chronic inflammation. Acute inflammation is characterised by a buildup of neutrophils; it has a rapid onset and lasts for a short period (from minutes to a few days). Chronic inflammation is characterised by a rush of macrophages and lymphocytes and is a prolonged event with a longer duration (days to years) (Chen et al., 2017; Newson et al., 2017).

The complement system can be activated in one of three pathways: classical, mannose-binding lectin and alternative pathways that trigger a cascade reaction like the coagulation cascade where components are activated in a specific order to reach an absolute result. The classical pathway is evolutionary, the youngest of the three and can be activated directly by pathogens or indirectly by binding C1q with an antibody (IgG or IgM) attached to an antigen and forming a complex. The mannose-binding lectin pathway is activated following mannose-binding lectin (MBL) binding with specific carbohydrates. The alternative pathway is evolutionary, the oldest complement pathway discovered, which can be activated by bacteria, fungi, viruses, parasites, cobra venom, polysaccharides, and immunoglobulin A (IgA) (Bain et al., 2020; Dodds, 2002). All three pathways converge at the formation of complement C3 (central protein of complement), which functions as a master switch that controls all downstream effectors that ultimately cause cell destruction and induce inflammation-mediated tissue damage. Complement component C5 is another important complement protein downstream of this

pathway, which kills pathogens through the membrane attack complex (MAC). Complement C5 also contributes to inflammation by binding to mast cells to increase inflammation (Zhang and Kurashima, 2021).

Complement activation is tightly regulated by various soluble and membrane-bound complement regulatory proteins (CRPs) to circumvent self-harm (Zipfel and Skerka, 2009). Activation of C3 and C5 form C3a and C3b, C5a and C5b respectively. C3a and C5a are potent anaphylatoxins that promote inflammation and prompt multiple effects, including attracting neutrophils and monocytes to the site of complement activation. C3a and C5a further bind their respective receptors, C3aR and C5aR, that belong to the family of transmembrane G protein-coupled receptors and were recently identified as a new class of immune checkpoint receptors that can be targeted for cancer immunotherapy (Wang et al., 2019). Complement signalling by C3aR and C5aR expressed on T effector cells was shown to block cytokine interleukin 10 (IL-10) production, associated with an immunosuppressive role (Wang et al., 2016).

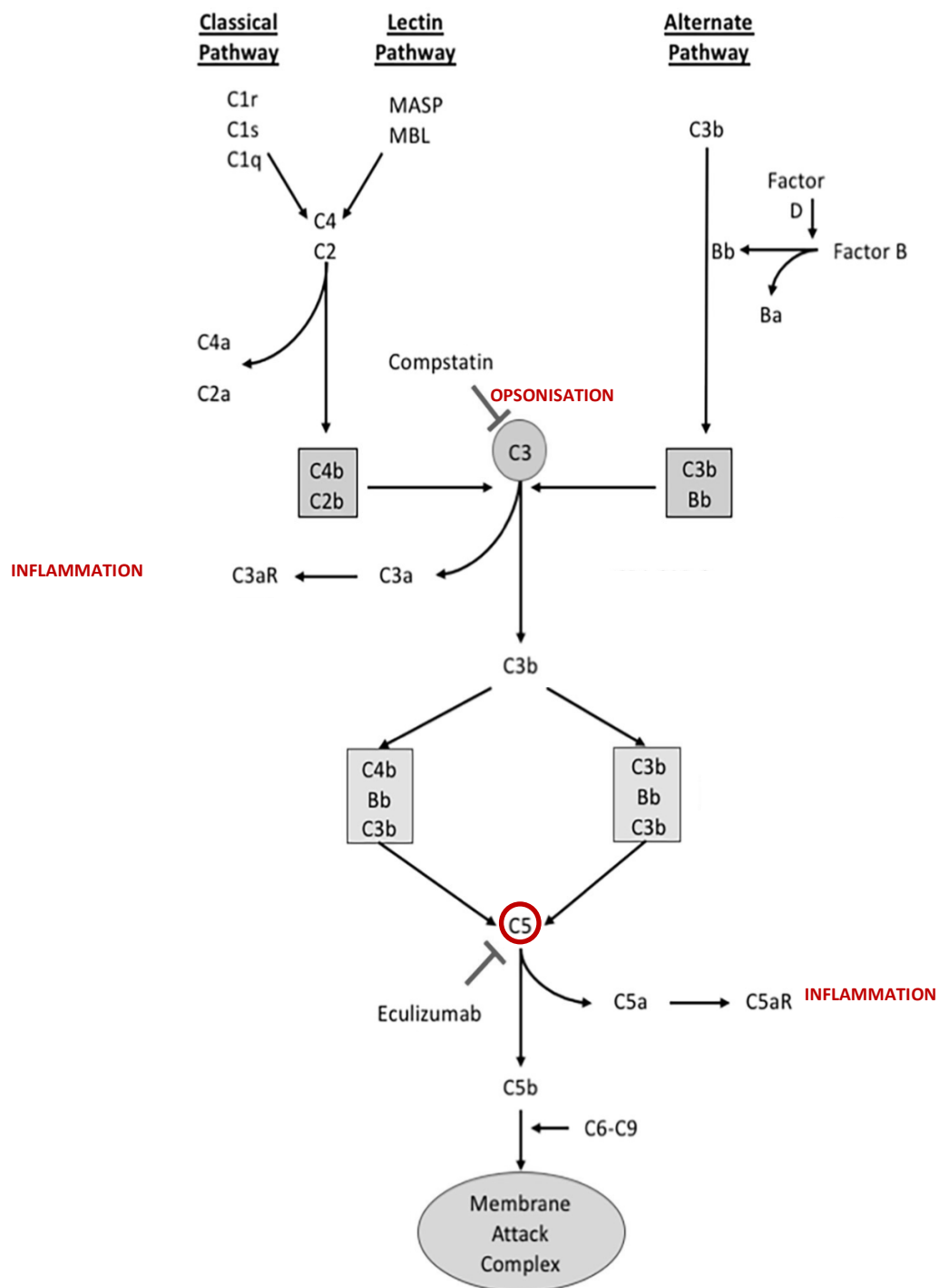


Figure 3.1: The complement cascade/pathway. The complement cascade consists of three pathways: classical, lectin/mannose-binding, lectin and alternative pathway, which all merge at C3. Complement C3 plays a crucial role in activating complement and inflammation.

Due to the crucial role of the complement system, it has been a critical target for immunotherapy and most inhibitors of different complement components developed for various immunological diseases. Some complement-targeting drugs approved by the Food and Drug

Administrator (FDA) for treating immunological diseases such as paroxysmal nocturnal haemoglobinuria and thrombotic microangiopathy include Compstatin and eculizumab (Cassol et al., 2019; Ricklin and Lambris, 2007). Compstatin is a safe and effective inhibitor of complement activation. It does this by binding C3 and inhibiting convertase from forming C3a and C3b and all downstream effects, including forming C5 and the membrane attack complex (MAC). Eculizumab inhibits the cleavage of C5 into C5a and C5b and prevents the generation of the terminal complement complex C5b-9 (Talaat et al., 2022). Previously, the complement system was considered to function only as an antibacterial mediator; however, recent studies have identified its role within the tumour microenvironment (TME) (Kleczko et al., 2019; Ricklin et al., 2010). The role of complement in humoral innate immunity and its ability to switch from anti-tumoural to pro-tumoural depending on the type of cancer provides an opportunity to explore it further. A thorough understanding of the complement system and the role of its components in cancer and the TME could lead to a significant advancement in cancer immunotherapy. Given the overexpression of complement C3 and C5 in tissues, this chapter aims to investigate the expression profile of complement C3 and C5 in the circulation of PDAC patients in different stages of the disease.

Upon activation of the complement system, infiltrating cells together with tissues locally secrete complement system components and induce an intracellular activation of the complement pathway. Determining the levels of these complement system components as PDAC progresses will help establish a link between these components and their potential role in PDAC progression. Importantly, due to genetic or environmental factors, the circulating levels of molecules linked to tumourigenic processes may differ across different population groups. An increased incidence of PDAC is expected in individuals of African descent compared to other ethnic groups (Hu et al., 2021). Therefore, in this study, the circulating levels of complement components were evaluated in patients of African ancestry who had different stages of PDAC.

3.2 METHODS

3.2.1 Patient selection and recruitment

Patients were recruited from the hepatopancreatobiliary (HPB) unit of Chris Hani Baragwanath Academic Hospital (CHBAH) in Johannesburg between March 2019 and March 2020. Criteria for selection of PDAC patients are described in section 2.2.1.

Plasma: A separate cohort of twenty-five PDAC patients (Thirteen resectable, eight locally advanced and four metastatic) categorised according to the National Comprehensive Cancer

Network guidelines (NCCN) (Tempero et al., 2021) and six chronic pancreatitis patients (an inflammatory disease affecting the pancreas) were recruited as control group as it has been identified as a risk factor for PDAC (Kim et al., 2023). Individuals (three) who self-reported being in good health and not taking any medication were recruited as healthy control participants. Acknowledging the limited sample sizes in these studies is crucial, especially given the challenges posed by sample collection, particularly during the COVID-19 pandemic. Sourcing samples, especially from patients, has indeed been difficult, and the associated costs of analyses further compounded the issue. Regrettably, the timing constraints imposed by the pandemic necessitated a balance between study completion and sample availability.

3.2.2 Sample processing

Whole blood (10ml) was collected into anti-coagulant Ethylenediaminetetraacetic acid EDTA vacutainer tubes (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) by venepuncture and processed within two hours of collection. The blood was separated by gravity separation and then centrifuged at 3000xg at 4°C for 30 minutes, and the isolated plasma was aliquoted (500 µl) into Eppendorf tubes. All samples were stored in a -80° C freezer until required, and analysis was conducted within six months.

3.2.3 Quantification of circulating levels of complement proteins using ELISA

The human complement C3 and C5 ELISA kits (Cat no. ab108822 and ab125963, Abcam, Cambridge, United Kingdom) were used to measure plasma C3 and C5 quantitatively across PDAC patients in different stages (resectable, locally advanced and metastatic), chronic pancreatitis patients and healthy individuals. The enzyme-linked immunosorbent assay (ELISA) is an immunoassay that is identified as a gold standard for immunoassays (Alhajj and Farhana, 2022). It is used in many research areas. ELISA is a highly sensitive plate-based technique commonly used to detect and quantify the expression of soluble substances such as proteins, peptides, antigens, antibodies and hormones in blood serum or plasma. The principle of an ELISA involves the binding of a primary antibody to a specific antigen then, a secondary antibody binds to the primary antibody and the substrate added is converted by an enzyme-linked antibody to produce a coloured product which can be measured by a colourimeter.

Procedure

The human complement C3 and C5 ELISA kits (Cat no. ab108822 and ab125963, Abcam, Cambridge, United Kingdom) were used to measure plasma C3 and C5 quantitatively across PDAC patients in different stages (resectable, locally advanced and metastatic), chronic pancreatitis patients and healthy individuals. Based on the manufacturer's recommendation, plasma samples were diluted 1:800 into 1X diluent M for C3 and 1:20 000 into 1X diluent N

for C5. Wash buffer, streptavidin-peroxidase conjugate and 1X biotinylated complement C3/C5 (1X), seven standards and a blank were prepared according to the manufacturer's protocol.

Samples: 25µl, 50 µl and standards were added to each well of a pre-coated and blocked 96-well microtiter plate for C3 and C5, respectively. For C3, 25µl of 1X biotinylated complement C3 antibody was added and incubated at room temperature for 2 hours. For C5, 50µl of 1X biotinylated complement C5 antibody was added and incubated for 1 hour. Plates were washed as per the manufacturer's guidelines, followed by the addition of 50µl 1X streptavidin-peroxidase conjugate to all wells for 30 minutes at RT. Stop solution (50µl) was added to each well, and the colour change from blue to yellow and absorbance was measured at 450nm immediately using the Biochrom Anthos 2010 Microplate reader (Biochrom Ltd, Cambridge, United Kingdom).

3.2.4 Statistical analysis

The clinical data for patients who gave written consent was collected using the REDCap v9.0 electronic data capture tools hosted at the University of the Witwatersrand (Harris et al., 2019, 2009) between March 2019 and March 2020. Data was analysed using the statistical software SPSS v.26 and STATISTICA v.13.5 with ELISA quantified data to generate graphs. The Kruskal-Wallis rank sum and Fisher's Exact tests were employed for the ELISA analysis to assess the differences between continuous and categorical variables, respectively. Due to the small sample size, the Mann-Whitney U test was performed for *post-hoc* analysis. Spearman correlation analyses were conducted, and the median concentration test looked at C3 and C5 ratios, C3 and C5 averages and the C-reactive protein levels in different stages of PDAC observed. The differences were regarded as significant at $p < 0.05$ for all tests.

3.3 RESULTS

3.3.1 Demographic and clinical characteristics of the patient cohort

3.3.1.1 Demographic information of patients

The demographic information of chronic pancreatitis and PDAC patients included age, gender, smoker, alcohol, and HIV status. There was no statistical difference observed for all demographic parameters ($p > 0.05$), indicating that the groups were well-matched. Of the chronic pancreatitis patients, 83.3% were smokers. Fifty percent of chronic pancreatitis, locally advanced and metastatic patients self-reported consuming alcohol. In the resectable group, only 38.5% consumed alcohol.

Table 3.1: Demographic information for chronic pancreatitis and PDAC patients

Parameters	Chronic Pancreatitis (n=6)	Resectable (n=13)	Locally Advanced (n=8)	Metastasis (n=4)	p-value
Age, Median [Range]	51 [46 57]	65 [54 66]	56.5 [43 61]	56 [46 76]	0.355
Gender					0.367
Male, n (%)	6 (100)	10 (77)	6 (75)	2 (50)	
Female n (%)	0 (0)	3 (23)	2 (25)	2 (50)	
Smoker					0.685
Yes n (%)	5 (83.3)	7 (53.2)	4 (50)	2 (50)	
No n (%)	1 (16.7)	6 (46.2)	4 (50)	2 (50)	
HIV Status					0.650
Positive n (%)	1 (16.7)	1 (7.7))	2 (25)	0 (0)	
Negative n (%)	5 (83.3)	12 (92.3)	6 (75)	4 (100)	
Alcohol					0.957
Yes n (%)	3 (50)	5 (38.5)	4 (50)	2 (50)	
No n (%)	3 (50)	8 (61.5)	4 (50)	2 (50)	

Clinicopathological characteristics investigated included parameters such as the full blood count, blood chemistry, and liver function test. Statistical analysis of these clinicopathological characteristics showed no significance ($p>0.05$) in almost all of them except for some liver function tests. The liver function tests interrogated included eight variables, of which four were statistically significant including total bilirubin ($p=0.010$) and conjugated bilirubin ($p=0.013$), aspartate transaminase ($p=0.040$) and alkaline phosphate ($p=0.034$), which were reduced in chronic pancreatitis and significantly increased in PDAC patients (Table 3.2).

Table 3.2: Clinical characteristics of chronic pancreatitis and PDAC patients

Parameters	Chronic Pancreatitis Median [IQR]	Resectable Median [IQR]	Locally Advanced Median [IQR]	Metastasis Median [IQR]	p-value
FULL BLOOD COUNT					
Haematocrit	0.39 [0.23-0.52]	0.32 [0.28-0.37]	0.30 [0.24-0.35]	0.34 [0.22-0.45]	0.522
White cell count (/L)^9	6.20 [3.12-9.19]	9.24 [5.98-15.81]	10.85 [7.22-11.96]	9.91 [-3.48-26.74]	0.296
Red cell count (/L) ^12	4.09 [2.45-5.54]	3.68 [2.50-6.26]	3.33 [2.65-3.93]	3.77 [2.32-5.18]	0.703
Platelet count (/L) ^9	310.50 [153.76-439.24]	359 [267.3-545.81]	436.50 [274.98-575.27]	358 [247.18-482.82]	0.181
Haemoglobin (g/DL)	12.70 [8.13-16.47]	10.30 [9.23-12.28]	9.40 [7.94-11.42]	10.95 [7.03-14.27]	0.498
Prothrombin time (sec)	12.10 [10.31-14.64]	12.80 [12.20-14.45]	14.35 [12.86-15.02]	14.20 [10.57-20.18]	0.098
INR	0.94 [0.85-1.09]	1.01 [0.97-1.15]	1.17 [1.05-1.20]	1.17 [0.85-1.70]	0.210
MCV (fL)	92.95 [90.51-96.74]	92.70 [89.05-97.53]	89.45 [85.06-95.99]	91.25 [81.08-100.22]	0.756
MCH (pg)	30.95 [29.25-32.71]	30.20 [29.15-32.78]	29.59 [28.17-30.98]	28.5 [26.53-30.57]	0.332
MCHC (g/dl)	33.00 [31.40-34.75]	33.50 [32.40-33.95]	33.10 [34.35-31.10]	31.05 [29.38-33.67]	0.358
MPV (fL)	9.75 [8.27-11.13]	10.50 [9.89-10.94]	10.40 [9.34-11.39]	10.90 [9.59-12.21]	0.348
BLOOD CHEMISTRY					
Creatinine (μmol/L)	90.00 [48.47-146.87]	63.00 [56.66-101.67]	64.50 [29.22-127.78]	76.50 [62.72-90.28]	0.582
C-reactive protein (mg/L)	70 [-10.47-209.81]	44.50 [25.4-136.76]	62 [22.78-116.47]	164.50 [83.24-417.74]	0.745
Sodium (mmol/L)	138 [135.03-140.97]	137.5 [134.57-140.59]	135.5 [128.52-141.48]	139.5 [135.32-144.68]	0.279
Potassium (mmol/L)	4.05 [3.54-4.09]	3.55 [3.31-3.90]	3.55 [3.04-4.46]	3.70 [2.44-5.01]	0.535
Chloride (mmol/L)	97.50 [93.95-12.72]	95.50 [93.67-96.83]	93.50 [89.67-98.58]	90.50 [82.03-100.47]	0.119
Bicarbonate (mmol/L)	19.50 [17.03-22.97]	23.50 [18.14-25.86]	20.00 [18.66-22.34]	24.50 [12.67-35.83]	0.775
Anion Gap (mmol/L)	24.50 [18.69-29.31]	26.00 [20.69-28.48]	23.00 [21.74-26.51]	29.50 [19.58-38.92]	0.430
Calcium (mmol/L)	2.26 [2.05-2.50]	2.20 [1.66-2.40]	2.11 [1.98-2.29]	2.14 [2.11-2.17]	0.467
Magnesium (mmol/L)	0.87 [0.68-1.02]	0.73 [0.66-0.82]	0.77 [0.66-0.95]	0.78 [0.01-2.19]	0.525
Inorganic Phosphate (mmol/L)	1.18 [0.96-1.38]	1.02 [0.87-1.14]	1.09 [0.87-1.41]	1.14 [0.61-2.04]	0.248
Urea (mmol/L)	4.00 [2.03-7.44]	3.80 [2.23-6.22]	3.50 [1.45-9.77]	4.35 [0.85-9.20]	0.851
LIVER FUNCTION TEST					
Albumin (g/L)	36.50 [27.60-45.73]	30.00 [28.67-35.51]	25.50 [21.30-34.70]	39.00 [15.60-54.40]	0.383

Alanine Transaminase (U/L)	18.00 [11.36-25.64]	92.00 [41.81-145.64]	42.50 [-49.59-233.25]	30.00 [-16.30-75.63]	0.078
Alkaline Phosphate (U/L)	74.00 [-97.80-478.13]	526.00 [297.57-1256.43]	426.00 [53.73-1255.27]	302.00 [-78.53-559.86]	0.034
Gamma Glutamyl Transferase (U/L)	61.50 [-239.63-871.30]	441.00 [302.99-1480.64]	350.00 [-160.94-1712.61]	400.00 [-293.63-979.68]	0.141
Total Protein (g/L)	66.00 [55.05-82.28]	59.00 [53.01-67.71]	66.00 [53.03-76.64]	69.00 [59.08-83.59]	0.262
Total Bilirubin (Tbil) (μmol/L)	5.00 [2.45-11.88]	153.00 [67.23-290.40]	131 [19.71-271.62]	56 [-19.97-106.63]	0.010
Conjugated Bilirubin (Dbil) (μmol/L)	2.00 [-0.93-8.59]	141.00 [59.41-266.78]	120.50 [18.52-230.48]	39.00 [-24.17-88.83]	0.013
Aspartate Transaminase (U/L)	28.50 [20.72-36.95]	92.00 [59.20-154.62]	87.00 [20.44-147.56]	42.00 [-55.78-169.78]	0.040
HIV TEST					
CD4 Lymphocytes (%)	-	-	18.03 [-128.41-164.46]	-	0.407
CD4 Count (cells/μL)	-	-	245 [-644.43-1134.43]	-	0.259
CD45 + White Cell Count	-	-	9.11 [3.39-14.83]	-	0.103
ENZYMES					
Lipase (U/L)	163.00 [-307.13-633.13]	823.50 [-1241.26-2888.26]	-	-	0.165
Amylase (U/L)	-	285 [40-1780]	-	-	-

3.3.2 Complement C3 and C5 are elevated in the plasma of early-stage PDAC patients

Complement C3 and C5 levels were investigated in the plasma of PDAC patients at different disease stages and compared to chronic pancreatitis patients and healthy individuals. The levels of C3 in resectable patients were significantly higher compared to chronic pancreatitis and healthy control. The late-stage patients also had significantly higher levels compared to healthy individuals. There were no observed significant changes across the different tumour stages; however, a significant reduction ($p=0.047$) in C3 levels were observed when patients with early-stage (resectable) PDAC ($1099 \pm [851-1253]$ ng/ml) when compared to those with advanced-stage disease (locally advanced and metastatic) ($511 \pm [430-821]$ ng/ml) (Figure 3.2a). The levels of C5 were increased in all patient groups, including cancer and chronic pancreatitis patients, compared to healthy individuals. Additionally, C5 was shown to be significantly elevated in patients with resectable PDAC ($25.3 \pm [10.4-26.6]$ ng/ml) compared to healthy individuals ($6.7 \pm [4.9-10.5]$ ng/ml) ($p=0.001$) (Figure 3.2b). Although there was a reduction in its levels in late-stage disease compared to early-stage, this was not significant (Figure 3.2b).

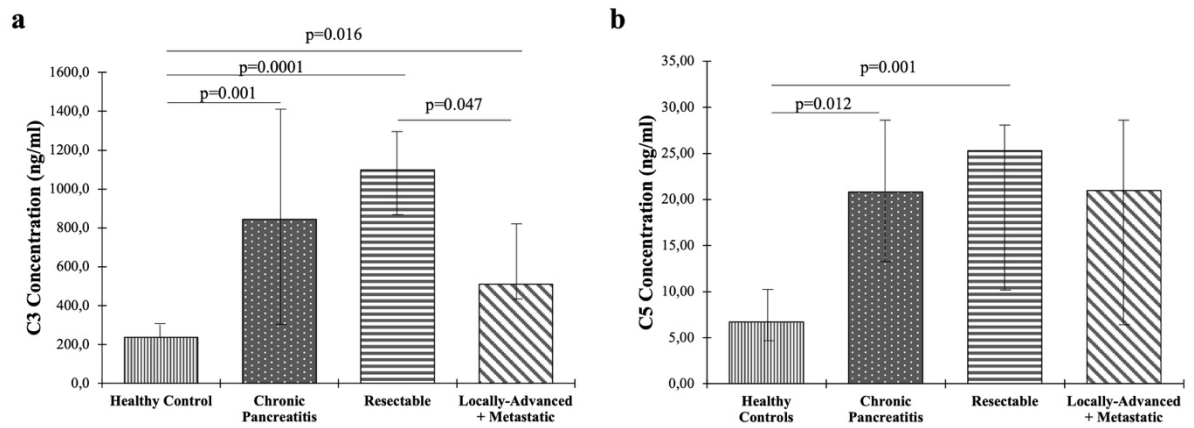


Figure 3.2: Protein expression levels of C3 and C5 in plasma of PDAC patients. (a-b) A decreasing trend is observed in all PDAC samples from early- to late-stage disease. Healthy individuals express lower levels of C3 and C5 compared to other groups. The experiment was conducted twice $n=2$. Values are indicated in median [IQR].

The Spearman correlation test was conducted using patient data extracted from patient files. and it showed a low but positive correlation ($r=0.45669$) between the complement C5 and the clinical inflammatory marker C-reactive protein (CRP) ($p=0.0098$) (Figure 3.3).

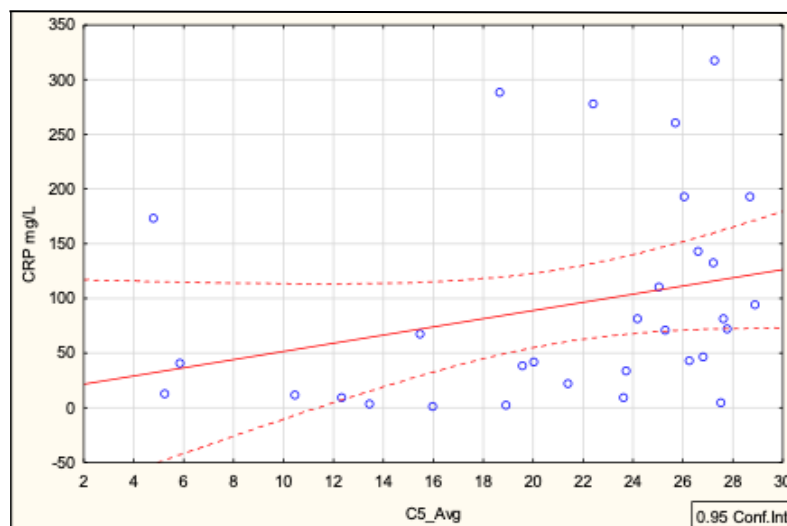


Figure 3.3: Correlation analysis between C5 and CRP. Scatter plot showing a positive correlation ($r=0.45669$) between C-reactive protein and the complement C5 which are both inflammatory markers ($p=0.0098$).

3.4 DISCUSSION

PDAC is closely linked with disruptions in both inflammatory and immune responses, highlighting the critical interplay between the tumour microenvironment and the intricate balance of cellular signaling pathways. The bloodstream serves as a vital channel for the spread of inflammatory and immune response targets. Within this dynamic environment, a cascade of events unfolds, leading to the secretion of different bioactive molecules. Components of the complement system, including complement proteins and antibodies, are released into the bloodstream, enhancing the recognition and elimination of foreign pathogens (Marshall et al., 2018). Assessing the status of this release can provide crucial insights about the intensity of the inflammatory and immune responses within the body. Following verification of targets (complement C3 and C5) in PDAC tissues, their circulating levels were investigated further on 25 patients in different stages of PDAC (13 resectable, 8 locally advanced and 4 metastatic) with the ELISA assay. The demographic and clinicopathological data of these patients were also recorded.

Liver function tests showed a significant increase in conjugated bilirubin ($p=0.010$), total bilirubin ($p=0.13$), alkaline phosphate ($p=0.034$) and aspartate transaminase ($p=0.040$) across groups. PDAC patients often present with obstructive jaundice (Gouma et al., 2006; Shen et al., 2018), which in most cases is caused by a tumour that blocks the common bile duct and results in elevated bilirubin. These results can, therefore, be expected. Most studies have proven bilirubin as a marker for liver diseases and PDAC (Gong et al., 2017; Yagyu et al., 2019; Zhang et al., 2020). This study corroborates that PDAC is associated with increased levels of bilirubin.

3.4.1 Complement C3 and C5 are elevated in early PDAC

The complement C3 and C5 are overexpressed in the pancreatic tumour microenvironment. Their expression was further investigated in the circulation of PDAC patients in different stages of the disease. These complement proteins were found to be elevated in chronic pancreatitis and in resectable PDAC patients. Chronic pancreatitis is characterized by persistent inflammation of the pancreas, which can lead to ongoing tissue damage and dysfunction (Ashraf et al., 2021; Barry, 2018). Increased C3 levels in chronic pancreatitis and early cancer might indicate an active immune response against any foreign invaders and cancer cells. However, as cancer progresses, the immune system becomes compromised and less effective in combating the tumour, leading to a decrease in C3 levels. Liver function is compromised in metastatic disease, which affects the synthesis of complement primarily originating from the liver, resulting in reduced circulating levels. Another potential factor is whether tumours induce obstructive jaundice, potentially causing bile duct obstruction, cholangitis, and infection, which

could elevate complement levels. Furthermore, these levels could indicate tumour growth and invasion. Cancer cells can manipulate and evade the immune system by hijacking various mechanisms, including the complement system (Messerschmidt et al., 2016). In early cancer stages, an increase in C3 levels could imply the activation of the complement system against tumour cells, inhibiting their growth and invasion. Alternatively, tumour cells might find ways to downregulate the complement system in advanced cancer stages, allowing them to proliferate and spread more easily.

Monitoring changes in complement C3 levels can potentially serve as a prognostic marker in the cancer. An increase in C3 levels in early PDAC might suggest a more favourable prognosis, as it could indicate an ongoing effective immune response against the tumour. On the other hand, a decrease in C3 levels in late-stage cancer may suggest a poorer prognosis, reflecting a compromised immune system unable to control tumour growth. Taken together, it is possible that tumour cells might find ways to downregulate the complement system, allowing them to proliferate and spread more easily.

3.4.2 Elevated complement C5 may be linked to increased inflammation

The principal role of inflammation is to protect the host from foreign invaders. However, the continuous presence of inflammation causes injury and can be problematic, especially in cancer, where it is considered a hallmark of the disease (Winstead, 2022). The presence of inflammation triggers an anti-inflammatory response, which could prompt hepatocytes to increase the synthesis of C-reactive protein, a well-known clinical inflammatory marker (Sproston and Ashworth, 2018). This study showed a positive correlation between CRP and complement C5, both of which are inflammatory markers. This correlation may suggest that these proteins interact with one another either to induce an anti-inflammatory response in PDAC or the CRP influences the production of C5 in response to infection to combat inflammation. Although correlation does not always mean causation, some studies have shown C5 to be a key player in inflammatory pathways (Giorgio et al., 2021; Stennett et al., 2023), suggesting that it may be involved in the inflammatory pathways linked to PDAC progression. Additionally, a strong positive correlation between the white cell count (WCC) and CRP observed could indicate an increase in inflammation as these are clinical markers for inflammation. Inflammation increases CRP levels, the risks of acute, chronic pancreatitis and cancer (Zhu et al., 2022). Elevated circulating levels of CRP are associated with poor prognosis in patients with several types of solid cancers, including PDAC, whereas in healthy controls, elevated levels of CRP are associated with increased future risk of cancer (Suthahar et al.,

2023). Taken together, the elevation of the complement C5, CRP and the white cell count could be indicative of increased inflammation in PDAC progression.

The hypothesised mechanism of action in which these clinical markers interact with one another could be as follows: post-infection, hepatocytes accelerate the synthesis of CRP, while white blood cells e.g (macrophages) are increased as a response to infection. CRP binds bacterial polysaccharides and ligands exposed on damaged cells, thereby causing re-uptake by phagocytes (Slaats et al., 2016). CRP binding to bacterial polysaccharides results in complement activation. Activation of complement further increases inflammation by C5 (Figure 3.4).

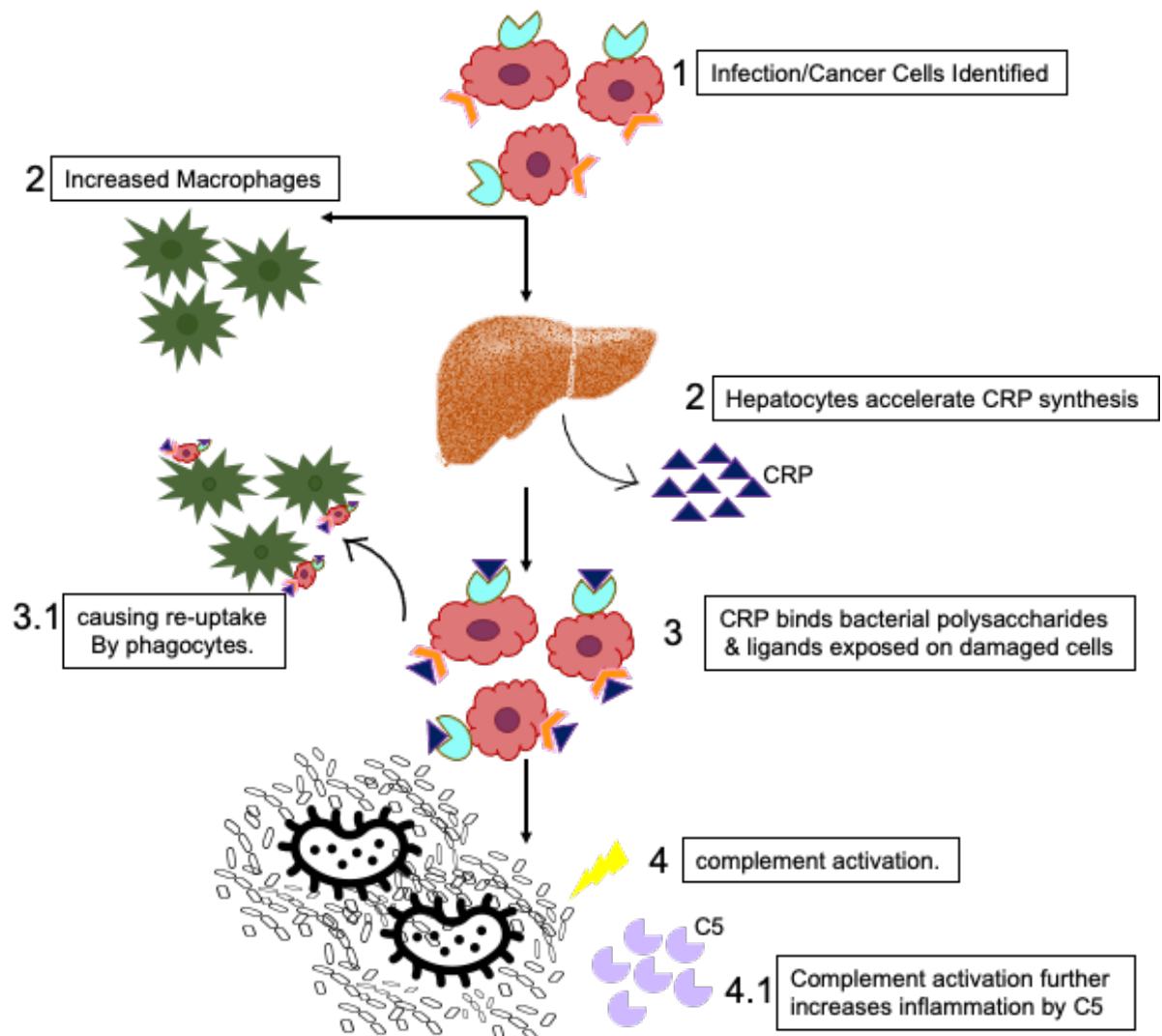


Figure 3.4: Proposed mechanism of action in which C5 and CRP increase inflammation.

3.4.3 Expression of C3 and C5 is associated with PDAC staging and patient survival

The expression of complement C3 and C5 were observed to be elevated within the tumours and plasma of resectable PDAC patients. However, as the cancer progresses, their levels reduced, possibly due to the reduced immune activation that occurs. This suggestion is made following that elevated levels of the complement C3 can contribute to immunosuppression by inhibiting T cell activation and other immune effector cells that aid tumour immune evasion (Kolev et al., 2022). Additionally, chronic pancreatitis, a risk factor of PDAC development, shows an increased expression of these proteins (Roumenina et al., 2019). Chronic pancreatitis is mainly characterised by inflammation, and the immune response in these patients is constantly activated which explains the increase of complement proteins (Inman, 2014; Manohar et al., 2017; Zhang et al., 2021). However, terminal patients presenting with metastatic disease tend to have reduced immunity due to cancer cells having evolved mechanisms to evade the immune system. Metastatic cancer cells can suppress complement activation to avoid detection and attack by the immune system, resulting in decreased complement activity (Kim and Cho, 2022; Sandoval-Valencia, 2019). This immune evasion allows the cancer to spread to other parts of the body.

3.5 CONCLUSION

This study demonstrated that complement C3 and C5 are elevated in the plasma of resectable PDAC patients. However, as PDAC progresses, C3 reduces significantly, which could be of a loss of the innate immune response as these patients become immunocompromised. A positive correlation between the complement C5 and CRP observed could suggest a link between these molecules. Complement C5 may be involved in the inflammatory processes that result in the progression of PDAC. More studies are required to validate these expression findings in a larger cohort.

CHAPTER 4

4. EFFECTS OF PHARMACOLOGICAL INHIBITION OF THE COMPLEMENT PATHWAY ON PDAC CELLS (MIA PACA-2)

4.1 INTRODUCTION

PDAC is a devastating malignancy characterized by its aggressive nature and resistance to conventional treatments (Halbrook et al., 2023). Despite significant advancements made in cancer and therapeutics, the overall survival rate for PDAC patients remains alarmingly low. To improve patient outcomes, it is important to understand the underlying mechanisms that contribute to its progression and identifying potential therapeutic targets is crucial for improving patient outcomes. The immune system is a powerful ally in the fight against cancer. Studying its interactions with cancer cells and the tumour microenvironment can uncover valuable insights into cancer biology and identify new therapeutic targets and pathways for the development of more effective cancer treatments.

The complement system is a key component of the innate immune system that comprises a complex network of proteins and enzymes that have significant roles in immune defence against pathogens, as well as tissue homeostasis. Complement components are synthesized primarily by the liver, yet it is crucial to acknowledge that both tumour and stromal cells can also synthesize them. Consequently, their levels within the tumour result from the combined influence of the systemic compartment and the local synthesis by various cell types (Revel et al., 2020). Complement activation results in a cascade that leads to the generation of various effector molecules, including anaphylatoxins (C3a and C5a) and membrane attack complexes (MAC) (Figure 3.1), which have been implicated in various pathological processes, including cancer. Emerging studies have shown that the complement system may also be involved in tumour development and modulation of the tumour microenvironment (Ain et al., 2021; Olcina et al., 2018; Yarmoska et al., 2021). Complement proteins are elevated in the serum of PDAC patients, indicating their potential involvement in its pathogenesis (Saraswat et al., 2019). Complement C3 is a central component of the complement system and is involved in both innate and adaptive immune responses.

The complement system has been associated with the recruitment of immune cells, promotion of inflammation, and regulation of angiogenesis (Markiewski et al., 2020; Zhao et al., 2021). These processes are critical for tumour growth and progression and understanding the role of

complement in them can provide important insights into the pathogenesis of PDAC and may lead to the development of novel therapeutic strategies. For instance, the production and secretion of complement C3 by PDAC cells have been reported (Sodji et al., 2022). This suggests that PDAC cells can autonomously activate the complement system and utilize complement proteins to promote their growth and survival.

The intricate relationship between the complement system and pancreatic tumour cells plays a critical role in the complex landscape of tumour progression. Recognised for its role in the immune system's defence against pathogens and damaged cells, the complement system involvement in cancer, particularly PDAC extends beyond only the immune response modulation (Afshar-Kharghan, 2017). The dynamics between the complement system and PDAC are multifaceted, impacting migration, proliferation, cell death, and evasion mechanisms crucial to tumour progression. Tumor cell migration is a hallmark of cancer metastasis, and the complement system has emerged as a key regulator in this process (Zhang et al., 2019b). Complement activation products, such as C5a, have been implicated in promoting tumour cell migration. PDAC strategically exploit complement-mediated signaling to enhance their migration and invasion into surrounding tissues. This interaction triggers intracellular signaling pathways, initiating cytoskeletal rearrangements that further facilitate migration (Hussain et al., 2022).

The complement system's impact on tumour cell proliferation is a complex interaction between pro- and anti-proliferative signals. Some complement components, such as C3a and C5a, can stimulate cell proliferation by activating downstream signaling pathways, including the membrane attack complex (MAC) which induces cell death. In PDAC, the balance between these opposing signals is disrupted, leading to uncontrolled proliferation. Targeted cell biology approaches aim to manipulate this delicate balance.

Evading cell death is a hallmark of cancer cells, and pancreatic tumours exploit complement evasion mechanisms to thrive. Complement-mediated cell death, or complement-dependent cytotoxicity (CDC), is often thwarted by tumour cells through the downregulation of complement receptors or the expression of complement regulatory proteins. This evasion allows pancreatic tumours to resist immune-mediated destruction.

Targeted cell biology interventions to disrupt complement-mediated migration, restoring the sensitivity of tumour cells to complement-induced cell death and enhance complement activation or inhibit the expression of complement inhibitors on tumour cells are being explored. Researchers are investigating the development of novel therapeutics that specifically

target complement receptors or block signaling pathways involved in complement-associated diseases. Complement-targeting drugs have been developed over the years, with some already in clinical trials while some used for disease treatment. For example, C1 inhibitor (CINRYZE®) used in hereditary angioedema has been approved (Li et al., 2018). Complement therapeutics that target the initiation pathways in some diseases where complement is continuously activated, impeding its activation while leaving the defense role of the pathway unchanged may slow down the effect caused by overactivation. Examples of these include C1r, C1s and MASP inhibitors currently in different clinical trial stages to treat sepsis, kidney transplant, lupus and others (Ricklin et al., 2019). The amplification loop of the complement system can lead to excessive activation if not properly regulated. This excessive activation may result in inflammation, unintended tissue damage and an autoimmune response.

Therapeutic interventions targeting the amplification loop of the complement system aim to modulate or inhibit specific components or processes within this loop (Ricklin et al., 2019). By disrupting the amplification loop, these therapeutic interventions seek to achieve several goals including: reducing inflammation involved in chronic inflammation conditions, prevent tissue damage, while protecting organs and tissues from the harmful effects of an uncontrolled immune response, and modulate the immune response to restore balance and preventing inappropriate immune reactions. Examples of these include C3, FB, FD, convertase inhibitors used for the treatment of stroke, paroxysmal nocturnal hemoglobinuria, periodontal disease and nephroties among others. Complement effectors such as C5 and MAC contribute to clinical manifestation of complement-mediated diseases (Ricklin et al., 2019; Sturfelt and Truedsson, 2012).

Targeting effector pathways using a clinically approved monoclonal antibody such as anti-C5 mAb (Eculizumab) can block C5 effects (Zepek and Morgan, 2020) which may have several therapeutic implications such as reducing inflammation, keeping the body from attacking itself and improving patient outcomes by addressing the root cause of complement dysregulation. Considering the essential role of the complement system in defending the host against infections, it is crucial to carefully balance therapeutic benefits with the preservation of the immune system's protective functions.

Despite the increasing interest, there remains a lack of extensive research investigating the overall impact of complement inhibitors on cancer progression, resulting in substantial gaps in our comprehension of their effects in this specific context. Understanding the molecular complexities of migration, cell proliferation and apoptosis evasion induced by complement will make it possible to design therapeutic strategies aimed at impeding the metastatic capabilities

of pancreatic tumour cells. Compstatin is a tridecapeptide which acts as a highly selective protein-protein interaction inhibitor by binding native C3 to block the cleavage by convertase and thus inhibit the activation of the complement cascade (Reis et al., 2018). In this study, the complement C3 inhibitor (Compstatin) was employed to pharmacologically inhibit complement activation (Lamers et al., 2022; Luchini et al., 2019; Mastellos et al., 2015; Ricklin and Lambris, 2008) within MIA PaCa-2 cell lines, examining its impact on cell proliferation, the cell cycle, and cell migration.

4.2 METHODS

4.2.1 Cell culture and treatment

The PDAC cell line, MIA PaCa-2, representing a pancreatic cancer model, was used in this study. The cell line was obtained from the Japanese Cancer Research Resources Bank (JRCB) cell bank (cat no. JCRB0070) with the appropriate and extensive details confirming that they were MIA PaCa-2 cells including DNA profile (STR). (https://cellbank.nibiohn.go.jp/~cellbank/en/search_res_det.cgi?ID=245). MIA PaCa-2 cells were derived from a 65-year-old Caucasian male patient with pancreatic adenocarcinoma located at the head and tail of the pancreas (Deer et al., 2010). Upon obtaining the cell line, mycoplasma detection was conducted as previously published (Ligasová et al., 2019) (Figure S1). The MIA PaCa-2 were grown in Dulbecco's Modified Eagle Medium (DMEM) (cat no. D8900, Sigma Aldrich) with 10% fetal bovine serum (FBS) (cat no. 12107C, Sigma Aldrich) and 1% penicillin-streptomycin (cat no. P4333, Sigma Aldrich) and maintained in an incubator at 37 °C in 5% CO₂ and 95% air. Compstatin (Cat no. 2585, Tocris Bioscience, Bristol, United Kingdom) was used to inhibit the complement pathway pharmacologically (Lamers et al., 2022; Mastellos et al., 2015; Ricklin and Lambris, 2008), and dimethyl sulfoxide (DMSO) (cat no. 5.89569, Sigma Aldrich) as vehicle control and Gemcitabine was used as a positive control (cat no. Y0000675-50mg, Sigma Aldrich). Viable cell counts were determined using the Trypan blue exclusion assay, by adding 10µl cells to Trypan blue (1:1 ratio) to the cell suspension and counting on a TC20™ automated cell counter (cat no. 1450102, Bio-Rad Laboratories, Hercules, California, USA)

4.2.2 Cell proliferation assay using methoxynitrosulfophenyl-tetrazolium carboxanilide (XTT)

The cell proliferation kit II (XTT) (cat no. 11465015001, Sigma Aldrich) colourimetric assay was employed to quantify cell proliferation, viability and cytotoxicity in PDAC MIA PaCa-2

cells. Cells were cultured as described above and counted on a TC20™ automated cell counter (Bio-Rad Laboratories, Hercules, California, USA) by adding 10µl cell suspension to trypan blue (1:1 ratio). Minimum viability of seeded MIA PaCa-2 cells was 95%, cells seeded in different 96-well plates (ThermoFisher Scientific) at concentrations of 2.5×10^3 , 5×10^4 and 1×10^5 . Each well had a total volume of 200µl, comprising 2.5×10^3 , 5×10^4 and 1×10^5 cells in complete medium and incubated at 37°C, 5% CO₂ for 24 hours three times n=3. Once cells reached 60-70% confluence, they were treated with the complement inhibitor (compstatin), and controls (untreated DMEM + 10% FBS + 1% pen-strep and cells as negative control, and the vehicle control 0.1% DMSO and complete medium with no cells as a blank) for 24 and 48 hours using the range 0–100µM (Compstatin). Absorbance was measured on a spectrophotometer (MULTISKAN Sky, ThermoFisher Scientific) at 450 nm with a reference wavelength of 690 nm. Percentage survival for each sample was calculated as $100 \times [(\text{OD}_{450} \text{ of sample} - \text{OD}_{450} \text{ of negative control}) / (\text{OD}_{450} \text{ of positive control} - \text{OD}_{450} \text{ of negative control})]$ (Fan et al., 2020).

4.2.3 Total RNA extraction from cells

Total RNA was extracted using the TRIzol method (Chomczynski, 1993) following the cell culture method described in section (4.2.1). Cell media was discarded, the cell pellet dissolved in TRIzol (700 µl) (cat no. T9424, Sigma Aldrich, St. Louis, Missouri, United States) by aspiration, then transferred into 1.5 ml Eppendorf tubes and allowed to stand for 5 minutes at room temperature to allow cell lysis and nucleoprotein complexes dissociation. Chloroform (150 µl) (cat no. 02487, Sigma Aldrich) was added to promote phase separation and allow RNA isolation from DNA and proteins in the biological sample. The sample was shaken vigorously for 15 seconds until the mixture turned milky or cloudy, left to stand for 8 minutes, and centrifuged at 12 000 x g for 10 minutes at 4° C. The aqueous phase, comprising RNA, was transferred into sterile Eppendorf tubes, and isopropanol (350 µl) (cat no. 9516, Sigma Aldrich, St. Louis, Missouri, United States) was added. The sample was mixed by resuspension and allowed to stand for 7 minutes to allow RNA precipitation. The mixture was centrifuged at 12 000 x g for 8 minutes at 4° C. The supernatant was discarded, and the pellet was washed with 75% ice-cold ethanol (700 µl) (cat no. 34870, Sigma Aldrich, St. Louis, Missouri, United States) to dissolve and wash away soluble salts, leaving most of the RNA behind. The sample was vortexed for 5 seconds and centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was air-dried for 10 minutes and placed into a heating block for further drying. Nuclease-free (30 µl) water (cat no. 129317, Qiagen, Hilden Germany) was added to dissolve the pellet by repeatedly mixing while placed in the heating block for 5 minutes

at 55°C and immediately placed on ice. RNA concentration and quality were measured using the Nanodrop® (ND 1000) (Thermo Fisher, Waltham, Massachusetts, United States).

4.2.4 cDNA synthesis

The Invitrogen™ SuperScript™_VILO™ cDNA synthesis kit (ThermoFisher Scientific) was used to perform cDNA synthesis for real-time PCR. Into each tube placed on ice, 4µl 5X VILO™ reaction mix, 2µl 10X SuperScript™ enzyme mix, 2.5µg RNA and DEPC-treated water was added to make up to 20µl and gently mixed and incubated at 25°C for 10 minutes and further incubated at 42° C for one hour. The reaction was then terminated at 85°C for 5 minutes.

4.2.5 Real-Time PCR

The PCR reaction mix was prepared using validated predesigned TaqMan Gene Expression assays (Thermo Fisher Scientific) for the 3 genes; *C3* (Assay ID- Hs00163811_m1), *C5* (Assay ID- Hs01004342_m1) and *MRPL19* (Assay ID- Hs00608519_m1). The method involved adding 5 µl of TaqMan® Gene Expression Master Mix (2X), 0.5µl TaqMan® Gene Expression Assay 20X) for target gene and reference gene, 3 µl of cDNA template, Human Male Raji cDNA template (controls), and 1 µl nuclease-free water to a PCR plate. Samples were briefly vortexed to mix and centrifuged to collect contents at the bottom of the tube. 10 µl of the PCR reaction mix was then transferred into each well of an optical reaction plate twice, sealed with adhesive film and the plate centrifuged briefly to eliminate bubbles and collect contents to the bottom of the plate. The cycling conditions were as follows; UNG incubation and hold at 50° C for 2 minutes for optimal UNG activity, Polymerase activation and hold at 95° C for 10 minutes to activate AmpliTaq Gold™ DNA polymerase, denature at 95° C for 15 seconds and anneal or extend at 60° C for 1 minute (40 cycles) and run the PCR reaction plate on a real-time cycler (QuantStudio1; Thermo Scientific™). The targets were *C3* and *C5* with *MRPL-19* as a reference gene, Fold changes were determined using the $2^{-\Delta\Delta CT}$ method.

4.2.6 Cell cycle analysis

Measurements of the percentage of cells in the G0/G1, S and G2/M phases of the cell cycle were based on analysing DNA content using the single parameter-based propidium iodide staining technique (BD Biosciences, New Jersey, USA). MIA Paca-2 cells were counted by adding 10µl Trypan blue to cells (1:1 ratio), using a TC20™ automated cell counter (Bio-Rad Laboratories), and 2×10^6 viable cells were seeded twice and left to grow for 24 hours. Cells were treated with 6µM compstatin to inhibit the complement pathway and with 0.1% DMSO as vehicle control. Following trypsinisation, cells were centrifuged at 250xg for 5 minutes, fixed

in cold 70% ethanol and stored at -20°C for at least 24 hours. A working solution consisting of 2mg RNase A in 0.1% Triton-X100 reagent (BD Biosciences) was used to degrade the RNA, and 500µl of this solution was mixed with 400µl of a propidium iodide dye solution at a concentration of 500µg/ml, both in PBS. The mixture was incubated at room temperature for 30 minutes, and fluorescence was measured using the BD LSR Fortessa™ Analyser and FACS Diva software (BD Biosciences). The research question of this study does not directly incorporate the CST setting; thus, it was not included in the analysis. The cell cycle phase was estimated using the FlowJo™ v.10 software (BD Biosciences).

4.2.7 Cell migration (scratch assay)

PDAC MIA PaCa-2 cells were cultured and counted on a TC20™ automated cell counter (Bio-Rad Laboratories) by adding 10µl Trypan blue to cells (1:1 ratio). Viable MIA PaCa-2 cells (1×10^5) were seeded in a 24-well plate (ThermoFisher Scientific) three times. Plates were incubated at 37°C, 5% CO₂, for 24 hours. Once cells reached 80-100% confluence, the cell monolayer was imaged using the Olympus iX51 phase contrast microscope (Wirsam Scientific & Precision Equipment Ltd, Johannesburg, South Africa), followed by washing cells twice with 0.1M PBS. A scratch wound was made across the wells (Figure S3) on the second wash using a sterile 10µl pipette tip. The cells were then washed three times with PBS. Low serum media (DMEM + 1% FBS + 1% Pen-Strep) was added to ensure the cells were starved and to limit proliferation (Rashid and Coombs, 2019). Cells were treated with 6µM compstatin to inhibit complement activation; 0.1% DMSO was used as vehicle control. Images of the scratched area were captured using the Olympus iX51 phase contrast microscope with CellSens Software (Wirsam Scientific & Precision Equipment Ltd) at 0, 3, 6, 12 and 24 hours. Image J software v.2.9.0 was used to measure the distance between the scratched cells (Schindelin et al., 2012). The following calculation was used to measure the rate of cell migration $\frac{(D_{initial} - D_{final})}{D_{initial}} \times 100$ where D is distance.

4.2.7 Statistical analysis

The results were expressed as mean ± SD after conducting a one-way ANOVA and a Tukey post hoc test for the cell-based analysis, ensuring the assessment of normality. The software used to analyse data was GraphPad Prism 9/10 version 9.1.0 /10, and the differences were regarded as significant at p<0.05 for all tests.

4.3 RESULTS

4.3.1 Confirmation of Complement C3 inhibition using compstatin

Real-time PCR was used to verify complement inhibition after cellular treatment. Complement C3 and C5 were detected in the MIA PaCa-2 cell line before treatment. However, their expression was completely suppressed upon administration of 6 μ M of compstatin (Tocris Bioscience), as evidenced by a dose-response curve analysis (Figure S2). This confirms the subsequent inhibition of the complement cascade downstream (Figure 4.1).

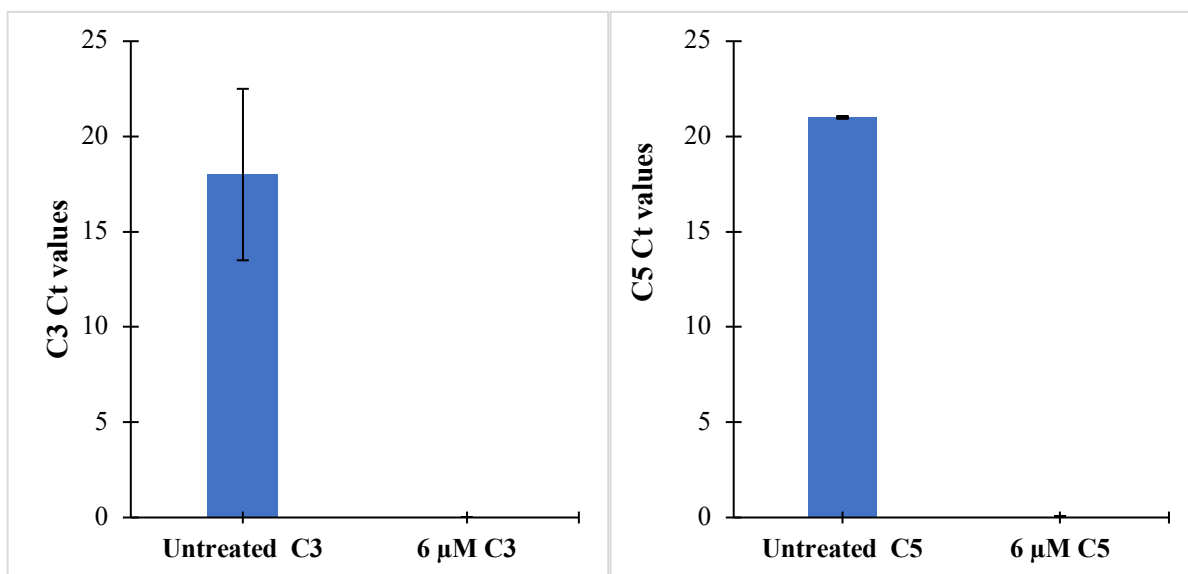


Figure 4.1: Confirmation of complement C3 and C5 inhibition using qPCR. Complement C3 and C5 are overexpressed in untreated MIA PaCa-2 cells and not expressed following treatment with compstatin. The experiment was performed twice (n=2). Values are indicated as mean $\pm$ SD.

4.3.2 Complement inhibition induces cell proliferation

The XTT cell proliferation assay was used to evaluate the viability and proliferation of MIA PaCa-2 cells after complement C3 inhibition. The compstatin inhibitor not only impeded C3 but also blocked all downstream effects of the complement pathway including complement C5. The experiment involved seeding different densities of cells (2.5×10^3 , 5×10^4 , and 1×10^5) to demonstrate that the proliferation observed was independent of the number of cells seeded (Figure 4.2 a and b). Cells were also exposed to gemcitabine as a positive control. Gemcitabine is commonly used as chemotherapy for numerous types of cancers, including PDAC. The results showed that complement C3 inhibition resulted in a significant increase ($p=0.001$) in the number of viable MIA PaCa-2 cells, thus indicative of heightened cell proliferation, compared to untreated cells (Figure 4.2). Treatment with gemcitabine, used as a positive control, led to a

reduction in cell viability in cancer cells. The following formula was used to calculate cell viability: $\text{cell viability} = (A_{\text{treatment}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\%$ (where A = absorbance).

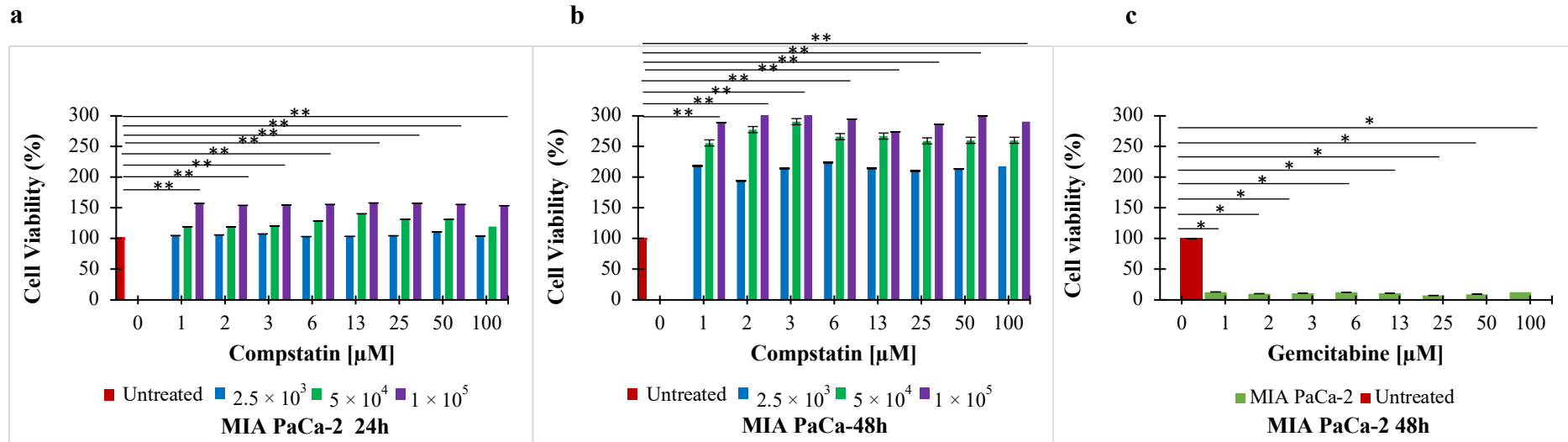


Figure 4.2: Complement inhibition on MIA PaCA-2 cells increases cell proliferation. (a-b) Inhibition of the complement pathway significantly increased ($p=0.001$) cell proliferation as evidenced by increased viability ($>100\%$) in MIA PaCa-2 cells over 48 hours, whereas (c) gemcitabine was toxic to MIA PaCa-2 (1×10^5). The experiment was conducted three times $n=3$. Values are indicated as mean $\pm$ SD. *, **, and *** indicate $p<0.05$, $p<0.001$, $p<0.0001$, respectively.

Flow cytometry was used to investigate the effect of complement inhibition on the cell cycle of MIA PaCa-2 cells. Propidium iodide (PI), a fluorescent dye that bind with DNA was used to stain cells, enabling the measurement of DNA content in individual cells and facilitate the differentiation of various stages of the cell cycle based on the relative DNA content. The findings showed that untreated cells were significantly higher ($p < 0.0001$) in the G1 ($63.4 \pm 6.84\%$) compared to the G0 phase ($0.52 \pm 6.84\%$). However, after compstatin treatment, the cells exhibited a significant reduction ($p = 0.004$) in the G1 phase ($42.0 \pm 42.6\%$) and an increase in the S phase ($21.4 \pm 42.6\%$) necessary for DNA replication and repair, and also increased in the G0 phase ($13.9 \pm 42.6\%$), although the increase was not statistically significant ($p = 0.5055$).

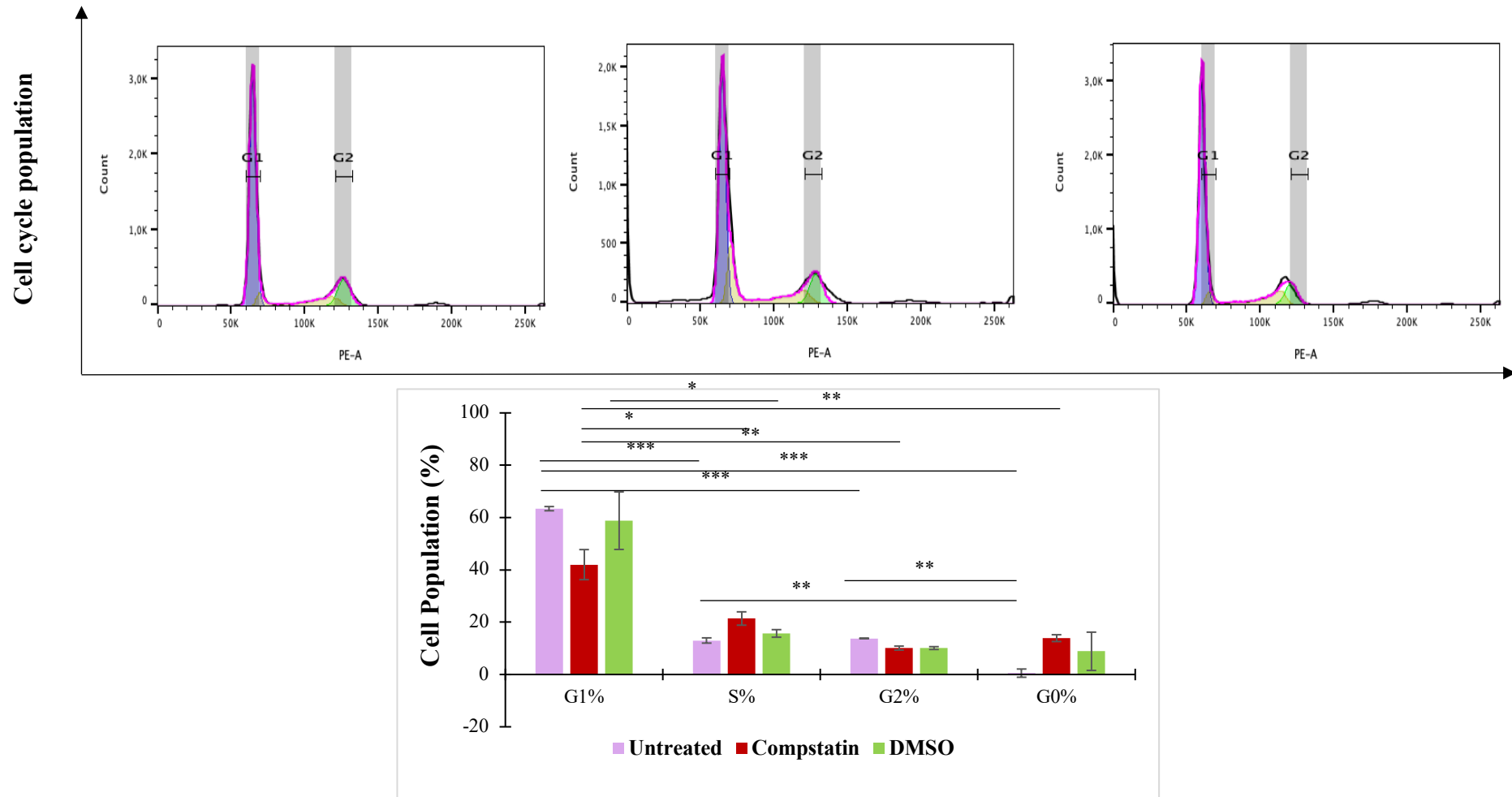


Figure 4.3: Enhanced cell division induced by complement inhibition in MIA PaCA-2 cells. Complement inhibition allowed cells to transition from the G1 to the S phase, showing increased cell division. The experiment was conducted twice (n=2). Values are indicated as mean $\pm$ SD. *, **, and *** indicate $p < 0.05$, $p < 0.001$, $p < 0.0001$, respectively.

4.3.3 Complement inhibition induces cell migration

Cell migration was investigated on the MIA PaCa-2 cells by inducing a scratch on the cell monolayer and inhibiting the complement pathway using compstatin. Cells were exposed to a low serum medium supplemented with 1% foetal bovine serum (FBS) instead of the standard 10% FBS to redirect cellular focus from proliferation to migration while maintaining viability (Kauanova et al., 2021). This alteration effectively reduced nutrient levels, allowing the cells to prioritise migration over proliferation, albeit with some residual proliferation activity. The following calculation was used to measure the rate of cell migration $\frac{(D_{initial} - D_{final})}{D_{initial}} \times 100$

The scratch assay showed complement inhibition accelerates cell migration in MIA PaCa-2 cells. A significant increase ($p=0.002$) in migration was observed from 0h to 24h following treatment with compstatin (Figure 4.4 and Figure S3).

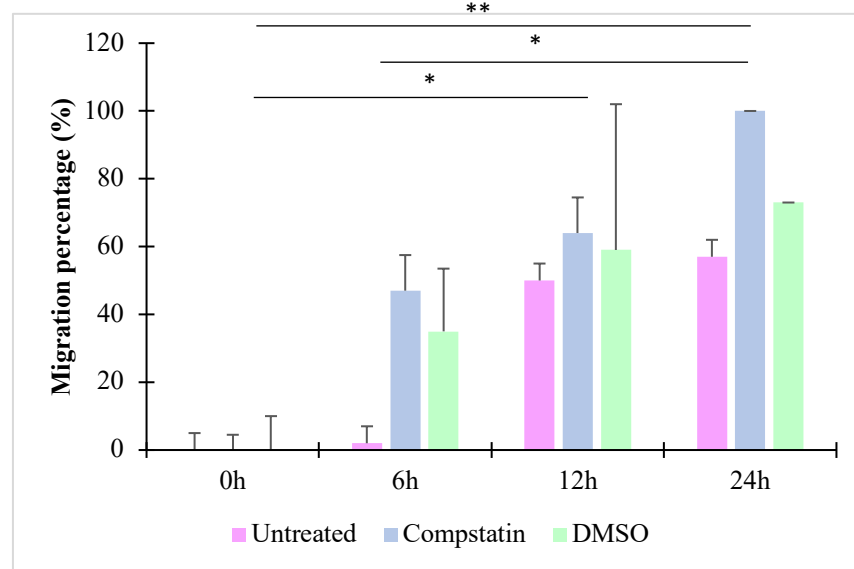


Figure 4.4: Enhanced migration induced by complement inhibition in MIA PaCa-2 cells. Complement inhibition enhances cell migration, compared to untreated and DMSO vehicle controls. The experiment was conducted twice ($n=2$). Values are indicated as mean $\pm$ SD. *, **, and *** indicate $p<0.05$, $p<0.001$, $p<0.0001$, respectively.

4.4 DISCUSSION

This study investigated the effects of complement pathway inhibition on the phenotype of PDAC cells, specifically focusing on cell proliferation, cell cycle, and cell migration. Pharmacological inhibition of the complement pathway using compstatin increased the proliferation, cell division, and migration of MIA PaCa-2 cells, suggesting that the reduction of complement pathway activity may promote tumour growth and metastasis in PDAC.

The complement system has multiple roles in different cancer types and based on different factors (e.g. tumour staging), it may exhibit different phenotypes. It exhibits a spectrum of effects: a protective role characterized by positive outcomes linked to heightened expression of complement genes, specifically associated with beneficial outcomes when C3 expression is high compared to other genes (Revel et al., 2020). Conversely, an aggressive complement profile is marked by adverse outcomes correlated with increased expression of complement genes. Additionally, there exists an ambiguous relevance of complement in cases where a discernible pattern is absent (Revel et al., 2020). For example, Tegla et al., (2011) found that complement factors are involved in cancer cell proliferation and disrupt apoptosis. Similarly, Ain et al. (2021) showed that in an ovarian cancer transgenic mouse model, knockout of C3 or C5aR suppressed tumour growth. Whereas Xie et al (2021) demonstrated that complement can promote tumour progression in a mouse model of cervical cancer by generating C5a in the tumour microenvironment and enhancing the activity of immune-suppressing cells for disease progression (O'Brien et al., 2021). It is still unclear the specific roles of components of the complement pathway in PDAC. This study confirms its overexpression in PDAC but has shown that this is reduced in late stage compared to the early stages of the disease. Furthermore, blocking this pathway induces an aggressive phenotype in PDAC. It is possible that due to the potential role of the complement pathway in immune response, shutting it down could lead to the aggressive nature observed.

4.4.1 Complement pathway inhibition increases cellular proliferation in PDAC cells

Due to their genetic modification to proliferate faster, cancer cells minimize the time in the G0 phase of cell replication (Yano et al., 2014). Cancer cells can exhibit increased proliferation during the S phase of the cell cycle due to underlying genetic mutations and dysregulation of cellular control mechanisms (Otto and Sicinski, 2017). This study has shown that inhibiting the complement pathway increases MIA PaCa-2 cells at the G0 phase. The G0 phase is a resting phase where cells are not actively dividing but are metabolically active and in a protective state. However, in some cases, cancer cells can escape this quiescent state and re-enter the cell cycle, leading to increased proliferation (Lindell et al., 2023). This may be attributed to gene mutations

that regulate cell cycle checkpoints, such as tumour suppressor genes like p53, that are commonly mutated in many cancers including PDAC (Marei et al., 2021). When these genes are mutated or dysfunctional, the normal mechanisms that prevent cells from entering the cell cycle inappropriately can be compromised. As a result, cancer cells may bypass the G0 phase and resume dividing, contributing to uncontrolled growth (Truskowski et al., 2023). Complement inhibition allowed the G1 to S phase transition seen in the increase of cells in the S phase following treatment. The S phase is the DNA synthesis phase where genetic material is replicated. Cancer cells may exhibit increased activity during this phase due to alterations in cell cycle regulators and DNA repair mechanisms. For instance, dysregulation of tumour suppressor p53 and Rb (retinoblastoma protein) can also lead to unchecked cell proliferation, activation of proto-oncogenes (e.g., Ras, Myc), and other critical regulators of the cell cycle, including cyclins, cyclin-dependent kinases (CDKs), and checkpoint control proteins which ultimately enable the cells to evade checkpoints and control mechanisms that normally prevent uncontrolled growth in S phase (Matthews et al., 2022; Thu et al., 2018). Additionally, alterations in DNA repair genes, such as *BRCA1* and *BRCA2*, can compromise the cell's ability to repair DNA damage, potentially contributing to the progression of cancer. In conclusion, the increased synthesis of cancer cells during the S phases observed in this study can be attributed to the genetic mutations brought about by inhibition, and inactivation of the tumour suppressor activity of complement. This can provide a favourable environment for accumulating genetic mutations and developing more aggressive cancer phenotypes.

4.4.2 Increased cell migration with complement pathway inhibition may be indicative of tumour metastasis

Cell migration constitutes a key determinant in cancer metastasis, particularly in PDAC cases. In this study, complement inhibition was observed to increase cell migration in MIA PaCa-2 cells, suggesting the role of complement pathway could play a critical role in this process. Within the tumour microenvironment, the complement system can influence various processes, including immune cell recruitment, angiogenesis, and extracellular matrix remodelling, which collectively contribute to tumour progression and metastasis. By inhibiting complement, the intricate balance of these activities may be disrupted, leading to alterations in cell migration patterns and potentially facilitating tumour cell migration (Kolev et al., 2022). Furthermore, complement components have been implicated in modulating the interactions between tumour cells and their surrounding stromal cells, fibroblasts, endothelial cells, and immune cells. Alterations in complement activity may influence the secretion of cytokines, chemokines, and

other signaling molecules, thereby shaping the tumour microenvironment and promoting an environment conducive to tumour growth and migration.

Complement C3 and C5 might have direct effects on the signaling pathways involved in cell adhesion and migration, mediated through the recruitment and activation of immune cells (Ostrycharz and Hukowska-Szematowicz, 2022). For instance, the activation of complement C5, C5a can trigger the assembly of MAC, which, in turn, leads to the disruption of the extracellular matrix and cellular membrane integrity. This disruption can facilitate the release of various bioactive molecules, such as cytokines and growth factors, that further regulate cell adhesion and migration processes. Additionally, the activation of downstream signaling cascades, such as the PI3K-Akt and MAPK pathways, can be influenced by complement C3 and C5, ultimately modulating cellular adhesion and migration behaviours (Rutkowski et al., 2010; Zhu et al., 2021). Inhibiting the C5a receptor can increase the expression of matrix metalloproteinases, triggering escalated degradation of the extracellular matrix and ultimately promoting cell migration (Ajona et al., 2018; Mastellos et al., 2018). This corresponds to the finding of this study that shows an increased migration rate of MIA PaCa-2 cells following complement inhibition via C3 which impedes all downstream complement activities including C5, C5a and the formation of the membrane attack complex. This could suggest that blocking the complement system might potentially alter the tumour microenvironment, encouraging cell migration through changes in critical signaling pathways like PI3K-Akt, MAPK/ERK, JAK-STAT, Notch signaling, and Wnt/ β -Catenin. These pathways, when dysregulated, contribute to cancer cell progression, by inducing cell proliferation, cell migration, and chemoresistance (Borlongan and Wang, 2023; Hussain et al., 2022; McCubrey et al., 2016; Yang et al., 2019). Kleczko's suggested that inhibition of complement C3a or C5a signaling inhibits cancer progression in lung cancer models and other malignancies. However, the opposite effect is observed in PDAC, indicating the context-dependent roles of complement in different cancers.

4.5 CONCLUSION

The findings of this study demonstrate that the pharmacological inhibition of the complement pathway may contribute to an aggressive phenotype which is characterized by increased cellular proliferation, migration, division in PDAC. These effects are likely mediated by disturbances in the regulatory signals that govern cell growth and mobility and changes in the tumour microenvironment. Future studies should be conducted using models such as 3D models, which provide a physicochemical environment *in vitro* that is similar to the *in vivo* environment by facilitating cell–cell and cell–matrix interaction commonly associated with conventional

monolayer cell culture, or using *in vivo* animal models which better represent the TME of PDAC. Understanding the intricate interplay between the complement system and cancer cells will pave the way for developing targeted therapies to modulate complement activation. This approach could help preserve the beneficial functions of the complement system while minimising its negative impact on cancer progression.

CHAPTER 5

5. PROTEOMIC ANALYSIS OF COMPLEMENT PATHWAY INHIBITION ON PANCREATIC CANCER CELLS

5.1 INTRODUCTION

PDAC is currently a devastating disease with a mortality rate that equals its incidence (Wang et al., 2021) contributing to approximately 10 million deaths in 2020 alone (Raji et al., 2022). The high mortality rate of PDAC can be attributed to several factors, including late diagnosis (Zhang et al., 2018). PDAC is notorious for its silent progression, often remaining asymptomatic in its early stages and leading to late diagnoses in most patients. Given this challenging diagnostic landscape, efforts have been concentrated on improving early detection methods, including the exploration of novel biomarkers and advanced imaging techniques, to enable timely intervention and improve patient survival rates. Additionally, the limited treatment options available for PDAC, coupled with the inherent resistance of this cancer type to conventional therapies, have prompted a shift towards the development of more targeted and personalized treatment strategies. This involves the elucidation of molecular mechanisms associated with the disease including delineating key signaling pathways.

Several major signaling pathways which play crucial roles in tumour progression have also been implicated in the response to treatment. For example, aberrations in the KRAS signaling pathway are prevalent in PDAC and have been associated with tumour progression and resistance to chemotherapy (Waters and Der, 2018). Also, the activation of pathways such as those involved in Notch, TGF- β and PI3K/AKT/mTOR signaling has been further linked to cell survival, proliferation, and resistance to chemotherapy in PDAC, underscoring its importance in mediating treatment outcomes (Bou Antoun and Chioni, 2023; Hussain et al., 2022; Kumar et al., 2021). Over the years, there have been efforts to target several of these pathways towards effective treatment outcomes; however, these have not yielded the expected results. Although these are well-known signalling pathways linked to PDAC, it is crucial to study and identify other relevant ones that could be linked to tumour progression and treatment resistance to reveal potential key proteins.

5.1.1 Mitochondrial proteins, pathways and PDAC: establishing the link

The mitochondrion, also known as the "powerhouse" of a cell, is an important organelle that is responsible for energy production and many other important cellular processes. Mitochondria are well known for energy production through oxidative phosphorylation (OXPHOS) and other

processes such as lipid metabolism, iron-sulphur cluster assembly, reactive oxygen species (ROS) management, and cell signaling (Fuhrmann and Brüne, 2017; Ježek et al., 2018; Napolitano et al., 2021). Based on the roles they play, mitochondrial proteins can be grouped into multiple families with examples including OXPHOS proteins, responsible for the electron transport chain and ATP synthesis (Yin and O'Neill, 2021); TCA cycle enzymes, functioning in the citric acid cycle for energy metabolism (Kang et al., 2021); mitochondrial ribosomal proteins, required for mitochondrial protein synthesis (Rudler et al., 2021); transporter proteins, which facilitate the import and export of molecules across mitochondrial membranes (Eaglesfield and Tokatlidis, 2021) and lastly fusion and fission proteins, which control mitochondrial dynamics, ensuring proper function and maintenance (Adebayo et al., 2021).

Malfunctions in mitochondrial proteins can lead to severe health issues. In PDAC, mitochondria undergo significant changes which include disruptions in mitochondrial dynamics, impaired oxidative phosphorylation, and increased production of reactive oxygen species (ROS) (Chen et al., 2023; Reyes-Castellanos et al., 2020). Such changes contribute to the metabolic reprogramming observed in cancer cells, promoting their growth and survival. Mitochondrial proteins play a substantial role in the development and progression of pancreatic cancer, offering critical insights into the disease's pathophysiology and potential avenues for diagnosis, treatment, and research. Mutations in these proteins in the context of PDAC can trigger metabolic alterations that facilitate tumour growth (Padinharayil et al., 2023). Several mitochondrial proteins have been identified as significant contributors to the onset and progression of pancreatic cancer. These include mitochondrial metabolism regulators, which are proteins involved in the regulation of mitochondrial metabolism, e.g. succinate dehydrogenase and isocitrate dehydrogenase. Mutations in these proteins in PDAC can trigger metabolic alterations that facilitate tumour growth (Di Gregorio et al., 2022).

Dysregulation of mitochondrial ROS scavenging proteins can result in increased oxidative stress, which is known to promote cancer development (Snezhkina et al., 2019). Additionally, mitochondrial apoptosis regulators like the Bcl-2 family members may influence cell survival and apoptosis (Hussar, 2022). Altered expression of these proteins in PDAC cells can confer resistance to cell death, whereas mutations in mitochondrial DNA have been observed in PDAC, potentially impacting the function of mitochondrial proteins encoded by this DNA (Moro, 2021).

Dysregulation of mitochondrial proteins can lead to changes in energy metabolism, promoting the enhanced growth and survival of PDAC cells. Mitochondrial dysfunction may drive cancer cells to favour glycolysis over oxidative phosphorylation, a phenomenon known as the Warburg

effect (Liu et al., 2021; Warburg, 1956). The altered metabolism in cancer cells, favouring glycolysis, can lead to an increased production of ROS as a by-product which often contribute to oxidative stress within cancer cells as a result of mitochondrial dysfunction and increased metabolic activity. Elevated ROS levels can damage DNA and contribute to genomic instability, a hallmark of cancer. ROS-induced DNA damage from dysfunctional mitochondria can lead to an increased mutational burden, potentially driving the accumulation of genetic alterations in PDAC cells (Puente-Cobacho et al., 2023; Zhou et al., 2020). PDAC has been associated with mutations in mitochondrial DNA, impacting the function of mitochondrial proteins encoded by the mitochondrial genome. Such mutations can affect mitochondrial integrity and function.

PDAC tumours often create a hypoxic microenvironment, whereas mitochondrial pathways are essential for the cell's response to hypoxia, allowing for adaptive mechanisms that enhance cancer cell survival and invasiveness (Tao et al., 2021). In summary, mitochondrial proteins and pathways have significant implications in pancreatic cancer development, affecting energy metabolism, oxidative stress, apoptosis resistance, and genomic stability. A comprehensive understanding of the role of these pathways is essential for developing effective therapeutic strategies to combat this aggressive disease.

5.1.2 Histone acetylases and their role in PDAC

Histone deacetylases (HDAC) are important enzymes that coordinate the intricate process of histone modification within cells. In normal physiological contexts, these enzymes regulate gene expression by adding acetyl groups to specific lysine residues on histone proteins, thereby relaxing chromatin structure and facilitating transcriptional activation (Luan et al., 2015). The modulation of chromatin structure by histone acetylases is important in regulating essential cellular processes, including cell cycle regulation, DNA repair, and differentiation (Kim et al., 2023). However, when the intricate balance of histone acetylase activity becomes disrupted, it can yield significant implications, particularly in the context of cancer. Dysregulation of histone acetylases have been significantly associated with the onset and progression of various cancers, including PDAC (Sim et al., 2022). In PDAC, dysregulation of histone deacetylases disrupts the delicate balance of histone modifications, leading to aberrant gene expression profiles associated with tumour initiation, progression, and metastasis (Sim et al., 2022). When histone acetylases are dysregulated, they often results in perturbed chromatin structure and aberrant gene expression. This can lead to the silencing of tumour suppressor genes or the activation of oncogenes, fuelling the aggressive behaviour of cancer cells (Sim et al., 2022). Additionally, such dysfunctions may impair DNA repair mechanisms and perturb the delicate equilibrium of

cellular processes essential for maintaining genomic stability, further exacerbating the malignant phenotype observed in PDAC.

Understanding the intricate roles of histone acetylases in maintaining normal cellular function, their dysregulation in cancer contexts, particularly in PDAC, and the subsequent molecular consequences of their malfunction is crucial. This comprehension not only sheds light on the underlying mechanisms driving PDAC but also presents promising avenues for targeted therapeutic interventions aimed at restoring histone acetylation balance to impede or reverse the malignant progression of PDAC.

5.1.3 The case for proteomics in elucidating key pathways in PDAC

Proteomics has proven to be a valuable technique for understanding disease processes at the molecular level. The identification of biomarkers has become critical in this field, as they can serve as early indicators of cancer, allow for more accurate diagnosis, and guide treatment decisions. Through proteomic techniques, biomarkers can be identified through the analysis of protein expression levels, post-translational modifications (PTMs), and protein interactions (Neagu et al., 2023). For example, proteomic analysis has been utilized to identify novel biomarkers in breast, prostate, and ovarian cancer, among others (Beretov et al., 2015; Ryu and Thomas, 2021). Proteomics has also been applied to study drug resistance mechanisms in cancer cells and to identify potential therapeutic targets. Furthermore, proteomic analysis can provide a comprehensive understanding of the functional changes that occur in cancer cells and can be used to elucidate the signaling pathways involved in cancer development and progression (Kwon et al., 2021).

The use of mass spectrometry in proteomic analysis offers several advantages for cancer research. These include high sensitivity, high throughput, comprehensive analysis, quantitative analysis, compatibility with clinical samples and the ability to identify drug targets. Mass spectrometry is a highly sensitive technique, capable of detecting low-abundance proteins and peptides (Boschetti and Righetti, 2023). This is critical in cancer research, where changes in protein expression levels can be subtle and difficult to detect using other techniques. It enables the simultaneous analysis of thousands of proteins in a single experiment, making it a valuable tool in cancer biomarker discovery enabling the identification of different types of protein modifications, including phosphorylation, acetylation, and glycosylation, providing a comprehensive view of protein expression and function (Bobalova et al., 2023). Additionally, mass spectrometry enables quantitative analysis of protein expression levels, allowing for the identification of differentially expressed proteins between cancer and normal tissues. It is compatible with a variety of clinical samples, including blood, urine, and tissue samples,

making it an attractive technique for biomarker discovery and clinical applications. Lastly, it can be used to identify drug targets and pathways involved in cancer development and progression, providing important insights for the development of targeted therapies (Aguilar-Valdés et al., 2021; Liyanage et al., 2021). This study used SWATH-MS-based proteomics to investigate the effect of complement inhibition in the MIA PaCa-2 cells.

5.2 METHODS

5.2.1 Cell treatment and preparation

MIA PaCa-2 cells were counted as previously described, and 1×10^5 viable cells were seeded in duplicate in a 24-well plate and left to grow for 24 hours before being treated with $6\mu\text{M}$ compstatin to inhibit the complement pathway by targeting C3. A cell pellet was prepared by washing adherent cells with PBS and adding trypsin-EDTA to detach, collect and centrifuge cells at 250xg for 5 minutes. The pellet was washed at least four times with PBS and stored at -80°C until processing. Treated and untreated cell pellets were resuspended in 50mM Tris-HCl pH 8.0, containing 2% SDS. The protein concentration was measured using the Pierce Bicinchoninic assay (ThermoFisher Scientific), per the manufacturer's instruction. Protein samples ($10\mu\text{g}$ per sample) were reduced with 5mM tris (2-carboxyethyl) phosphine and alkylated with 10mM 2-chloroacetamide at room temperature for 20 minutes.

5.2.2 Proteomic analysis using SWATH-MS

Samples were purified of detergents and salts using MagReSyn™ HILIC beads (ReSyn Biosciences) as previously described (Baichan et al., 2023; Nweke et al., 2020). On-bead protein digestion was performed using a 1:20 and 1:100 protease:protein ratio for sequencing-grade trypsin and Lys-C, respectively. The resultant peptides were dried and stored at -80°C before LC-MS analysis.

Approximately $1\mu\text{g}$ of peptides per sample was analysed using a Dionex Ultimate 3000 RSLC system coupled to a Sciex 5600 TripleTOF mass spectrometer. Injected peptides were de-salted using an Acclaim PepMap C18 trap column ($75\mu\text{M} \times 2\text{cm}$: 2 min at $5\mu\text{l}.\text{min}^{-1}$ using 2% ACN/0.2% FA). Trapped peptides were gradient eluted and separated on a Waters Acquity CSH C18 NanoEase column ($75\mu\text{M} \times 25\text{cm}$, $1.7\mu\text{m}$ particle size) at a flow rate of $0.3\mu\text{l}.\text{min}^{-1}$ with a gradient of 6-40% B over 90 minutes (A: 0.1% FA; B: 80% ACN/0.1% FA). For sequential window acquisition of all theoretical mass spectra (SWATH), precursor scans were acquired from $400\text{-}1100m/z$ with 50 msec accumulation time. 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.

SWATH data was processed using Spectronaut v17 software (Biognosys). The default direct DIA identification and quantification settings were used for data processing. Carbamidomethylation was added as a fixed modification, and N-terminal acetylation and methionine oxidation were added as variable modifications. Swiss-Prot Human sequences (downloaded on 03 March 2023 from www.uniprot.org) and common contaminating proteins were used as the search database. 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) ≥ 0.58 .

5.2.3 Statistical and bioinformatic analyses

Functional enrichment analyses were performed on all the dysregulated proteins. Interaction network analysis and visualisation were performed using Cytoscape v3.8.2 (Shannon et al., 2003). The StringApp v2.0.1 (Doncheva et al., 2019) and Reactome v8.0.5 (Fabregat et al., 2017) plugins on Cytoscape were used to determine the molecular functions, cell compartments and pathways enriched by the dysregulated proteins. The proteins were queried with filters including *homo sapiens* as the reference species, a confidence cut-off score of 0.4 and zero additional interactors. Single non-interacting proteins were excluded from the interaction network.

5.3 RESULTS

5.3.1 Proteins dysregulated with inhibition of the complement pathway

Cells were exposed to compstatin for 24 hours and SWATH-MS performed to delineate further the molecular mechanisms linked with the inhibition of the complement pathway in PDAC. Following treatment, SWATH-MS was used to conduct a proteomic analysis, and the results identified a total of 2814 protein groups. A comparison of the compstatin-treated cells with untreated cells showed 53 upregulated and 60 downregulated proteins (Table S6). Furthermore, the enriched upregulated proteins included a variety of crucial proteins such as the Cysteine and Glycine Rich Protein 1 (CSRP), Mitochondrial Ribosomal Protein (MRPL4, MRPL47), Mitochondrial Ribosome Recycling Factor (MRRF), Glutamate Cysteine Ligase catalytic subunit (GCLC), Euchromatin Histone Methyltransferase II (EHMT2) and RNA Polymerase I and III subunit D (POLR1D). These proteins play different roles in several cellular functions:

development, cellular differentiation, mitochondrial processes (energy metabolism/ATP synthesis, respiration), RNA synthesis, cell cycle exit, DNA damage and cell stress response. Moreover, the H2A clustered histone family of molecules (H2AJ, H2AC7, H2AC12, H2AC14, H2AC17, H2AC19 and H2AC20), which are fundamental nuclear proteins that participate in chromatin assembly and gene expression control, were among the top five enriched downregulated proteins.

Network analysis demonstrated that the proteins are intricately linked by a series on common interactions such as biological processes and pathways (Figure 5.1). The top three upregulated pathways included the MTF1-activated gene expression pathway, response to metal ions pathway and Mitochondrial translation termination pathway while the top three downregulated pathways included the RMTs methylate histone arginines pathway, metalloprotease DUBs pathway and the packaging of telomere ends pathway (Table 5.1).

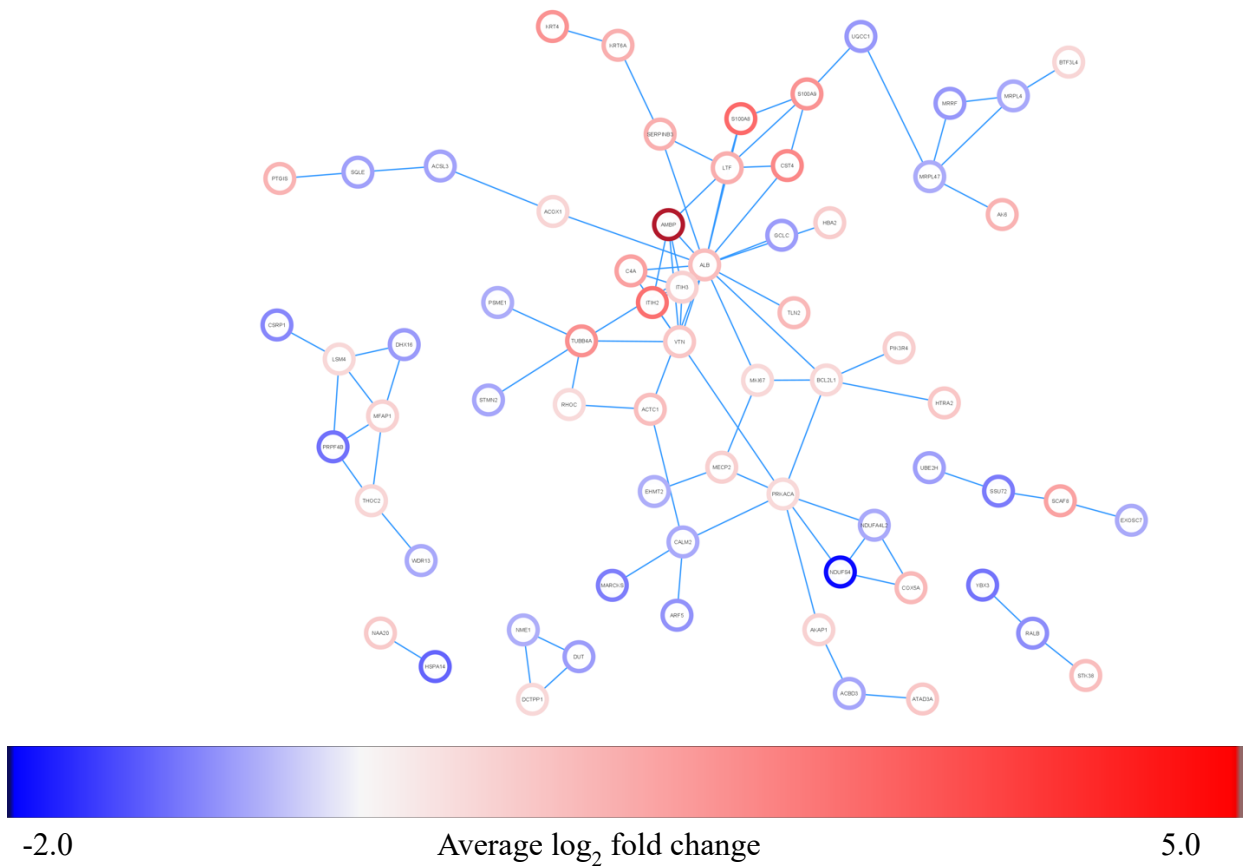


Figure 5.2: Network analysis showing the intricate interaction of dysregulated proteins. Blue and red represent up-and downregulated proteins, respectively, in Compstatin-treated vs untreated pancreatic cancer cells.

Table 5.1: Top 10 pathways enriched by the upregulated and downregulated proteins

Upregulated			Downregulated		
Pathway name	p-value	Proteins enriched	Pathway name	p-value	Proteins enriched
MTF1 activated gene expression	3.95×10^{-6}	CSRP1	RMTs methylate histone arginines	7.96×10^{-10}	H2AC12, H2AC19, H2AJ, H2AC14, H2AC20, H2AC17, H2AC7
Response to metal ions	0.004	CSRP1	Metalloprotease DUBs	3.58×10^{-8}	H2AC12, H2AC19, H2AC14, H2AC20, H2AC17, H2AC7
Mitochondrial translation termination	0.007	MRPL4, MRPL47, MRRF	Packaging of Telomere ends	1.46×10^{-6}	H2AC12, H2AC19, H2AJ, H2AC14, H2AC20, H2AC7
NFE2L2 regulating antioxidant/detoxification enzymes	0.009	GCLC	HDACs deacetylate histones	1.81×10^{-6}	H2AC12, H2AC19, H2AC14, H2AC20, H2AC17, H2AC7
RNA polymerase I transcription initiation	0.019	EHMT2, POLR1D	DNA methylation	1.95×10^{-6}	H2AC12, H2AC19, H2AJ, H2AC14, H2AC20, H2AC7
Synthesis of very long-chain fatty acyl-CoAs	0.019	ACSL3, HACD3	RHO GTPase effectors	2.43×10^{-6}	PIK3R4, S100A8, PRKACA, S100A9, RHOC, TUBB4A, H2AC12, H2AC19, H2AJ, H2AC14, H2AC20, H2AC7
Membrane trafficking	0.021	ACBD3, CALM, SCOC, AP4S1, MCFD2, ARF5, RABGAP1	Recognition and association of DNA glycosylase with site containing an affected purine	2.90×10^{-6}	H2AC12, H2AC19, H2AJ, H2AC14, H2AC20, H2AC7
KEAP1-NFE2L2 pathway	0.023	GCLC, PSME1	Regulation of MECP2 expression and activity	3.29×10^{-6}	MECP2
Loss of phosphorylation of MECP2 at T308	0.029	CALM1	Assembly of the ORC complex at the origin of replication	3.29×10^{-6}	H2AC12, H2AC19, H2AJ, H2AC14, H2AC20, H2AC7
RUNX1 regulates transcription of genes involved in BCR signaling	0.029	ELF2	HATs acetylate histones	3.43×10^{-6}	H2AC12, H2AC19, H2AJ, H2AC14, H2AC20, H2AC17, H2AC7, TAF9

5.4 DISCUSSION

5.4.1 Complement inhibition upregulates mitochondrial ribosomal proteins

Proteomic analysis showed that pharmacologically inhibiting the complement pathway increased the expression of mitochondrial ribosomal proteins (MRPL4, MRPL47) and mitochondrial ribosome recycling factor (MRRF), which play crucial roles in protein synthesis, energy metabolism, respiration and ATP synthesis (Huang et al., 2020; Pfanner et al., 2019). Most cancers alter mitochondrial functions to stimulate the Warburg effect, causing an increase in glucose production and uptake, and elevated levels of ROS species that damage DNA, proteins and lipids, driving a variety of cancer-associated processes that include inflammation, DNA damage and metabolic reprogramming (Jaworska et al., 2023; Kennedy et al., 2020; Liberti and Locasale, 2016). Hypoxia, characterized by low oxygen levels in tumours, intricately links to both the Warburg effect and the generation of reactive oxygen species (ROS) in cancer cells. Under oxygen-deprived conditions, hypoxia-inducible factor 1- α (HIF-1 α) becomes stabilized, prompting cancer cells to preferentially utilize glycolysis for energy even in the presence of oxygen—the hallmark of the Warburg effect. Simultaneously, hypoxia disrupts the cellular machinery, especially in mitochondria, leading to an altered electron transport chain and increased leakage of electrons, thereby elevating ROS production. This dual impact of hypoxia drives cancer cells towards altered metabolism and heightened ROS levels, contributing significantly to their survival, aggressive behaviour, and adaptation to the tumour microenvironment (Thomas and Ashcroft, 2021). The observed increase in complement C5 aligns with previous research and correlates with heightened inflammation. This increase in C5 levels potentially impacts cell proliferation and division within the cell cycle, potentially linking to mitochondrial function and pathways involved in cellular responses to inflammation. This impact might include altering energy availability and disrupting mitochondrial homeostasis, consequently influencing cellular activities related to proliferation and division.

The upregulation of genes implicated in mitochondrial metabolism have been shown in several tumours including PDAC (Gaude and Frezza, 2016; Thomas and Ashcroft, 2021). In Yuan and colleagues' study, data from 2658 malignancies across 38 tumour types, compiled from The International Cancer Genome Consortium (ICGC) and TCGA projects and analysed by the Pan-Cancer Analysis of Whole Genomes (PCAWG) Consortium, focused on mitochondrial somatic mutations and their link to nuclear alterations (Yuan et al., 2020). Notably, PDAC patients (n = 507) showed increased mtDNA copy numbers for 13 mitochondrial genes, while patients with kidney clear cell carcinoma, hepatocellular carcinoma, and myeloproliferative neoplasm displayed decreased copy numbers. These results, taken together, suggest that the complement

pathway may be involved in tumour progression in PDAC via the upregulation of certain mitochondrial proteins.

The field of cancer therapies targeting mitochondria or mitochondrial functions includes various approaches aimed at disrupting cancer cell survival and metabolism. One strategy involves targeting oxidative phosphorylation, inhibiting components of the electron transport chain (ETC) like complex I (e.g., metformin), complex III, and ATP synthase (oligomycin) to hinder ATP production, inducing energy stress in cancer cells (Nayak et al., 2018). Complementary approaches involve mitochondrial metabolism modulators, such as PGC-1 α modulators or mitophagy inducers, seeking to influence mitochondrial biogenesis, fusion/fission dynamics, or selective removal of dysfunctional mitochondria to impact cancer cell survival and metabolism. Additionally, some therapies exploit the role of mitochondria in apoptosis, targeting pathways like mitochondrial outer membrane permeabilization (MOMP) or Bcl-2 family proteins to trigger apoptosis in cancer cells (Kalkavan and Green, 2018). Strategies aiming at targeting or manipulating mitochondrial DNA (mtDNA) integrity or replication as potential cancer therapies are also being explored, as disruptions in mtDNA can induce cell death in cancer cells (Hsu and Sabatini, 2008). Moreover, investigations into therapies targeting reactive oxygen species (ROS) production within mitochondria are underway, aiming to influence signaling pathways crucial in cancer cell proliferation, survival, and DNA damage responses. Exploiting metabolic vulnerabilities specific to mitochondrial function in cancer cells emerges as a promising therapeutic avenue, recognizing that cancers with mitochondrial defects might exhibit heightened sensitivity to treatments addressing these vulnerabilities (Nguyen and Pandey, 2019).

While these mitochondrial-targeted therapies aim to exploit cancer cells' unique metabolic features while preserving healthy cells, the intricate role of mitochondria in both healthy and cancerous cells poses challenges in developing precise and effective therapies. Consequently, ongoing research endeavours strive to refine these strategies, seeking improved efficacy and reduced side effects in cancer treatment.

5.4.2 The histone family proteins are downregulated with complement pathway inhibition

The pharmacological inhibition of complement was demonstrated to downregulate H2A clustered histone molecules. It has been shown that the dysregulation of these molecules may result in aberrant cell cycle progression, genome instability, DNA damage response, and transcriptional regulation (Mei et al., 2017). Epigenetic modifications, such as DNA methylation and histone modifications, have been found to play a crucial role in tumour initiation and development (Wang et al., 2022). H2AC12, H2AC14, H2AC7, H2AC19,

H2AC20, H2AC17 and H2AJ are some of the proteins that were found to be downregulated in response to complement inhibition, potentially contributing to dysregulated gene transcription. Histone acetylation, regulated by histone acetyltransferases and histone deacetylases, is a key epigenetic modification that affects gene expression by loosening or compacting chromatin structure (Jahangirian et al., 2020; Mustafa et al., 2018; Ou et al., 2015). Moreover, histone modifications influence chromatin accessibility, regulating the accessibility of genes to transcriptional machinery (Zhao et al., 2019). Complement inhibition-induced histone downregulation might alter this accessibility, potentially activating genes associated with cancer cell proliferation, and migration.

The interplay between complement inhibition and histone regulation could therefore exert epigenetic changes that drive PDAC towards a more aggressive phenotype. Research has shown that aberrant histone deacetylation, which disrupts the balance between acetylation and deacetylation processes, can lead to chromatin remodeling and the functional loss of tumour-suppressor genes or the activation of oncogenes (Wang et al., 2014). In PDAC, this alteration might affect genes responsible for tumour suppression, DNA repair, or cell cycle regulation, thereby fostering an environment conducive to tumour growth and spread. Some studies suggest that PDAC cells may downregulate or decrease the expression of specific chromatin-remodelling proteins critical for proper DNA duplication and repair (Hasan and Ahuja, 2019a; Sobanski et al., 2021; Wang et al., 2021). By doing so, these cancer cells may be able to tolerate and even exploit DNA damage and other mutations that arise during the cell cycle, which can contribute to cancer progression (Hasan and Ahuja, 2019).

The development of cancer therapies targeting histone modifications has gained significant attention in recent years, focusing on the manipulation of epigenetic mechanisms crucial in regulating gene expression. These therapies encompass various approaches, including histone deacetylase (HDAC) inhibitors like Vorinostat, which have found utility in treating cutaneous T-cell lymphoma and multiple myeloma (Eckschlager et al., 2017). HDAC inhibitors work by targeting enzymes responsible for removing acetyl groups from histone proteins, thereby altering chromatin structure and gene expression. Research endeavours are actively pursuing histone methyltransferase inhibitors to create specific drugs that modulate histone methylation patterns, aiming to influence gene expression in cancer therapy (Marzochi et al., 2023). Additionally, bromodomain inhibitors have demonstrated promise in both preclinical and clinical settings for treating diverse cancers, including haematological malignancies and solid tumours (Ameratunga et al., 2020; Spriano et al., 2020), by targeting proteins that recognize acetylated lysine residues on histones, thereby affecting gene transcription. Other strategies

involve histone demethylase inhibitors that target enzymes removing methyl groups from histones, potentially altering the epigenetic landscape and gene expression in cancer cells. Moreover, the targeting of histone variants implicated in cancer, such as H2A.X and H3.3, or the pathways regulating their incorporation into chromatin, has emerged as a potential therapeutic intervention (Ghiraldini et al., 2021).

These therapies aim to alter the epigenetic landscape of cancer cells, thereby influencing gene expression patterns that can either drive or suppress tumorigenesis. While early studies show promise, the efficacy and specificity of these interventions across various cancer types are subjects of ongoing investigation. Additionally, exploring combination therapies involving histone-targeting agents with conventional treatments like chemotherapy or immunotherapy holds promise for enhancing the efficacy of cancer treatments.

5.5 CONCLUSION

This study has shown the dysregulation of mitochondrial and histone molecules following complement inhibition. The upregulation and downregulation of mitochondrial proteins and histone-family proteins, respectively in cancer cells may contribute to tumour progression. Additionally, understanding of these mechanisms linked to cancer progression has the potential for developing targeted therapies aimed at disrupting these dysregulated molecules, thus potentially impeding cancer progression.

CHAPTER 6

6. OVERALL CONCLUSION AND FUTURE WORK

The findings of this study demonstrated that early PDAC exhibits high expression levels of C3 and C5, which decrease as the severity of the tumour increases. Expression levels of C3 and C5 could potentially serve as early-stage PDAC biomarkers, aiding in early PDAC diagnosis and severity assessment. As these levels decrease with tumour progression, this trend might reveal therapeutic possibilities—targeting these proteins or their associated pathways might offer strategies to impede tumour advancement. Future studies should investigate the specific roles of C3 and C5 in PDAC progression, which could identify detailed mechanisms and validate them as biomarkers through larger patient cohorts or exploring targeted therapies based on these complement proteins. However, it seems that they could have both pro and anti-tumourigenic roles depending on the context. The corresponding *in vitro* component of the study, showed that inhibition of the complement pathway resulted in a more aggressive phenotype, by stimulating cellular growth, proliferation, and migration, indicating the involvement of complement C3 and C5 in tumour progression. Our findings suggest the dysregulation of the complement pathway in PDAC progression may further help in understanding the context-specific role of the complement pathway in carcinogenesis. This was highlighted by our study showing complement inhibition upregulates mitochondrial ribosomal proteins which play major roles in protein synthesis and metabolism, thereby potentiating tumour progression. Moreover, the findings that key proteins involved in histone modifications are downregulated with complement inhibition, indicate an additional route by which PDAC is driven toward a more aggressive phenotype. Thus while *in vitro* findings in this study have identified proteins of interest, caution should be taken prior to targeting the complement pathway without a comprehensive understanding of context-specific outcomes. Therapies directed at complement C3 and C5 need to assess potential unintended consequences on tumour behaviour. Future studies could focus on unravelling the specific mechanisms with which complement inhibition leads to increased cellular growth, proliferation, and migration. Investigating the downstream pathways affected by C3 and C5 in tumour progression can pave the way for more precise therapies. Additionally, exploring combination therapies that merge complement pathway targeting with conventional treatments might offer insights into overcoming limitations observed with individual pathway inhibition. Furthermore, additional studies should be conducted using models such as 3D models or *in vivo* which better represent the TME of PDAC.

Lastly, investigations into pathways that are linked to the regulation of the complement pathway would be beneficial in further elucidating its role in PDAC.

REFERENCES

- Adebayo, M., Singh, S., Singh, A.P., Dasgupta, S., 2021. Mitochondrial Fusion and Fission: The fine-tune balance for cellular homeostasis. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 35, e21620. <https://doi.org/10.1096/fj.202100067R>
- Afshar-Kharghan, V., 2017. The role of the complement system in cancer. *J. Clin. Invest.* 127, 780–789. <https://doi.org/10.1172/JCI90962>
- Aguilar-Valdés, A., Noriega, L.G., Tovar, A.R., Ibarra-Sánchez, M. de J., Sosa-Hernández, V.A., Maravillas-Montero, J.L., Martínez-Aguilar, J., 2021. SWATH-MS proteomics of PANC-1 and MIA PaCa-2 pancreatic cancer cells allows identification of drug targets alternative to MEK and PI3K inhibition. *Biochem. Biophys. Res. Commun.* 552, 23–29. <https://doi.org/10.1016/j.bbrc.2021.03.018>
- Ahn, H.R., Baek, G.O., Yoon, M.G., Son, J.A., You, D., Yoon, J.H., Cho, H.J., Kim, S.S., Cheong, J.Y., Eun, J.W., 2021. *HMBS* is the most suitable reference gene for RT-qPCR in human HCC tissues and blood samples. *Oncol. Lett.* 22, 1–9. <https://doi.org/10.3892/ol.2021.13052>
- Ain, D., Shaikh, T., Manimala, S., Ghebrehwet, B., 2021. The role of complement in the tumor microenvironment. *Fac. Rev.* 10, 80. <https://doi.org/10.12703/r/10-80>
- Ajona, D., Zandueta, C., Corrales, L., Moreno, H., Pajares, M.J., Ortiz-Espinosa, S., Martínez-Terroba, E., Perurena, N., de Miguel, F.J., Jantus-Lewintre, E., Camps, C., Vicent, S., Agorreta, J., Montuenga, L.M., Pio, R., Lecanda, F., 2018. Blockade of the Complement C5a/C5aR1 Axis Impairs Lung Cancer Bone Metastasis by CXCL16-mediated Effects. *Am. J. Respir. Crit. Care Med.* 197, 1164–1176. <https://doi.org/10.1164/rccm.201703-0660OC>
- Alhajj, M., Farhana, A., 2022. Enzyme Linked Immunosorbent Assay, in: *StatPearls*. StatPearls Publishing, Treasure Island (FL).
- Amakye, D., Jagani, Z., Dorsch, M., 2013. Unraveling the therapeutic potential of the Hedgehog pathway in cancer. *Nat. Med.* 19, 1410–1422. <https://doi.org/10.1038/nm.3389>
- Ameratunga, M., Braña, I., Bono, P., Postel-Vinay, S., Plummer, R., Aspegren, J., Korjamo, T., Snapir, A., de Bono, J.S., 2020. First-in-human Phase 1 open label study of the BET inhibitor ODM-207 in patients with selected solid tumours. *Br. J. Cancer* 123, 1730–1736. <https://doi.org/10.1038/s41416-020-01077-z>
- Arias-Pinilla, G.A., Modjtahedi, H., 2021. Therapeutic Application of Monoclonal Antibodies in Pancreatic Cancer: Advances, Challenges and Future Opportunities. *Cancers* 13, 1781. <https://doi.org/10.3390/cancers13081781>
- Artero-Castro, A., Castellvi, J., García, A., Hernández, J., Ramón y Cajal, S., Leonart, M.E., 2011. Expression of the ribosomal proteins Rplp0, Rplp1, and Rplp2 in gynecologic tumors. *Hum. Pathol.* 42, 194–203. <https://doi.org/10.1016/j.humpath.2010.04.020>
- Ashraf, H., Colombo, J.P., Marcucci, V., Rhoton, J., Olowoyo, O., 2021. A Clinical Overview of Acute and Chronic Pancreatitis: The Medical and Surgical Management. *Cureus* 13. <https://doi.org/10.7759/cureus.19764>

- Baichan, P., Naicker, P., Augustine, T.N., Smith, M., Candy, G., Devar, J., Nweke, E.E., 2023. Proteomic analysis identifies dysregulated proteins and associated molecular pathways in a cohort of gallbladder cancer patients of African ancestry. *Clin. Proteomics* 20, 8. <https://doi.org/10.1186/s12014-023-09399-9>
- Bailey, P., Chang, D.K., Forget, M.-A., Lucas, F.A.S., Alvarez, H.A., Haymaker, C., Chattopadhyay, C., Kim, S.-H., Ekmekcioglu, S., Grimm, E.A., Biankin, A.V., Hwu, P., Maitra, A., Roszik, J., 2016. Exploiting the neoantigen landscape for immunotherapy of pancreatic ductal adenocarcinoma. *Sci. Rep.* 6. <https://doi.org/10.1038/srep35848>
- Bain, W., Li, H., Van Der Geest, R., Moore, S.R., Olonisakin, T.F., Ahn, B., Papke, E., Moghbeli, K., DeSensi, R., Rapport, S., Saul, M., Hulver, M., Xiong, Z., Mallampalli, R.K., Ray, P., Morris, A., Ma, L., Doi, Y., Zhang, Y., Kitsios, G.D., Kulkarni, H.S., McVerry, B.J., Ferreira, V.P., Nouraie, M., Lee, J.S., 2020. Increased Alternative Complement Pathway Function and Improved Survival during Critical Illness. *Am. J. Respir. Crit. Care Med.* 202, 230–240. <https://doi.org/10.1164/rccm.201910-2083OC>
- Balkwill, F.R., Mantovani, A., 2012. Cancer-related inflammation: Common themes and therapeutic opportunities. *Cancer Immunother.* 22, 33–40. <https://doi.org/10.1016/j.semcancer.2011.12.005>
- Barry, K., 2018. Chronic Pancreatitis: Diagnosis and Treatment. *Am. Fam. Physician* 97, 385–393.
- Bengsch, F., Knoblock, D.M., Liu, A., McAllister, F., Beatty, G.L., 2017. CTLA-4/CD80 pathway regulates T cell infiltration into pancreatic cancer. *Cancer Immunol. Immunother.* 66, 1609–1617. <https://doi.org/10.1007/s00262-017-2053-4>
- Benjamin, O., Lappin, S.L., 2023. Chronic Pancreatitis, in: StatPearls. StatPearls Publishing, Treasure Island (FL).
- Bennett, K.M., Rooijackers, S.H.M., Gorham, R.D., 2017. Let's Tie the Knot: Marriage of Complement and Adaptive Immunity in Pathogen Evasion, for Better or Worse. *Front. Microbiol.* 8. <https://doi.org/10.3389/fmicb.2017.00089>
- Beretov, J., Wasinger, V.C., Millar, E.K.A., Schwartz, P., Graham, P.H., Li, Y., 2015. Proteomic Analysis of Urine to Identify Breast Cancer Biomarker Candidates Using a Label-Free LC-MS/MS Approach. *PLOS ONE* 10, e0141876. <https://doi.org/10.1371/journal.pone.0141876>
- Bhatia, A., Kumar, Y., 2011. Cancer-Immune Equilibrium: Questions Unanswered. *Cancer Microenviron.* 4, 209–217. <https://doi.org/10.1007/s12307-011-0065-8>
- Biocom, A., 2019. Targovax reports 100% one-year survival in pancreatic cancer trial [WWW Document]. URL <https://european-biotechnology.com/up-to-date/latest-news/news/targovax-reports-100-one-year-survival-in-pancreatic-cancer-trial.html> (accessed 2.1.20).
- Bobalova, J., Strouhalova, D., Bobal, P., 2023. Common Post-translational Modifications (PTMs) of Proteins: Analysis by Up-to-Date Analytical Techniques with an Emphasis on Barley. *J. Agric. Food Chem.* 71, 14825–14837. <https://doi.org/10.1021/acs.jafc.3c00886>
- Borlongan, M.C., Wang, H., 2023. Profiling and targeting cancer stem cell signaling pathways for cancer therapeutics. *Front. Cell Dev. Biol.* 11.

- Boschetti, E., Righetti, P.G., 2023. Low-Abundance Protein Enrichment for Medical Applications: The Involvement of Combinatorial Peptide Library Technique. *Int. J. Mol. Sci.* 24, 10329. <https://doi.org/10.3390/ijms241210329>
- Bou Antoun, N., Chioni, A.-M., 2023. Dysregulated Signalling Pathways Driving Anticancer Drug Resistance. *Int. J. Mol. Sci.* 24, 12222. <https://doi.org/10.3390/ijms241512222>
- Boutillier, A.J., Elsawa, S.F., 2021. Macrophage Polarization States in the Tumor Microenvironment. *Int. J. Mol. Sci.* 22, 6995. <https://doi.org/10.3390/ijms22136995>
- Bowcock, S.J., Shee, C.D., Rassam, S.M.B., Harper, P.G., 2004. Chemotherapy for cancer patients who present late. *BMJ* 328, 1430–1432.
- Bremnes, R.M., Dønnem, T., Al-Saad, S., Al-Shibli, K., Andersen, S., Sirera, R., Camps, C., Marinez, I., Busund, L.-T., 2011. The Role of Tumor Stroma in Cancer Progression and Prognosis: Emphasis on Carcinoma-Associated Fibroblasts and Non-small Cell Lung Cancer. *J. Thorac. Oncol.* 6, 209–217. <https://doi.org/10.1097/JTO.0b013e3181f8a1bd>
- Bulla, R., Tripodo, C., Rami, D., Ling, G.S., Agostinis, C., Guarnotta, C., Zorzet, S., Durigutto, P., Botto, M., Tedesco, F., 2016. C1q acts in the tumour microenvironment as a cancer-promoting factor independently of complement activation. *Nat. Commun.* 7, 10346. <https://doi.org/10.1038/ncomms10346>
- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622. <https://doi.org/10.1373/clinchem.2008.112797>
- Canto, M.I., Goggins, M., Hruban, R.H., Petersen, G.M., Giardiello, F.M., Yeo, C., Fishman, E.K., Brune, K., Axilbund, J., Griffin, C., Ali, S., Richman, J., Jagannath, S., Kantsevov, S.V., Kalloo, A.N., 2006. Screening for early pancreatic neoplasia in high-risk individuals: a prospective controlled study. *Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc.* 4, 766–781; quiz 665. <https://doi.org/10.1016/j.cgh.2006.02.005>
- Carvelli, J., Demaria, O., Vély, F., Batista, L., Chouaki Benmansour, N., Fares, J., Carpentier, S., Thibult, M.-L., Morel, A., Remark, R., André, P., Represa, A., Piperoglou, C., Cordier, P.Y., Le Dault, E., Guervilly, C., Simeone, P., Gainnier, M., Morel, Y., Ebbo, M., Schleinitz, N., Vivier, E., 2020. Association of COVID-19 inflammation with activation of the C5a–C5aR1 axis. *Nature* 588, 146–150. <https://doi.org/10.1038/s41586-020-2600-6>
- Cassol, C.A., Brodsky, S.V., Satoskar, A.A., Blissett, A.R., Cataland, S., Nadasdy, T., 2019. Eculizumab deposits in vessel walls in thrombotic microangiopathy. *Kidney Int.* 96, 761–768. <https://doi.org/10.1016/j.kint.2019.05.008>
- Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A.P., Sander, C., Schultz, N., 2012. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discov.* 2, 401–404. <https://doi.org/10.1158/2159-8290.CD-12-0095>
- Chang, J.H., Jiang, Y., Pillarisetty, V.G., 2016. Role of immune cells in pancreatic cancer from bench to clinical application: An updated review. *Medicine (Baltimore)* 95, e5541. <https://doi.org/10.1097/MD.0000000000005541>

- Chen, Huey-Ling, Wu, S.-H., Hsu, S.-H., Liou, B.-Y., Chen, Hui-Ling, Chang, M.-H., 2018. Jaundice revisited: recent advances in the diagnosis and treatment of inherited cholestatic liver diseases. *J. Biomed. Sci.* 25, 75. <https://doi.org/10.1186/s12929-018-0475-8>
- Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., Zhao, L., 2017. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* 9, 7204–7218. <https://doi.org/10.18632/oncotarget.23208>
- Chen, W., Zhao, H., Li, Y., 2023. Mitochondrial dynamics in health and disease: mechanisms and potential targets. *Signal Transduct. Target. Ther.* 8, 1–25. <https://doi.org/10.1038/s41392-023-01547-9>
- Cheng, H., Luo, G., Lu, Y., Jin, K., Guo, M., Xu, J., Long, J., Liu, L., Yu, X., Liu, C., 2016. The combination of systemic inflammation-based marker NLR and circulating regulatory T cells predicts the prognosis of resectable pancreatic cancer patients. *Pancreatol. Off. J. Int. Assoc. Pancreatol. IAP AI* 16, 1080–1084. <https://doi.org/10.1016/j.pan.2016.09.007>
- Chomczynski, P., 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* 15, 532–4, 536–7.
- Conroy, T., Desseigne, F., Ychou, M., Bouché, O., Guimbaud, R., Bécouarn, Y., Adenis, A., Raoul, J.-L., Gourgou-Bourgade, S., de la Fouchardière, C., Bennouna, J., Bachet, J.-B., Khemissa-Akouz, F., Péré-Vergé, D., Delbaldo, C., Assenat, E., Chauffert, B., Michel, P., Montoto-Grillot, C., Ducreux, M., 2011. FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer. *N. Engl. J. Med.* 364, 1817–1825. <https://doi.org/10.1056/NEJMoa1011923>
- Conroy, T., Hammel, P., Hebbar, M., Ben Abdelghani, M., Wei, A.C., Raoul, J.-L., Choné, L., Francois, E., Artru, P., Biagi, J.J., Lecomte, T., Assenat, E., Faroux, R., Ychou, M., Volet, J., Sauvanet, A., Breysacher, G., Di Fiore, F., Cripps, C., Kavan, P., Texereau, P., Bouhier-Leporrier, K., Khemissa-Akouz, F., Legoux, J.-L., Juzyna, B., Gourgou, S., O’Callaghan, C.J., Jouffroy-Zeller, C., Rat, P., Malka, D., Castan, F., Bachet, J.-B., 2018. FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer. *N. Engl. J. Med.* 379, 2395–2406. <https://doi.org/10.1056/NEJMoa1809775>
- Corcoran, R.B., Contino, G., Deshpande, V., Tzatsos, A., Conrad, C., Benes, C.H., Settleman, J., Engelman, J.A., Bardeesy, N., 2011. STAT3 plays a critical role in KRAS-induced pancreatic tumorigenesis. *Cancer Res.* 71, 5020–5029. <https://doi.org/10.1158/0008-5472.CAN-11-0908>
- Corrales, L., Ajona, D., Rafail, S., Lasarte, J.J., Riezu-Boj, J.I., Lambris, J.D., Rouzaut, A., Pajares, M.J., Montuenga, L.M., Pio, R., 2012. Anaphylatoxin C5a Creates a Favorable Microenvironment for Lung Cancer Progression. *J. Immunol.* 189, 4674–4683. <https://doi.org/10.4049/jimmunol.1201654>
- Cruz-Ramos, M., García-Foncillas, J., 2019. CAR-T cell and Personalized Medicine. *Adv. Exp. Med. Biol.* 1168, 131–145. https://doi.org/10.1007/978-3-030-24100-1_9
- Danenberg, E., Bardwell, H., Zanutelli, V.R.T., Provenzano, E., Chin, S.-F., Rueda, O.M., Green, A., Rakha, E., Aparicio, S., Ellis, I.O., Bodenmiller, B., Caldas, C., Ali, H.R., 2022. Breast tumor microenvironment structures are associated with genomic features and clinical outcome. *Nat. Genet.* 54, 660–669. <https://doi.org/10.1038/s41588-022-01041-y>

- de Streel, G., Lucas, S., 2021. Targeting immunosuppression by TGF- β 1 for cancer immunotherapy. *Biochem. Pharmacol.* 192, 114697. <https://doi.org/10.1016/j.bcp.2021.114697>
- Deer, E.L., González-Hernández, J., Coursen, J.D., Shea, J.E., Ngatia, J., Scaife, C.L., Firpo, M.A., Mulvihill, S.J., 2010. Phenotype and Genotype of Pancreatic Cancer Cell Lines. *Pancreas* 39, 425–435. <https://doi.org/10.1097/MPA.0b013e3181c15963>
- Dhatchinamoorthy, K., Colbert, J.D., Rock, K.L., 2021. Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Front. Immunol.* 12, 636568. <https://doi.org/10.3389/fimmu.2021.636568>
- Di Gregorio, J., Petricca, S., Iorio, R., Toniato, E., Flati, V., 2022. Mitochondrial and metabolic alterations in cancer cells. *Eur. J. Cell Biol.* 101, 151225. <https://doi.org/10.1016/j.ejcb.2022.151225>
- Dodds, A.W., 2002. Which Came First, the Lectin/Classical Pathway or the Alternative Pathway of Complement? *Immunobiology* 205, 340–354. <https://doi.org/10.1078/0171-2985-00137>
- Doncheva, N.T., Morris, J.H., Gorodkin, J., Jensen, L.J., 2019. Cytoscape StringApp: Network Analysis and Visualization of Proteomics Data. *J. Proteome Res.* 18, 623–632. <https://doi.org/10.1021/acs.jproteome.8b00702>
- Duan, W., Wang, Z., Qian, W., Qi, D., Ma, Q., Wu, E., 2022. Editorial: The Microenvironment in Pancreatic Cancer and Therapeutic Strategies Targeting Microenvironment. *Front. Oncol.* 12.
- Eaglesfield, R., Tokatlidis, K., 2021. Targeting and Insertion of Membrane Proteins in Mitochondria. *Front. Cell Dev. Biol.* 9.
- Eckschlager, T., Plch, J., Stiborova, M., Hrabeta, J., 2017. Histone Deacetylase Inhibitors as Anticancer Drugs. *Int. J. Mol. Sci.* 18, 1414. <https://doi.org/10.3390/ijms18071414>
- El Khoury, W., Nasr, Z., 2021. Deregulation of ribosomal proteins in human cancers. *Biosci. Rep.* 41, BSR20211577. <https://doi.org/10.1042/BSR20211577>
- Ene-Obong, A., Clear, A.J., Watt, J., Wang, J., Fatah, R., Riches, J.C., Marshall, J.F., Chin-Aleong, J., Chelala, C., Gribben, J.G., Ramsay, A.G., Kocher, H.M., 2013. Activated pancreatic stellate cells sequester CD8⁺ T cells to reduce their infiltration of the juxtatumoral compartment of pancreatic ductal adenocarcinoma. *Gastroenterology* 145, 1121–1132. <https://doi.org/10.1053/j.gastro.2013.07.025>
- Fabregat, A., Sidiropoulos, K., Viteri, G., Forner, O., Marin-Garcia, P., Arnau, V., D'Eustachio, P., Stein, L., Hermjakob, H., 2017. Reactome pathway analysis: a high-performance in-memory approach. *BMC Bioinformatics* 18, 142. <https://doi.org/10.1186/s12859-017-1559-2>
- Fan, X., Yao, H., Liu, X., Shi, Q., Lv, L., Li, P., Wang, R., Tang, T., Qi, K., 2020. High-Fat Diet Alters the Expression of Reference Genes in Male Mice. *Front. Nutr.* 7.
- Feig, C., Jones, J.O., Kraman, M., Wells, R.J.B., Deonaraine, A., Chan, D.S., Connell, C.M., Roberts, E.W., Zhao, Q., Caballero, O.L., Teichmann, S.A., Janowitz, T., Jodrell, D.I., Tuveson, D.A., Fearon, D.T., 2013. Targeting CXCL12 from FAP-expressing carcinoma-

- associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc. Natl. Acad. Sci. U. S. A.* 110, 20212–20217. <https://doi.org/10.1073/pnas.1320318110>
- Feng, Y., Ye, Z., Song, F., He, Y., Liu, J., 2022. The Role of TAMs in Tumor Microenvironment and New Research Progress. *Stem Cells Int.* 2022, 5775696. <https://doi.org/10.1155/2022/5775696>
- Fogar, P., Basso, D., Fadi, E., Greco, E., Pantano, G., Padoan, A., Bozzato, D., Facco, M., Sanzari, M.C., Teolato, S., Zambon, C.-F., Navaglia, F., Semenzato, G., Pedrazzoli, S., Plebani, M., 2011. Pancreatic Cancer Alters Human CD4⁺ T Lymphocyte Function: A Piece in the Immune Evasion Puzzle. *Pancreas* 40, 1131–1137. <https://doi.org/10.1097/MPA.0b013e31822077b8>
- Fuhrmann, D.C., Brüne, B., 2017. Mitochondrial composition and function under the control of hypoxia. *Redox Biol.* 12, 208–215. <https://doi.org/10.1016/j.redox.2017.02.012>
- Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., Ferrucci, L., Gilroy, D.W., Fasano, A., Miller, G.W., Miller, A.H., Mantovani, A., Weyand, C.M., Barzilai, N., Goronzy, J.J., Rando, T.A., Effros, R.B., Lucia, A., Kleinstreuer, N., Slavich, G.M., 2019. Chronic inflammation in the etiology of disease across the life span. *Nat. Med.* 25, 1822–1832. <https://doi.org/10.1038/s41591-019-0675-0>
- Gaida, M.M., Welsch, T., Herpel, E., Tschaharganeh, D.F., Fischer, L., Schirmacher, P., Hänsch, G.M., Bergmann, F., 2012. MHC class II expression in pancreatic tumors: a link to intratumoral inflammation. *Virchows Arch. Int. J. Pathol.* 460, 47–60. <https://doi.org/10.1007/s00428-011-1175-x>
- Gandhi, S., de la Fuente, J., Murad, M.H., Majumder, S., 2022. Chronic Pancreatitis Is a Risk Factor for Pancreatic Cancer, and Incidence Increases With Duration of Disease: A Systematic Review and Meta-analysis. *Clin. Transl. Gastroenterol.* 13, e00463. <https://doi.org/10.14309/ctg.0000000000000463>
- Gao, Z., Zhang, Q., Zhang, X., Song, Y., 2022. Advance of T regulatory cells in tumor microenvironment remodeling and immunotherapy in pancreatic cancer. *Eur. J. Inflamm.* 20, 1721727X221092900. <https://doi.org/10.1177/1721727X221092900>
- Garrido, F., Aptsiauri, N., Doorduijn, E.M., Garcia Lora, A.M., van Hall, T., 2016. The urgent need to recover MHC class I in cancers for effective immunotherapy. *Curr. Opin. Immunol.* 39, 44–51. <https://doi.org/10.1016/j.coi.2015.12.007>
- Gaude, E., Frezza, C., 2016. Tissue-specific and convergent metabolic transformation of cancer correlates with metastatic potential and patient survival. *Nat. Commun.* 7, 13041. <https://doi.org/10.1038/ncomms13041>
- Geindreau, M., Bruchard, M., Vegran, F., 2022. Role of Cytokines and Chemokines in Angiogenesis in a Tumor Context. *Cancers* 14, 2446. <https://doi.org/10.3390/cancers14102446>
- Geng, L., Huang, D., Liu, J., Qian, Y., Deng, J., Li, D., Hu, Z., Zhang, J., Jiang, G., Zheng, S., 2008. B7-H1 up-regulated expression in human pancreatic carcinoma tissue associates with tumor progression. *J. Cancer Res. Clin. Oncol.* 134, 1021–1027. <https://doi.org/10.1007/s00432-008-0364-8>

- Ghiraldini, F.G., Filipescu, D., Bernstein, E., 2021. Solid tumours hijack the histone variant network. *Nat. Rev. Cancer* 21, 257–275. <https://doi.org/10.1038/s41568-020-00330-0>
- Giorgio, C., Zippoli, M., Cocchiaro, P., Castelli, V., Varrassi, G., Aramini, A., Allegretti, M., Brandolini, L., Cesta, M.C., 2021. Emerging Role of C5 Complement Pathway in Peripheral Neuropathies: Current Treatments and Future Perspectives. *Biomedicines* 9, 399. <https://doi.org/10.3390/biomedicines9040399>
- Golay, J., Taylor, R.P., 2020. The Role of Complement in the Mechanism of Action of Therapeutic Anti-Cancer mAbs. *Antibodies* 9, 58. <https://doi.org/10.3390/antib9040058>
- Gong, J., Cho, M., Fakih, M., 2017. Chemotherapy in patients with hepatobiliary cancers and abnormal hepatic function. *J. Gastrointest. Oncol.* 8. <https://doi.org/10.21037/jgo.2016.09.17>
- Gorhls, L., Kaipe, H., 2021. Interactions between Cancer-Associated Fibroblasts and T Cells in the Pancreatic Tumor Microenvironment and the Role of Chemokines. *Cancers* 13, 2995. <https://doi.org/10.3390/cancers13122995>
- Gordon-Dseagu, V.L., Devesa, S.S., Goggins, M., Stolzenberg-Solomon, R., 2018. Pancreatic cancer incidence trends: evidence from the Surveillance, Epidemiology and End Results (SEER) population-based data. *Int. J. Epidemiol.* 47, 427–439. <https://doi.org/10.1093/ije/dyx232>
- Goulart, M.R., Stasinos, K., Fincham, R.E.A., Delvecchio, F.R., Kocher, H.M., 2021. T cells in pancreatic cancer stroma. *World J. Gastroenterol.* 27, 7956–7968. <https://doi.org/10.3748/wjg.v27.i46.7956>
- Gouma, D.J., Busch, O.R.C., Van Gulik, T.M., 2006. Pancreatic carcinoma: Palliative surgical and endoscopic treatment. *HPB* 8, 369–376. <https://doi.org/10.1080/13651820600804021>
- Grossberg, A.J., Chu, L.C., Deig, C.R., Fishman, E.K., Hwang, W.L., Maitra, A., Marks, D.L., Mehta, A., Nabavizadeh, N., Simeone, D.M., Weekes, C.D., Thomas Jr, C.R., 2020. Multidisciplinary standards of care and recent progress in pancreatic ductal adenocarcinoma. *CA. Cancer J. Clin.* 70, 375–403. <https://doi.org/10.3322/caac.21626>
- Gutierrez, O.I.B., Lennon, A.M., 2019. Chronic pancreatitis-like changes in individuals at high risk for the development of pancreatic cancer: What is the significance? *Gastrointest. Endosc.* 89, 852–854. <https://doi.org/10.1016/j.gie.2019.01.023>
- Halbrook, C.J., Lyssiotis, C.A., Pasca Di Magliano, M., Maitra, A., 2023. Pancreatic cancer: Advances and challenges. *Cell* 186, 1729–1754. <https://doi.org/10.1016/j.cell.2023.02.014>
- Hanahan, D., 2022. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* 12, 31–46. <https://doi.org/10.1158/2159-8290.CD-21-1059>
- Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *cell* 144, 646–674.
- Hanahan, D., Weinberg, R.A., 2000. The hallmarks of cancer. *Cell* 100.
- Harris, P.A., Taylor, R., Minor, B.L., Elliott, V., Fernandez, M., O’Neal, L., McLeod, L., Delacqua, G., Delacqua, F., Kirby, J., Duda, S.N., REDCap Consortium, 2019. The

- REDCap consortium: Building an international community of software platform partners. *J. Biomed. Inform.* 95, 103208. <https://doi.org/10.1016/j.jbi.2019.103208>
- Harris, P.A., Taylor, R., Thielke, R., Payne, J., Gonzalez, N., Conde, J.G., 2009. Research Electronic Data Capture (REDCap) - A metadata-driven methodology and workflow process for providing translational research informatics support. *J. Biomed. Inform.* 42, 377–381. <https://doi.org/10.1016/j.jbi.2008.08.010>
- Hasan, N., Ahuja, N., 2019a. The Emerging Roles of ATP-Dependent Chromatin Remodeling Complexes in Pancreatic Cancer. *Cancers* 11, 1859. <https://doi.org/10.3390/cancers11121859>
- Hasan, N., Ahuja, N., 2019b. The Emerging Roles of ATP-Dependent Chromatin Remodeling Complexes in Pancreatic Cancer. *Cancers* 11, 1859. <https://doi.org/10.3390/cancers11121859>
- Hassan, R., Blumenschein, G.R., Moore, K.N., Santin, A.D., Kindler, H.L., Nemunaitis, J.J., Seward, S.M., Thomas, A., Kim, S.K., Rajagopalan, P., Walter, A.O., Laurent, D., Childs, B.H., Sarapa, N., Elbi, C., Bendell, J.C., 2020. First-in-Human, Multicenter, Phase I Dose-Escalation and Expansion Study of Anti-Mesothelin Antibody–Drug Conjugate Anetumab Ravtansine in Advanced or Metastatic Solid Tumors. *J. Clin. Oncol.* 38, 1824–1835. <https://doi.org/10.1200/JCO.19.02085>
- Hassid, B.G., Lucas, A.L., Salomao, M., Weng, C., Liu, F., Khanna, L.G., Kumar, S., Hwang, C., Chabot, J.A., Frucht, H., 2014. Absence of Pancreatic Intraepithelial Neoplasia (PanIN) Predicts Poor Survival Following Resection of Pancreatic Cancer. *Pancreas* 43, 1073–1077. <https://doi.org/10.1097/MPA.0000000000000161>
- He, X., Xu, C., 2020. Immune checkpoint signaling and cancer immunotherapy. *Cell Res.* 30, 660–669. <https://doi.org/10.1038/s41422-020-0343-4>
- Hegde, S., Krisnawan, V.E., Herzog, B.H., Zuo, C., Breden, M.A., Knolhoff, B.L., Hogg, G.D., Tang, J.P., Baer, J.M., Mpoy, C., Lee, K.B., Alexander, K.A., Rogers, B.E., Murphy, K.M., Hawkins, W.G., Fields, R.C., DeSelm, C.J., Schwarz, J.K., DeNardo, D.G., 2020. Dendritic Cell Paucity Leads to Dysfunctional Immune Surveillance in Pancreatic Cancer. *Cancer Cell* 37, 289-307.e9. <https://doi.org/10.1016/j.ccell.2020.02.008>
- Himmel, M.E., Saibil, S.D., Saltman, A.P., 2020. Immune checkpoint inhibitors in cancer immunotherapy. *CMAJ Can. Med. Assoc. J.* 192, E651. <https://doi.org/10.1503/cmaj.191231>
- Homma, Y., Taniguchi, K., Nakazawa, M., Matsuyama, R., Mori, R., Takeda, K., Ichikawa, Y., Tanaka, K., Endo, I., 2014. Changes in the immune cell population and cell proliferation in peripheral blood after gemcitabine-based chemotherapy for pancreatic cancer. *Clin. Transl. Oncol. Off. Publ. Fed. Span. Oncol. Soc. Natl. Cancer Inst. Mex.* 16, 330–335. <https://doi.org/10.1007/s12094-013-1079-0>
- Houg, D.S., Bijlsma, M.F., 2018. The hepatic pre-metastatic niche in pancreatic ductal adenocarcinoma. *Mol. Cancer* 17, 95. <https://doi.org/10.1186/s12943-018-0842-9>
- Hsu, P.P., Sabatini, D.M., 2008. Leaders in Pharmaceutical Business Intelligence (LPBI) Group. *Cell* 134, 703–707.

- Hu, J.-X., Zhao, C.-F., Chen, W.-B., Liu, Q.-C., Li, Q.-W., Lin, Y.-Y., Gao, F., 2021. Pancreatic cancer: A review of epidemiology, trend, and risk factors. *World J. Gastroenterol.* 27, 4298–4321. <https://doi.org/10.3748/wjg.v27.i27.4298>
- Huang, G., Li, H., Zhang, H., 2020. Abnormal Expression of Mitochondrial Ribosomal Proteins and Their Encoding Genes with Cell Apoptosis and Diseases. *Int. J. Mol. Sci.* 21, 8879. <https://doi.org/10.3390/ijms21228879>
- Huang, X., Zhang, X., Bai, X., Liang, T., 2020. Eating self for not be eaten: Pancreatic cancer suppresses self-immunogenicity by autophagy-mediated MHC-I degradation. *Signal Transduct. Target. Ther.* 5, 1–2. <https://doi.org/10.1038/s41392-020-0209-8>
- Huggett, M.T., Pereira, S.P., 2011. Diagnosing and managing pancreatic cancer. *The Practitioner* 255, 21–3.
- Huppert, L.A., Green, M.D., Kim, L., Chow, C., Leyfman, Y., Daud, A.I., Lee, J.C., 2022. Tissue-specific Tregs in cancer metastasis: opportunities for precision immunotherapy. *Cell. Mol. Immunol.* 19, 33–45. <https://doi.org/10.1038/s41423-021-00742-4>
- Hussain, N., Das, D., Pramanik, A., Pandey, M.K., Joshi, V., Pramanik, K.C., 2022. Targeting the complement system in pancreatic cancer drug resistance: a novel therapeutic approach. *Cancer Drug Resist.* 5, 317–327. <https://doi.org/10.20517/cdr.2021.150>
- Hussar, P., 2022. Apoptosis Regulators Bcl-2 and Caspase-3. *Encyclopedia* 2, 1624–1636. <https://doi.org/10.3390/encyclopedia2040111>
- Inman, K.S., 2014. Complex role for the immune system in initiation and progression of pancreatic cancer. *World J. Gastroenterol.* 20, 11160. <https://doi.org/10.3748/wjg.v20.i32.11160>
- Jahangirian, H., Saleh, B., Kalantari, K., Rafiee-Moghaddam, R., Nikpey, B., Jahangirian, S., Webster, T.J., 2020. Enzymatic Synthesis of Ricinoleyl Hydroxamic Acid Based on Commercial Castor Oil, Cytotoxicity Properties and Application as a New Anticancer Agent. *Int. J. Nanomedicine* 15, 2935–2945. <https://doi.org/10.2147/IJN.S223796>
- Jaworska, M., Szczudło, J., Pietrzyk, A., Shah, J., Trojan, S.E., Ostrowska, B., Kocemba-Pilarczyk, K.A., 2023. The Warburg effect: a score for many instruments in the concert of cancer and cancer niche cells. *Pharmacol. Rep.* 75, 876–890. <https://doi.org/10.1007/s43440-023-00504-1>
- Ježek, J., Cooper, K.F., Strich, R., 2018. Reactive Oxygen Species and Mitochondrial Dynamics: The Yin and Yang of Mitochondrial Dysfunction and Cancer Progression. *Antioxidants* 7, 13. <https://doi.org/10.3390/antiox7010013>
- Joshi, S., Sharabi, A., 2022. Targeting myeloid-derived suppressor cells to enhance natural killer cell-based immunotherapy. *Pharmacol. Ther.* 235, 108114. <https://doi.org/10.1016/j.pharmthera.2022.108114>
- Kabacaoglu, D., Ciecieski, K.J., Ruess, D.A., Algül, H., 2018. Immune Checkpoint Inhibition for Pancreatic Ductal Adenocarcinoma: Current Limitations and Future Options. *Front. Immunol.* 9, 1878. <https://doi.org/10.3389/fimmu.2018.01878>
- Kalkavan, H., Green, D.R., 2018. MOMP, cell suicide as a BCL-2 family business. *Cell Death Differ.* 25, 46–55. <https://doi.org/10.1038/cdd.2017.179>

- Kallungal, A., Olszewski, M., Maciejewska, N., Brankiewicz, W., Baginski, M., 2023. Cancer immune escape: the role of antigen presentation machinery. *J. Cancer Res. Clin. Oncol.* 149, 8131–8141. <https://doi.org/10.1007/s00432-023-04737-8>
- Kandikattu, H.K., Venkateshaiah, S.U., Mishra, A., 2020. Chronic Pancreatitis and the Development of Pancreatic Cancer. *Endocr. Metab. Immune Disord. Drug Targets* 20, 1182–1210. <https://doi.org/10.2174/1871530320666200423095700>
- Kandra, P., Nandigama, R., Eul, B., Huber, M., Kobold, S., Seeger, W., Grimminger, F., Savai, R., 2022. Utility and Drawbacks of Chimeric Antigen Receptor T Cell (CAR-T) Therapy in Lung Cancer. *Front. Immunol.* 13, 903562. <https://doi.org/10.3389/fimmu.2022.903562>
- Kang, W., Suzuki, M., Saito, T., Miyado, K., 2021. Emerging Role of TCA Cycle-Related Enzymes in Human Diseases. *Int. J. Mol. Sci.* 22, 13057. <https://doi.org/10.3390/ijms222313057>
- Kanji, Z.S., Gallinger, S., 2013. Diagnosis and management of pancreatic cancer. *Can. Med. Assoc. J.* 185, 1219–1226. <https://doi.org/10.1503/cmaj.121368>
- Karamitopoulou, E., Andreou, A., Pahud de Mortanges, A., Tinguely, M., Gloor, B., Perren, A., 2021. PD-1/PD-L1-Associated Immunoarchitectural Patterns Stratify Pancreatic Cancer Patients into Prognostic/Predictive Subgroups. *Cancer Immunol. Res.* 9, 1439–1450. <https://doi.org/10.1158/2326-6066.CIR-21-0144>
- Karpińska, M., Czauderna, M., 2022. Pancreas—Its Functions, Disorders, and Physiological Impact on the Mammals’ Organism. *Front. Physiol.* 13, 807632. <https://doi.org/10.3389/fphys.2022.807632>
- Kauanova, S., Urazbayev, A., Vorobjev, I., 2021. The Frequent Sampling of Wound Scratch Assay Reveals the “Opportunity” Window for Quantitative Evaluation of Cell Motility-Impeding Drugs. *Front. Cell Dev. Biol.* 9, 640972. <https://doi.org/10.3389/fcell.2021.640972>
- Kennedy, L., Sandhu, J.K., Harper, M.-E., Cuperlovic-Culf, M., 2020. Role of Glutathione in Cancer: From Mechanisms to Therapies. *Biomolecules* 10, 1429. <https://doi.org/10.3390/biom10101429>
- Kenner, B.J., Chari, S.T., Maitra, A., Srivastava, S., Cleeter, D.F., Go, V.L.W., Rothschild, L.J., Goldberg, A.E., 2016. Early Detection of Pancreatic Cancer—a Defined Future Using Lessons From Other Cancers: A White Paper. *Pancreas* 45, 1073–1079. <https://doi.org/10.1097/MPA.0000000000000701>
- Khanam, A., Ghosh, A., Chua, J.V., Kottlil, S., 2023. Blockade of CCR4 breaks immune tolerance in chronic hepatitis B patients by modulating regulatory pathways. *J. Transl. Med.* 21, 271. <https://doi.org/10.1186/s12967-023-04104-8>
- Kim, H.-M., Zheng, X., Lee, E., 2023. Experimental Insights into the Interplay between Histone Modifiers and p53 in Regulating Gene Expression. *Int. J. Mol. Sci.* 24, 11032. <https://doi.org/10.3390/ijms241311032>
- Kim, S.K., Cho, S.W., 2022. The Evasion Mechanisms of Cancer Immunity and Drug Intervention in the Tumor Microenvironment. *Front. Pharmacol.* 13, 868695. <https://doi.org/10.3389/fphar.2022.868695>

- King, M.E., Zhang, J., Lin, J.Q., Garza, K.Y., DeHoog, R.J., Feider, C.L., Bensussan, A., Sans, M., Krieger, A., Badal, S., Keating, M.F., Woody, S., Dhingra, S., Yu, W., Pirko, C., Brahmabhatt, K.A., Van Buren, G., Fisher, W.E., Suliburk, J., Eberlin, L.S., 2021. Rapid diagnosis and tumor margin assessment during pancreatic cancer surgery with the MasSpec Pen technology. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2104411118. <https://doi.org/10.1073/pnas.2104411118>
- Kleczko, E.K., Kwak, J.W., Schenk, E.L., Nemenoff, R.A., 2019. Targeting the Complement Pathway as a Therapeutic Strategy in Lung Cancer. *Front. Immunol.* 10, 954. <https://doi.org/10.3389/fimmu.2019.00954>
- Kleeff, J., Korc, M., Apte, M., La Vecchia, C., Johnson, C.D., Biankin, A.V., Neale, R.E., Tempero, M., Tuveson, D.A., Hruban, R.H., Neoptolemos, J.P., 2016. Pancreatic cancer. *Nat. Rev. Dis. Primer* 2, 1–22. <https://doi.org/10.1038/nrdp.2016.22>
- Klochkov, A., Kudaravalli, P., Lim, Y., Sun, Y., 2023. Alcoholic Pancreatitis, in: StatPearls. StatPearls Publishing, Treasure Island (FL).
- Kolev, M., Das, M., Gerber, M., Baver, S., Deschatelets, P., Markiewski, M.M., 2022. Inside-Out of Complement in Cancer. *Front. Immunol.* 13.
- Kolev, M., Kemper, C., 2017. Keeping It All Going—Complement Meets Metabolism. *Front. Immunol.* 8. <https://doi.org/10.3389/fimmu.2017.00001>
- Kolev, M., Markiewski, M.M., 2018. Targeting complement-mediated immunoregulation for cancer immunotherapy. *Semin. Immunol.* 37, 85–97. <https://doi.org/10.1016/j.smim.2018.02.003>
- Kumar, V., Vashishta, M., Kong, L., Wu, X., Lu, J.J., Guha, C., Dwarakanath, B.S., 2021. The Role of Notch, Hedgehog, and Wnt Signaling Pathways in the Resistance of Tumors to Anticancer Therapies. *Front. Cell Dev. Biol.* 9.
- Kurihara, R., Yamaoka, K., Sawamukai, N., Shimajiri, S., Oshita, K., Yukawa, S., Tokunaga, M., Iwata, S., Saito, K., Chiba, K., Tanaka, Y., 2010. C5a promotes migration, proliferation, and vessel formation in endothelial cells. *Inflamm. Res.* 59, 659–666. <https://doi.org/10.1007/s00011-010-0178-4>
- Kwon, Y.W., Jo, H.-S., Bae, S., Seo, Y., Song, P., Song, M., Yoon, J.H., 2021. Application of Proteomics in Cancer: Recent Trends and Approaches for Biomarkers Discovery. *Front. Med.* 8, 747333. <https://doi.org/10.3389/fmed.2021.747333>
- Laheru, D., Lutz, E., Burke, J., Biedrzycki, B., Solt, S., Onners, B., Tartakovsky, I., Nemunaitis, J., Le, D., Sugar, E., Hege, K., Jaffee, E., 2008. Allogeneic GM-CSF Secreting Tumor Immunotherapy (GVAX®) Alone or in Sequence with Cyclophosphamide for Metastatic Pancreatic Cancer: A Pilot Study of Safety, Feasibility and Immune Activation. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 14, 1455–1463. <https://doi.org/10.1158/1078-0432.CCR-07-0371>
- Lamers, C., Mastellos, D.C., Ricklin, D., Lambris, J.D., 2022. Compstatins: the dawn of clinical C3-targeted complement inhibition. *Trends Pharmacol. Sci.* 43, 629–640. <https://doi.org/10.1016/j.tips.2022.01.004>

- Le Cosquer, G., Maulat, C., Bournet, B., Cordelier, P., Buscail, E., Buscail, L., 2023. Pancreatic Cancer in Chronic Pancreatitis: Pathogenesis and Diagnostic Approach. *Cancers* 15, 761. <https://doi.org/10.3390/cancers15030761>
- Le, D.T., Lutz, E., Uram, J.N., Sugar, E.A., Onners, B., Solt, S., Zheng, L., Diaz, L.A., Donehower, R.C., Jaffee, E.M., Laheru, D.A., 2013. Evaluation of Ipilimumab in combination with allogeneic pancreatic tumor cells transfected with a GM-CSF gene in previously treated pancreatic cancer. *J. Immunother. Hagerstown Md* 1997 36, 382–389. <https://doi.org/10.1097/CJI.0b013e31829fb7a2>
- Le, I., Dhandayuthapani, S., Chacon, J., Eiring, A.M., Gadad, S.S., 2022. Harnessing the Immune System with Cancer Vaccines: From Prevention to Therapeutics. *Vaccines* 10, 816. <https://doi.org/10.3390/vaccines10050816>
- Lee, M.G., Ohana, E., Park, H.W., Yang, D., Muallem, S., 2012. Molecular Mechanism of Pancreatic and Salivary Glands Fluid and HCO₃[−] Secretion. *Physiol. Rev.* 92, 39–74. <https://doi.org/10.1152/physrev.00011.2011>
- Lee, S., Chang, P., Chen, P., Lu, C., Hung, Y., Tsang, N., Hung, C., Chen, J., Hsu, H., Chen, Y., Chou, W., 2019. Association of time interval between cancer diagnosis and initiation of palliative chemotherapy with overall survival in patients with unresectable pancreatic cancer. *Cancer Med.* 8, 3471–3478. <https://doi.org/10.1002/cam4.2254>
- Li, C., Jiang, P., Wei, S., Xu, X., Wang, J., 2020. Regulatory T cells in tumor microenvironment: new mechanisms, potential therapeutic strategies and future prospects. *Mol. Cancer* 19, 116. <https://doi.org/10.1186/s12943-020-01234-1>
- Li, H.-B., Yang, Z.-H., Guo, Q.-Q., 2021. Immune checkpoint inhibition for pancreatic ductal adenocarcinoma: limitations and prospects: a systematic review. *Cell Commun. Signal.* 19, 117. <https://doi.org/10.1186/s12964-021-00789-w>
- Li, H.H., Mycroft, S., Christiansen, S., Wood, D.N., Feuersenger, H., Pawaskar, D., Jacobs, I., 2018. Subcutaneous C1-esterase inhibitor to prevent hereditary angioedema attacks: Safety findings from the COMPACT trial. *Allergy Asthma Proc.* 39, 365–370. <https://doi.org/10.2500/aap.2018.39.4164>
- Liberti, M.V., Locasale, J.W., 2016. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* 41, 211–218. <https://doi.org/10.1016/j.tibs.2015.12.001>
- Ligasová, A., Vydržalová, M., Buriánová, R., Brůčková, L., Večeřová, R., Janošťáková, A., Koberna, K., 2019. A New Sensitive Method for the Detection of Mycoplasmas Using Fluorescence Microscopy. *Cells* 8. <https://doi.org/10.3390/cells8121510>
- Lindell, E., Zhong, L., Zhang, X., 2023. Quiescent Cancer Cells—A Potential Therapeutic Target to Overcome Tumor Resistance and Relapse. *Int. J. Mol. Sci.* 24, 3762. <https://doi.org/10.3390/ijms24043762>
- Liu, C., Cheng, H., Luo, G., Lu, Y., Jin, K., Guo, M., Ni, Q., Yu, X., 2017. Circulating regulatory T cell subsets predict overall survival of patients with unresectable pancreatic cancer. *Int. J. Oncol.* 51, 686–694. <https://doi.org/10.3892/ijo.2017.4032>
- Liu, C., Jin, Y., Fan, Z., 2021. The Mechanism of Warburg Effect-Induced Chemoresistance in Cancer. *Front. Oncol.* 11.

- Liu, C., Yang, Z., Li, D., Liu, Z., Miao, X., Yang, L., Zou, Q., Yuan, Y., 2015. Overexpression of B2M and loss of ALK7 expression are associated with invasion, metastasis, and poor-prognosis of the pancreatic ductal adenocarcinoma. *Cancer Biomark. Sect. Dis. Markers* 15, 735–743. <https://doi.org/10.3233/CBM-150515>
- Liu, J., Fu, M., Wang, M., Wan, D., Wei, Y., Wei, X., 2022. Cancer vaccines as promising immuno-therapeutics: platforms and current progress. *J. Hematol. Oncol.* *J Hematol Oncol* 15, 28. <https://doi.org/10.1186/s13045-022-01247-x>
- Liu, J., Geng, X., Hou, J., Wu, G., 2021. New insights into M1/M2 macrophages: key modulators in cancer progression. *Cancer Cell Int.* 21, 389. <https://doi.org/10.1186/s12935-021-02089-2>
- Liu, S., Sun, Q., Ren, X., 2023. Novel strategies for cancer immunotherapy: counter-immunoediting therapy. *J. Hematol. Oncol.* *J Hematol Oncol* 16, 38. <https://doi.org/10.1186/s13045-023-01430-8>
- Liu, X., Xu, J., Zhang, B., Liu, J., Liang, C., Meng, Q., Hua, J., Yu, X., Shi, S., 2019. The reciprocal regulation between host tissue and immune cells in pancreatic ductal adenocarcinoma: new insights and therapeutic implications. *Mol. Cancer* 18, 184. <https://doi.org/10.1186/s12943-019-1117-9>
- Liu, Z., Gou, A., Wu, X., 2023. Liver metastasis of pancreatic cancer: the new choice at the crossroads. *Hepatobiliary Surg. Nutr.* 12, 88–91. <https://doi.org/10.21037/hbsn-22-489>
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta CT$ Method. *Methods* 25, 402–408. <https://doi.org/10.1006/meth.2001.1262>
- Liyanage, C., Malik, A., Abeysinghe, P., Clements, J., Batra, J., 2021. SWATH-MS Based Proteomic Profiling of Prostate Cancer Cells Reveals Adaptive Molecular Mechanisms in Response to Anti-Androgen Therapy. *Cancers* 13, 715. <https://doi.org/10.3390/cancers13040715>
- Luan, Y., Ngo, L., Han, Z., Wang, X., Qu, M., Zheng, Y.G., 2015. Chapter 14 - Histone Acetyltransferases: Enzymes, Assays, and Inhibitors, in: Zheng, Y.G. (Ed.), *Epigenetic Technological Applications*. Academic Press, Boston, pp. 291–317. <https://doi.org/10.1016/B978-0-12-801080-8.00014-4>
- Luchini, L.S.G., Pidde, G., Squaiella-Baptistão, C.C., Tambourgi, D.V., 2019. Complement System Inhibition Modulates the Pro-Inflammatory Effects of a Snake Venom Metalloproteinase. *Front. Immunol.* 10, 1137. <https://doi.org/10.3389/fimmu.2019.01137>
- Lwin, T.M., Turner, M.A., Nishino, H., Amirfakhri, S., Hernot, S., Hoffman, R.M., Bouvet, M., 2022. Fluorescent Anti-CEA Nanobody for Rapid Tumor-Targeting and Imaging in Mouse Models of Pancreatic Cancer. *Biomolecules* 12, 711. <https://doi.org/10.3390/biom12050711>
- Malchiodi, Z.X., Weiner, L.M., 2021. Understanding and Targeting Natural Killer Cell-Cancer-Associated Fibroblast Interactions in Pancreatic Ductal Adenocarcinoma. *Cancers* 13, 405. <https://doi.org/10.3390/cancers13030405>

- Manohar, M., Verma, A.K., Venkateshaiah, S.U., Sanders, N.L., Mishra, A., 2017. Pathogenic mechanisms of pancreatitis. *World J. Gastrointest. Pharmacol. Ther.* 8, 10–25. <https://doi.org/10.4292/wjgpt.v8.i1.10>
- Marei, H.E., Althani, A., Afifi, N., Hasan, A., Caceci, T., Pozzoli, G., Morrione, A., Giordano, A., Cenciarelli, C., 2021. p53 signaling in cancer progression and therapy. *Cancer Cell Int.* 21, 703. <https://doi.org/10.1186/s12935-021-02396-8>
- Markiewski, M.M., Daugherty, E., Reese, B., Karbowiczek, M., 2020. The Role of Complement in Angiogenesis. *Antibodies* 9, 67. <https://doi.org/10.3390/antib9040067>
- Marshall, J.S., Warrington, R., Watson, W., Kim, H.L., 2018. An introduction to immunology and immunopathology. *Allergy Asthma Clin. Immunol.* 14, 49. <https://doi.org/10.1186/s13223-018-0278-1>
- Martinez-Sanchez, M.E., Huerta, L., Alvarez-Buylla, E.R., Villarreal Luján, C., 2018. Role of Cytokine Combinations on CD4+ T Cell Differentiation, Partial Polarization, and Plasticity: Continuous Network Modeling Approach. *Front. Physiol.* 9.
- Marzochi, L.L., Cuzziol, C.I., Nascimento Filho, C.H.V.D., Dos Santos, J.A., Castanhole-Nunes, M.M.U., Pavarino, É.C., Guerra, E.N.S., Goloni-Bertollo, E.M., 2023. Use of histone methyltransferase inhibitors in cancer treatment: A systematic review. *Eur. J. Pharmacol.* 944, 175590. <https://doi.org/10.1016/j.ejphar.2023.175590>
- Mastellos, D.C., Reis, E.S., Lambris, J.D., 2018. Complement C5a-Mediated TAM-ing of Antitumor Immunity Drives Squamous Carcinogenesis. *Cancer Cell* 34, 531–533. <https://doi.org/10.1016/j.ccell.2018.09.005>
- Mastellos, D.C., Yancopoulou, D., Kokkinos, P., Huber-Lang, M., Hajishengallis, G., Biglarnia, A.R., Lupu, F., Nilsson, B., Risitano, A.M., Ricklin, D., Lambris, J.D., 2015. Compstatin: a C3-targeted complement inhibitor reaching its prime for bedside intervention. *Eur. J. Clin. Invest.* 45, 423–440. <https://doi.org/10.1111/eci.12419>
- Matthews, H.K., Bertoli, C., De Bruin, R.A.M., 2022. Cell cycle control in cancer. *Nat. Rev. Mol. Cell Biol.* 23, 74–88. <https://doi.org/10.1038/s41580-021-00404-3>
- McCoach, C.E., Bivona, T.G., 2018. The evolving understanding of immunoediting and the clinical impact of immune escape. *J. Thorac. Dis.* 10, 1248–1252. <https://doi.org/10.21037/jtd.2018.03.60>
- McCubrey, J.A., Rakus, D., Gizak, A., Steelman, L.S., Abrams, S.L., Lertpiriyapong, K., Fitzgerald, T.L., Yang, L.V., Montalto, G., Cervello, M., Libra, M., Nicoletti, F., Scalisi, A., Torino, F., Fenga, C., Neri, L.M., Marmioli, S., Cocco, L., Martelli, A.M., 2016. Effects of mutations in Wnt/ β -catenin, hedgehog, Notch and PI3K pathways on GSK-3 activity—Diverse effects on cell growth, metabolism and cancer. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* 1863, 2942–2976. <https://doi.org/10.1016/j.bbamcr.2016.09.004>
- Mei, Q., Huang, J., Chen, W., Tang, J., Xu, C., Yu, Q., Cheng, Y., Ma, L., Yu, X., Li, S., 2017. Regulation of DNA replication-coupled histone gene expression. *Oncotarget* 8, 95005–95022. <https://doi.org/10.18632/oncotarget.21887>
- Messerschmidt, J.L., Prendergast, G.C., Messerschmidt, G.L., 2016. How Cancers Escape Immune Destruction and Mechanisms of Action for the New Significantly Active Immune

- Therapies: Helping Nonimmunologists Decipher Recent Advances. *The Oncologist* 21, 233–243. <https://doi.org/10.1634/theoncologist.2015-0282>
- Mizrahi, J.D., Surana, R., Valle, J.W., Shroff, R.T., 2020. Pancreatic cancer. *The Lancet* 395, 2008–2020. [https://doi.org/10.1016/S0140-6736\(20\)30974-0](https://doi.org/10.1016/S0140-6736(20)30974-0)
- Molina-Montes, E., Gomez-Rubio, P., Márquez, M., Rava, M., Löhr, M., Michalski, C.W., Molero, X., Farré, A., Perea, J., Greenhalf, W., Ilzarbe, L., O'Rorke, M., Tardón, A., Gress, T., Barberà, V.M., Crnogorac-Jurcevic, T., Domínguez-Muñoz, E., Muñoz-Bellví, L., Balsells, J., Costello, E., Huang, J., Iglesias, M., Kleeff, J., Kong, B., Mora, J., Murray, L., O'Driscoll, D., Poves, I., Scarpa, A., Ye, W., Hidalgo, M., Sharp, L., Carrato, A., Real, F.X., Malats, N., the PanGenEU Study Investigators, 2018. Risk of pancreatic cancer associated with family history of cancer and other medical conditions by accounting for smoking among relatives. *Int. J. Epidemiol.* 47, 473–483. <https://doi.org/10.1093/ije/dyx269>
- MOMI, N., KAUR, S., KRISHN, S.R., BATRA, S.K., 2012. Discovering the route from inflammation to pancreatic cancer. *Minerva Gastroenterol. Dietol.* 58, 283–297.
- Moore, M., 1996. Activity of gemcitabine in patients with advanced pancreatic carcinoma: A review. *Cancer* 78, 633–638. [https://doi.org/10.1002/\(SICI\)1097-0142\(19960801\)78:3<633::AID-CNCR44>3.0.CO;2-X](https://doi.org/10.1002/(SICI)1097-0142(19960801)78:3<633::AID-CNCR44>3.0.CO;2-X)
- Moro, L., 2021. Mitochondrial DNA and MitomiR Variations in Pancreatic Cancer: Potential Diagnostic and Prognostic Biomarkers. *Int. J. Mol. Sci.* 22, 9692. <https://doi.org/10.3390/ijms22189692>
- Mucileanu, A., Chira, R., Mircea, P.A., 2021. PD-1/PD-L1 expression in pancreatic cancer and its implication in novel therapies. *Med. Pharm. Rep.* 94, 402–410. <https://doi.org/10.15386/mpr-2116>
- Muller, M., Haghnejad, V., Schaefer, M., Gauchotte, G., Caron, B., Peyrin-Biroulet, L., Bronowicki, J.-P., Neuzillet, C., Lopez, A., 2022. The Immune Landscape of Human Pancreatic Ductal Carcinoma: Key Players, Clinical Implications, and Challenges. *Cancers* 14, 995. <https://doi.org/10.3390/cancers14040995>
- Murakami, T., Hiroshima, Y., Matsuyama, R., Homma, Y., Hoffman, R.M., Endo, I., 2019. Role of the tumor microenvironment in pancreatic cancer. *Ann. Gastroenterol. Surg.* 3, 130–137. <https://doi.org/10.1002/ags3.12225>
- Mustafa, S., Jamil, K., Swamy, N., 2018. The mutations identified in human HDAC-10 gene and their prognosis in cancer cases. *Int. J. Pharm. Sci. Health* 2249 – 5738 3. <https://doi.org/10.26808/rs.ph.i8v3.04>
- Napolitano, G., Fasciolo, G., Venditti, P., 2021. Mitochondrial Management of Reactive Oxygen Species. *Antioxidants* 10, 1824. <https://doi.org/10.3390/antiox10111824>
- Narayanan, S., Vicent, S., Ponz-Sarvisé, M., 2021. PDAC as an Immune Evasive Disease: Can 3D Model Systems Aid to Tackle This Clinical Problem? *Front. Cell Dev. Biol.* 9.
- Nayak, A.P., Kapur, A., Barroilhet, L., Patankar, M.S., 2018. Oxidative Phosphorylation: A Target for Novel Therapeutic Strategies Against Ovarian Cancer. *Cancers* 10, 337. <https://doi.org/10.3390/cancers10090337>

- Neagu, A.-N., Whitham, D., Seymour, L., Haaker, N., Pelkey, I., Darie, C.C., 2023. Proteomics-Based Identification of Dysregulated Proteins and Biomarker Discovery in Invasive Ductal Carcinoma, the Most Common Breast Cancer Subtype. *Proteomes* 11, 13. <https://doi.org/10.3390/proteomes11020013>
- Newson, J., Motwani, M.P., Kendall, A.C., Nicolaou, A., Muccioli, G.G., Alhouayek, M., Bennett, M., Van De Merwe, R., James, S., De Maeyer, R.P.H., Gilroy, D.W., 2017. Inflammatory Resolution Triggers a Prolonged Phase of Immune Suppression through COX-1/mPGES-1-Derived Prostaglandin E2. *Cell Rep.* 20, 3162–3175. <https://doi.org/10.1016/j.celrep.2017.08.098>
- Nguyen, C., Pandey, S., 2019. Exploiting Mitochondrial Vulnerabilities to Trigger Apoptosis Selectively in Cancer Cells. *Cancers* 11, 916. <https://doi.org/10.3390/cancers11070916>
- Nomi, T., Sho, M., Akahori, T., Hamada, K., Kubo, A., Kanehiro, H., Nakamura, S., Enomoto, K., Yagita, H., Azuma, M., Nakajima, Y., 2007. Clinical Significance and Therapeutic Potential of the Programmed Death-1 Ligand/Programmed Death-1 Pathway in Human Pancreatic Cancer. *Clin. Cancer Res.* 13, 2151–2157. <https://doi.org/10.1158/1078-0432.CCR-06-2746>
- Noyes, D., Bag, A., Oseni, S., Semidey-Hurtado, J., Cen, L., Sarnaik, A.A., Sondak, V.K., Adeegbe, D., 2022. Tumor-associated Tregs obstruct antitumor immunity by promoting T cell dysfunction and restricting clonal diversity in tumor-infiltrating CD8+ T cells. *J. Immunother. Cancer* 10, e004605. <https://doi.org/10.1136/jitc-2022-004605>
- Nsingwane, Z., Candy, G., Devar, J., Omoshoro-Jones, J., Smith, M., Nweke, E., 2020. Immunotherapeutic strategies in pancreatic ductal adenocarcinoma (PDAC): current perspectives and future prospects. *Mol. Biol. Rep.* 47, 6269–6280. <https://doi.org/10.1007/s11033-020-05648-4>
- Nweke, E.E., Naicker, P., Aron, S., Stoychev, S., Devar, J., Tabb, D.L., Omoshoro-Jones, J., Smith, M., Candy, G., 2020. SWATH-MS based proteomic profiling of pancreatic ductal adenocarcinoma tumours reveals the interplay between the extracellular matrix and related intracellular pathways. *PLOS ONE* 15, e0240453. <https://doi.org/10.1371/journal.pone.0240453>
- O'Brien, R.M., Cannon, A., Reynolds, J.V., Lysaght, J., Lynam-Lennon, N., 2021. Complement in Tumourigenesis and the Response to Cancer Therapy. *Cancers* 13, 1209. <https://doi.org/10.3390/cancers13061209>
- O'Donnell, J.S., Teng, M.W.L., Smyth, M.J., 2019. Cancer immunoediting and resistance to T cell-based immunotherapy. *Nat. Rev. Clin. Oncol.* 16, 151–167. <https://doi.org/10.1038/s41571-018-0142-8>
- Olcina, M.M., Kim, R.K., Melemenidis, S., Graves, E.E., Giaccia, A.J., 2018. The tumour microenvironment links complement system dysregulation and hypoxic signalling. *Br. J. Radiol.* 20180069. <https://doi.org/10.1259/bjr.20180069>
- Orlacchio, A., Mazzone, P., 2021. The Role of Toll-like Receptors (TLRs) Mediated Inflammation in Pancreatic Cancer Pathophysiology. *Int. J. Mol. Sci.* 22, 12743. <https://doi.org/10.3390/ijms222312743>

- Ostrycharz, E., Hukowska-Szematowicz, B., 2022. New Insights into the Role of the Complement System in Human Viral Diseases. *Biomolecules* 12, 226. <https://doi.org/10.3390/biom12020226>
- Otto, T., Sicinski, P., 2017. Cell cycle proteins as promising targets in cancer therapy. *Nat. Rev. Cancer* 17, 93–115. <https://doi.org/10.1038/nrc.2016.138>
- Ou, W.-B., Zhu, J., Eilers, G., Li, X., Kuang, Y., Liu, L., Mariño-Enríquez, A., Yan, Z., Li, H., Meng, F., Zhou, H., Sheng, Q., Fletcher, J.A., 2015. HDACi inhibits liposarcoma via targeting of the MDM2-p53 signaling axis and PTEN, irrespective of p53 mutational status. *Oncotarget* 6, 10510–10520.
- Padinharayil, H., Rai, V., George, A., 2023. Mitochondrial Metabolism in Pancreatic Ductal Adenocarcinoma: From Mechanism-Based Perspectives to Therapy. *Cancers* 15, 1070. <https://doi.org/10.3390/cancers15041070>
- Pancreatic Cancer Europe, 2020. pancreatic Cancer Europe [WWW Document]. *Pancreat. Cancer Eur.* URL <https://pancreaticcancereurope.org/> (accessed 5.13.22).
- Park, J., Chung, Y.-J., 2019. Identification of neoantigens derived from alternative splicing and RNA modification. *Genomics Inform.* 17, e23. <https://doi.org/10.5808/GI.2019.17.3.e23>
- Paul, S., Lal, G., 2017. The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Front. Immunol.* 8.
- Pei, Q., Pan, J., Zhu, H., Ding, X., Liu, W., Lv, Y., Zou, X., Luo, H., 2014. Gemcitabine-treated pancreatic cancer cell medium induces the specific CTL antitumor activity by stimulating the maturation of dendritic cells. *Int. Immunopharmacol.* 19, 10–16. <https://doi.org/10.1016/j.intimp.2013.12.022>
- Peng, X., Chen, L., Jiao, Y., Wang, Y., Hao, Z., Zhan, X., 2021. Application of natural killer cells in pancreatic cancer (Review). *Oncol. Lett.* 22, 647. <https://doi.org/10.3892/ol.2021.12908>
- Pfanner, N., Warscheid, B., Wiedemann, N., 2019. Mitochondrial protein organization: from biogenesis to networks and function. *Nat. Rev. Mol. Cell Biol.* 20, 267–284. <https://doi.org/10.1038/s41580-018-0092-0>
- Principe, D.R., Underwood, P.W., Korc, M., Trevino, J.G., Munshi, H.G., Rana, A., 2021. The Current Treatment Paradigm for Pancreatic Ductal Adenocarcinoma and Barriers to Therapeutic Efficacy. *Front. Oncol.* 0. <https://doi.org/10.3389/fonc.2021.688377>
- Puente-Cobacho, B., Varela-López, A., Quiles, J.L., Vera-Ramirez, L., 2023. Involvement of redox signalling in tumour cell dormancy and metastasis. *Cancer Metastasis Rev.* 42, 49–85. <https://doi.org/10.1007/s10555-022-10077-9>
- Quante, A.S., Ming, C., Rottmann, M., Engel, J., Boeck, S., Heinemann, V., Westphalen, C.B., Strauch, K., 2016. Projections of cancer incidence and cancer-related deaths in Germany by 2020 and 2030. *Cancer Med.* 5, 2649–2656. <https://doi.org/10.1002/cam4.767>
- Rahib, L., Smith, B.D., Aizenberg, R., Rosenzweig, A.B., Fleshman, J.M., Matrisian, L.M., 2014. Projecting Cancer Incidence and Deaths to 2030: The Unexpected Burden of Thyroid, Liver, and Pancreas Cancers in the United States. *Cancer Res.* 74, 2913–2921. <https://doi.org/10.1158/0008-5472.CAN-14-0155>

- Raji, S., Sahranavard, M., Mottaghi, M., Sahebkar, A., 2022. MiR-212 value in prognosis and diagnosis of cancer and its association with patient characteristics: a systematic review and meta-analysis. *Cancer Cell Int.* 22, 163. <https://doi.org/10.1186/s12935-022-02584-0>
- Rashid, M., Coombs, K.M., 2019. Serum-reduced media impacts on cell viability and protein expression in human lung epithelial cells. *J. Cell. Physiol.* 234, 7718–7724. <https://doi.org/10.1002/jcp.27890>
- Rawla, P., Sunkara, T., Gaduputi, V., 2019. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. *World J. Oncol.* 10, 10–27. <https://doi.org/10.14740/wjon1166>
- Reis, E.S., Mastellos, D.C., Ricklin, D., Mantovani, A., Lambris, J.D., 2018. Complement in cancer: untangling an intricate relationship. *Nat. Rev. Immunol.* 18, 5–18. <https://doi.org/10.1038/nri.2017.97>
- Ren, J., Zhang, N., Li, X., Sun, X., Song, J., 2021. Identification of reference genes for gene expression studies among different developmental stages of murine hearts. *BMC Dev. Biol.* 21, 13. <https://doi.org/10.1186/s12861-021-00244-6>
- Revel, M., Daugan, M.V., Sautés-Fridman, C., Fridman, W.H., Roumenina, L.T., 2020. Complement System: Promoter or Suppressor of Cancer Progression? *Antibodies* 9, 57. <https://doi.org/10.3390/antib9040057>
- Reyes-Castellanos, G., Masoud, R., Carrier, A., 2020. Mitochondrial Metabolism in PDAC: From Better Knowledge to New Targeting Strategies. *Biomedicines* 8, 270. <https://doi.org/10.3390/biomedicines8080270>
- Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* 11, 785–797. <https://doi.org/10.1038/ni.1923>
- Ricklin, D., Lambris, J.D., 2008. Compstatin: A Complement Inhibitor on its Way to Clinical Application. *Adv. Exp. Med. Biol.* 632, 273–292.
- Ricklin, D., Lambris, J.D., 2007. Complement-targeted therapeutics. *Nat. Biotechnol.* 25, 1265–1275. <https://doi.org/10.1038/nbt1342>
- Ricklin, D., Mastellos, D.C., Lambris, J.D., 2019. Therapeutic targeting of the complement system. *Nat. Rev. Drug Discov.* 1–1. <https://doi.org/10.1038/s41573-019-0055-y>
- Rohaani, M.W., Wilgenhof, S., Haanen, J.B.A.G., 2019. Adoptive cellular therapies: the current landscape. *Virchows Arch.* 474, 449–461. <https://doi.org/10.1007/s00428-018-2484-0>
- Rojas, L.A., Sethna, Z., Soares, K.C., Olcese, C., Pang, N., Patterson, E., Lihm, J., Ceglia, N., Guasp, P., Chu, A., Yu, R., Chandra, A.K., Waters, T., Ruan, J., Amisaki, M., Zebboudj, A., Odgerel, Z., Payne, G., Derhovanessian, E., Müller, F., Rhee, I., Yadav, M., Dobrin, A., Sadelain, M., Łuksza, M., Cohen, N., Tang, L., Basturk, O., Gönen, M., Katz, S., Do, R.K., Epstein, A.S., Momtaz, P., Park, W., Sugarman, R., Varghese, A.M., Won, E., Desai, A., Wei, A.C., D’Angelica, M.I., Kingham, T.P., Mellman, I., Merghoub, T., Wolchok, J.D., Sahin, U., Türeci, Ö., Greenbaum, B.D., Jarnagin, W.R., Drebin, J., O’Reilly, E.M., Balachandran, V.P., 2023. Personalized RNA neoantigen vaccines stimulate T cells in pancreatic cancer. *Nature* 618, 144–150. <https://doi.org/10.1038/s41586-023-06063-y>

- Rolfe, B.E., Pio, R., Woodruff, T.M., Markiewski, M.M., Manthey, H.D., 2020. Editorial: The Role of Complement in Tumors. *Front. Immunol.* 11, 139. <https://doi.org/10.3389/fimmu.2020.00139>
- Roumenina, L.T., Daugan, M.V., Petitprez, F., Sautès-Fridman, C., Fridman, W.H., 2019. Context-dependent roles of complement in cancer. *Nat. Rev. Cancer* 19, 698–715. <https://doi.org/10.1038/s41568-019-0210-0>
- Rowshanravan, B., Halliday, N., Sansom, D.M., 2018. CTLA-4: a moving target in immunotherapy. *Blood* 131, 58–67. <https://doi.org/10.1182/blood-2017-06-741033>
- Rudler, D.L., Hughes, L.A., Viola, H.M., Hool, L.C., Rackham, O., Filipovska, A., 2021. Fidelity and coordination of mitochondrial protein synthesis in health and disease. *J. Physiol.* 599, 3449–3462. <https://doi.org/10.1113/JP280359>
- Rutkowski, M.J., Sughrue, M.E., Kane, A.J., Mills, S.A., Parsa, A.T., 2010. Cancer and the Complement Cascade. *Mol. Cancer Res.* 8, 1453–1465. <https://doi.org/10.1158/1541-7786.MCR-10-0225>
- Ryu, J., Thomas, S.N., 2021. Quantitative Mass Spectrometry-Based Proteomics for Biomarker Development in Ovarian Cancer. *Molecules* 26, 2674. <https://doi.org/10.3390/molecules26092674>
- Sandhu, V., Quan, M., 2017. SLE and Serum Complement: Causative, Concomitant or Coincidental? *Open Rheumatol. J.* 11, 113–122. <https://doi.org/10.2174/1874312901711010113>
- Sandoval-Valencia, R.R., 2019. Cancer immunotherapy: Role of the immune system in malignant transformation. *Rev. Médica Hosp. Gen. México* 82, 198–207. <https://doi.org/10.24875/hgmex.m19000029>
- Santos-Zas, I., Lemarié, J., Zlatanova, I., Cachanado, M., Seghezzi, J.-C., Benamer, H., Goube, P., Vandestienne, M., Cohen, R., Ezzo, M., Duval, V., Zhang, Y., Su, J.-B., Bizé, A., Sambin, L., Bonnin, P., Branchereau, M., Heymes, C., Tanchot, C., Vilar, J., Delacroix, C., Hulot, J.-S., Cochain, C., Bruneval, P., Danchin, N., Tedgui, A., Mallat, Z., Simon, T., Ghaleh, B., Silvestre, J.-S., Ait-Oufella, H., 2021. Cytotoxic CD8⁺ T cells promote granzyme B-dependent adverse post-ischemic cardiac remodeling. *Nat. Commun.* 12, 1483. <https://doi.org/10.1038/s41467-021-21737-9>
- Saraswat, M., Nieminen, H., Joenvaara, S., Tohmola, T., Seppänen, H., Ristimäki, A., Haglund, C., Renkonen, R., 2019. Label-free serum proteomics and multivariate data analysis identifies biomarkers and expression trends that differentiate Intraductal papillary mucinous neoplasia from pancreatic adenocarcinoma and healthy controls. *Transl. Med. Commun.* 4, 6. <https://doi.org/10.1186/s41231-019-0037-4>
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji - an Open Source platform for biological image analysis. *Nat. Methods* 9, 10.1038/nmeth.2019. <https://doi.org/10.1038/nmeth.2019>
- Seki, T., Suzuki, H., Yasukawa, S., Takase, A., Takayama, Y., Yoshinari, F., Matsui, A., Shirabe, K., 2023. A Rare Pancreatic Tumor with Adenomatoid Tumor-like Findings.

Intern. Med. Tokyo Jpn. 62, 2847–2853. <https://doi.org/10.2169/internalmedicine.1135-22>

- Sepand, M.R., Bigdelou, B., Ho, J.Q., Sharaf, M., Lannigan, A.J., Sullivan, I.M., da Silva, A.P., Barrett, L.O., McGoldrick, S., Lnu, Y., Lynch, S.E., Boisclair, J.M., Barnard-Pratt, D.D., Zanganeh, S., 2022. Long-Term Immunity and Antibody Response: Challenges for Developing Efficient COVID-19 Vaccines. *Antibodies* 11, 35. <https://doi.org/10.3390/antib11020035>
- Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* 13, 2498–2504. <https://doi.org/10.1101/gr.1239303>
- Sharma, V., Sachdeva, N., Gupta, V., Nada, R., Jacob, J., Sahni, D., Aggarwal, A., 2022. CCR4⁺ monocytic myeloid-derived suppressor cells are associated with the increased epithelial-mesenchymal transition in pancreatic adenocarcinoma patients. *Immunobiology* 227, 152210. <https://doi.org/10.1016/j.imbio.2022.152210>
- Shen, Z., Tian, L., Wang, X., 2018. Treatment of pancreatic head cancer with obstructive jaundice by endoscopy ultrasonography-guided gastrojejunostomy. *Medicine (Baltimore)* 97, e11476. <https://doi.org/10.1097/MD.00000000000011476>
- Shevchenko, I., Karakhanova, S., Soltek, S., Link, J., Bayry, J., Werner, J., Umansky, V., Bazhin, A.V., 2013. Low-dose gemcitabine depletes regulatory T cells and improves survival in the orthotopic Panc02 model of pancreatic cancer. *Int. J. Cancer* 133, 98–107. <https://doi.org/10.1002/ijc.27990>
- Sideras, K., Braat, H., Kwekkeboom, J., van Eijck, C.H., Peppelenbosch, M.P., Sleijfer, S., Bruno, M., 2014. Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies. *Cancer Treat. Rev.* 40, 513–522. <https://doi.org/10.1016/j.ctrv.2013.11.005>
- Siegel, R.L., Miller, K.D., Fuchs, H.E., Jemal, A., 2022. Cancer statistics, 2022. *CA. Cancer J. Clin.* 72, 7–33. <https://doi.org/10.3322/caac.21708>
- Sim, M.J.W., Sun, P.D., 2022. T Cell Recognition of Tumor Neoantigens and Insights Into T Cell Immunotherapy. *Front. Immunol.* 13, 833017. <https://doi.org/10.3389/fimmu.2022.833017>
- Sim, W., Lim, W.-M., Hii, L.-W., Leong, C.-O., Mai, C.-W., 2022. Targeting pancreatic cancer immune evasion by inhibiting histone deacetylases. *World J. Gastroenterol.* 28, 1934–1945. <https://doi.org/10.3748/wjg.v28.i18.1934>
- Singh, J., Bowne, W.B., Snook, A.E., 2021. Cancer Vaccines and Immunotherapy for Tumor Prevention and Treatment. *Vaccines* 9, 1298. <https://doi.org/10.3390/vaccines9111298>
- Slaats, J., ten Oever, J., van de Veerdonk, F.L., Netea, M.G., 2016. IL-1 β /IL-6/CRP and IL-18/ferritin: Distinct Inflammatory Programs in Infections. *PLoS Pathog.* 12, e1005973. <https://doi.org/10.1371/journal.ppat.1005973>
- Sliker, B.H., Goetz, B.T., Peters, H.L., Poelaert, B.J., Borgstahl, G.E.O., Solheim, J.C., 2019a. Beta 2-microglobulin regulates amyloid precursor-like protein 2 expression and the

- migration of pancreatic cancer cells. *Cancer Biol. Ther.* 20, 931–940. <https://doi.org/10.1080/15384047.2019.1580414>
- Sliker, B.H., Goetz, B.T., Peters, H.L., Poelaert, B.J., Borgstahl, G.E.O., Solheim, J.C., 2019b. Beta 2-microglobulin regulates amyloid precursor-like protein 2 expression and the migration of pancreatic cancer cells. *Cancer Biol. Ther.* 20, 931–940. <https://doi.org/10.1080/15384047.2019.1580414>
- Ślotwiński, R., Ślotwińska, S.M., 2017. Dysregulation of signaling pathways associated with innate antibacterial immunity in patients with pancreatic cancer. *Cent. Eur. J. Immunol.* 41, 404–418. <https://doi.org/10.5114/ceji.2016.65140>
- Snezhkina, A.V., Kudryavtseva, A.V., Kardymon, O.L., Savvateeva, M.V., Melnikova, N.V., Krasnov, G.S., Dmitriev, A.A., 2019. ROS Generation and Antioxidant Defense Systems in Normal and Malignant Cells. *Oxid. Med. Cell. Longev.* 2019, 6175804. <https://doi.org/10.1155/2019/6175804>
- Sobanski, T., Rose, M., Suraweera, A., O’Byrne, K., Richard, D.J., Bolderson, E., 2021. Cell Metabolism and DNA Repair Pathways: Implications for Cancer Therapy. *Front. Cell Dev. Biol.* 9.
- Sodji, Q.H., Nambiar, D.K., Viswanathan, V., von Eyben, R., Colburg, D., Binkley, M.S., Li, C.G., Olcina, M.M., Chang, D.T., Le, Q.-T., Giaccia, A.J., 2022. The Combination of Radiotherapy and Complement C3a Inhibition Potentiates Natural Killer cell Functions Against Pancreatic Cancer. *Cancer Res. Commun.* 2, 725–738. <https://doi.org/10.1158/2767-9764.crc-22-0069>
- Song, X., Liu, J., Lu, Y., Jin, H., Huang, D., 2014. Overexpression of B7-H1 correlates with malignant cell proliferation in pancreatic cancer. *Oncol. Rep.* 31, 1191–1198. <https://doi.org/10.3892/or.2013.2955>
- Song, X.-H., Liao, X.-Y., Zheng, X.-Y., Liu, J.-Q., Zhang, Z.-W., Zhang, L.-N., Yan, Y.-B., 2021. Human Ccr4 and Caf1 Deadenylation Regulate Proliferation and Tumorigenicity of Human Gastric Cancer Cells via Modulating Cell Cycle Progression. *Cancers* 13, 834. <https://doi.org/10.3390/cancers13040834>
- Sorbara, M., Cordelier, P., Bery, N., 2022. Antibody-Based Approaches to Target Pancreatic Tumours. *Antibodies* 11, 47. <https://doi.org/10.3390/antib11030047>
- Spriano, F., Stathis, A., Bertoni, F., 2020. Targeting BET bromodomain proteins in cancer: The example of lymphomas. *Pharmacol. Ther.* 215, 107631. <https://doi.org/10.1016/j.pharmthera.2020.107631>
- Sproston, N.R., Ashworth, J.J., 2018. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front. Immunol.* 9. <https://doi.org/10.3389/fimmu.2018.00754>
- Stennett, A., Friston, K., Harris, C.L., Wollman, A.J.M., Bronowska, A.K., Madden, K.S., 2023. The case for complement component 5 as a target in neurodegenerative disease. *Expert Opin. Ther. Targets* 27, 97–109. <https://doi.org/10.1080/14728222.2023.2177532>
- Sturfelt, G., Truedsson, L., 2012. Complement in the immunopathogenesis of rheumatic disease. *Nat. Rev. Rheumatol.* 8, 458–468. <https://doi.org/10.1038/nrrheum.2012.75>

- Sugiyama, D., Nishikawa, H., Maeda, Y., Nishioka, M., Tanemura, A., Katayama, I., Ezoe, S., Kanakura, Y., Sato, E., Fukumori, Y., Karbach, J., Jäger, E., Sakaguchi, S., 2013. Anti-CCR4 mAb selectively depletes effector-type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans. *Proc. Natl. Acad. Sci.* 110, 17945–17950. <https://doi.org/10.1073/pnas.1316796110>
- Sulciner, M.L., Clancy, T.E., 2023. Surgical Management of Pancreatic Neuroendocrine Tumors. *Cancers* 15, 2006. <https://doi.org/10.3390/cancers15072006>
- Suthahar, N., Wang, D., Aboumsallem, J.P., Shi, C., Wit, S. de, Liu, E.E., Lau, E.S., Bakker, S.J.L., Gansevoort, R.T., Vegt, B. van der, Jovani, M., Kreger, B.E., Splansky, G.L., Benjamin, E.J., Vasan, R.S., Larson, M.G., Levy, D., Ho, J.E., Boer, R.A. de, 2023. Association of Initial and Longitudinal Changes in C-reactive Protein With the Risk of Cardiovascular Disease, Cancer, and Mortality. *Mayo Clin. Proc.* 98, 549–558. <https://doi.org/10.1016/j.mayocp.2022.10.013>
- Syn, N.L., Teng, M.W.L., Mok, T.S.K., Soo, R.A., 2017. De-novo and acquired resistance to immune checkpoint targeting. *Lancet Oncol.* 18, e731–e741. [https://doi.org/10.1016/S1470-2045\(17\)30607-1](https://doi.org/10.1016/S1470-2045(17)30607-1)
- Takikawa, T., Kikuta, K., Hamada, S., Kume, K., Miura, S., Yoshida, N., Tanaka, Y., Matsumoto, R., Ikeda, M., Kataoka, F., Sasaki, A., Nakagawa, K., Unno, M., Masamune, A., 2022. Clinical features and prognostic impact of asymptomatic pancreatic cancer. *Sci. Rep.* 12, 4262. <https://doi.org/10.1038/s41598-022-08083-6>
- Talaat, I.M., Elemam, N.M., Saber-Ayad, M., 2022. Complement System: An Immunotherapy Target in Colorectal Cancer. *Front. Immunol.* 13.
- Tao, J., Yang, G., Zhou, W., Qiu, J., Chen, G., Luo, W., Zhao, F., You, L., Zheng, L., Zhang, T., Zhao, Y., 2021. Targeting hypoxic tumor microenvironment in pancreatic cancer. *J. Hematol. Oncol.* 14, 14. <https://doi.org/10.1186/s13045-020-01030-w>
- Taylor, B.C., Balko, J.M., 2022. Mechanisms of MHC-I Downregulation and Role in Immunotherapy Response. *Front. Immunol.* 13, 844866. <https://doi.org/10.3389/fimmu.2022.844866>
- Tegla, C.A., Cudrici, C., Patel, S., Trippe, R., Rus, V., Niculescu, F., Rus, H., 2011. Membrane attack by complement: the assembly and biology of terminal complement complexes. *Immunol. Res.* 51, 45–60. <https://doi.org/10.1007/s12026-011-8239-5>
- Teller, A., Jechorek, D., Hartig, R., Adolf, D., Reißig, K., Roessner, A., Franke, S., 2015. Dysregulation of apoptotic signaling pathways by interaction of RPLP0 and cathepsin X/Z in gastric cancer. *Pathol. - Res. Pract.* 211, 62–70. <https://doi.org/10.1016/j.prp.2014.09.005>
- Tempero, M.A., Malafa, M.P., Al-Hawary, M., Behrman, S.W., Benson, A.B., Cardin, D.B., Chiorean, E.G., Chung, V., Czito, B., Del Chiaro, M., Dillhoff, M., Donahue, T.R., Dotan, E., Ferrone, C.R., Fountzilas, C., Hardacre, J., Hawkins, W.G., Klute, K., Ko, A.H., Kunstman, J.W., LoConte, N., Lowy, A.M., Moravek, C., Nakakura, E.K., Narang, A.K., Obando, J., Polanco, P.M., Reddy, S., Reyngold, M., Scaife, C., Shen, J., Vollmer, C., Wolff, R.A., Wolpin, B.M., Lynn, B., George, G.V., 2021. Pancreatic Adenocarcinoma, Version 2.2021, NCCN Clinical Practice Guidelines in Oncology. *J. Natl. Compr. Cancer Netw. JNCCN* 19, 439–457. <https://doi.org/10.6004/jnccn.2021.0017>

- Thomas, D., Radhakrishnan, P., 2019. Tumor-stromal crosstalk in pancreatic cancer and tissue fibrosis. *Mol. Cancer* 18, 14. <https://doi.org/10.1186/s12943-018-0927-5>
- Thomas, L.W., Ashcroft, M., 2021. The Contextual Essentiality of Mitochondrial Genes in Cancer. *Front. Cell Dev. Biol.* 9.
- Thu, K., Soria-Bretones, I., Mak, T., Cescon, D., 2018. Targeting the cell cycle in breast cancer: towards the next phase. *Cell Cycle* 17, 1871–1885. <https://doi.org/10.1080/15384101.2018.1502567>
- Thurman, J.M., Laskowski, J., Nemenoff, R.A., 2020. Complement and Cancer—A Dysfunctional Relationship? *Antibodies* 9, 61. <https://doi.org/10.3390/antib9040061>
- Tonini, V., Zanni, M., 2021. Pancreatic cancer in 2021: What you need to know to win. *World J. Gastroenterol.* 27, 5851–5889. <https://doi.org/10.3748/wjg.v27.i35.5851>
- Torphy, R.J., Zhu, Y., Schulick, R.D., 2018. Immunotherapy for pancreatic cancer: Barriers and breakthroughs. *Ann. Gastroenterol. Surg.* 2, 274–281. <https://doi.org/10.1002/ags3.12176>
- Trovato, R., Fiore, A., Sartori, S., Canè, S., Giugno, R., Cascione, L., Paiella, S., Salvia, R., De Sanctis, F., Poffe, O., Anselmi, C., Hofer, F., Sartoris, S., Piro, G., Carbone, C., Corbo, V., Lawlor, R., Solito, S., Pinton, L., Mandruzzato, S., Bassi, C., Scarpa, A., Bronte, V., Ugel, S., 2019. Immunosuppression by monocytic myeloid-derived suppressor cells in patients with pancreatic ductal carcinoma is orchestrated by STAT3. *J. Immunother. Cancer* 7, 255. <https://doi.org/10.1186/s40425-019-0734-6>
- Truskowski, K., Amend, S.R., Pienta, K.J., 2023. Dormant cancer cells: programmed quiescence, senescence, or both? *Cancer Metastasis Rev.* 42, 37–47. <https://doi.org/10.1007/s10555-022-10073-z>
- Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyharto, C.A.-K., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P.-H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., Pontén, F., 2015. Proteomics. Tissue-based map of the human proteome. *Science* 347, 1260419. <https://doi.org/10.1126/science.1260419>
- Underwood, P.W., Herremans, K.M., Neal, D., Riner, A.N., Nassour, I., Hughes, S.J., Trevino, J.G., 2023. Changing Practice Patterns and Improving Survival for Patients with Pancreatic Ductal Adenocarcinoma. *Cancers* 15, 4464. <https://doi.org/10.3390/cancers15184464>
- Urban-Wojciuk, Z., Khan, M.M., Oyler, B.L., Fåhræus, R., Marek-Trzonkowska, N., Nita-Lazar, A., Hupp, T.R., Goodlett, D.R., 2019. The Role of TLRs in Anti-cancer Immunity and Tumor Rejection. *Front. Immunol.* 10, 2388. <https://doi.org/10.3389/fimmu.2019.02388>
- Verdon, D.J., Jenkins, M.R., 2021. Identification and Targeting of Mutant Peptide Neoantigens in Cancer Immunotherapy. *Cancers* 13, 4245. <https://doi.org/10.3390/cancers13164245>

- Verma, A., Mathur, R., Farooque, A., Kaul, V., Gupta, S., Dwarakanath, B.S., 2019. <p>T-Regulatory Cells In Tumor Progression And Therapy</p>. *Cancer Manag. Res.* 11, 10731–10747. <https://doi.org/10.2147/CMAR.S228887>
- Vesely, M.D., Schreiber, R.D., 2013. Cancer Immunoediting: antigens, mechanisms and implications to cancer immunotherapy. *Ann. N. Y. Acad. Sci.* 1284, 1–5. <https://doi.org/10.1111/nyas.12105>
- Vlavcheski, F., O'Neill, E.J., Gagacev, F., Tsiani, E., 2022. Effects of Berberine against Pancreatitis and Pancreatic Cancer. *Molecules* 27, 8630. <https://doi.org/10.3390/molecules27238630>
- Vogel, A., Römmeler-Zehrer, J., Li, J.S., McGovern, D., Romano, A., Stahl, M., 2016. Efficacy and safety profile of nab-paclitaxel plus gemcitabine in patients with metastatic pancreatic cancer treated to disease progression: a subanalysis from a phase 3 trial (MPACT). *BMC Cancer* 16, 817. <https://doi.org/10.1186/s12885-016-2798-8>
- von Ahrens, D., Bhagat, T.D., Negrath, D., Maitra, A., Verma, A., 2017. The role of stromal cancer-associated fibroblasts in pancreatic cancer. *J. Hematol. Oncol.* 10, 76. <https://doi.org/10.1186/s13045-017-0448-5>
- Von Hoff, D.D., Ervin, T., Arena, F.P., Chiorean, E.G., Infante, J., Moore, M., Seay, T., Tjulandin, S.A., Ma, W.W., Saleh, M.N., Harris, M., Reni, M., Dowden, S., Laheru, D., Bahary, N., Ramanathan, R.K., Tabernero, J., Hidalgo, M., Goldstein, D., Van Cutsem, E., Wei, X., Iglesias, J., Renschler, M.F., 2013. Increased Survival in Pancreatic Cancer with nab-Paclitaxel plus Gemcitabine. *N. Engl. J. Med.* 369, 1691–1703. <https://doi.org/10.1056/NEJMoa1304369>
- Wang, J., Elahi, A., Ajidahun, A., Clark, W., Hernandez, J., Achille, A., Hao, J., Seto, E., Shibata, D., 2014. The interplay between histone deacetylases and c-Myc in the transcriptional suppression of HPP1 in colon cancer. *Cancer Biol. Ther.* 15, 1198–1207. <https://doi.org/10.4161/cbt.29500>
- Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., Gong, Z., Zhang, S., Zhou, J., Cao, K., Li, X., Xiong, W., Li, G., Zeng, Z., Guo, C., 2017. Role of tumor microenvironment in tumorigenesis. *J. Cancer* 8, 761–773. <https://doi.org/10.7150/jca.17648>
- Wang, Q., Shao, X., Zhang, Y., Zhu, M., Wang, F.X.C., Mu, J., Li, J., Yao, H., Chen, K., 2023. Role of tumor microenvironment in cancer progression and therapeutic strategy. *Cancer Med.* 12, 11149–11165. <https://doi.org/10.1002/cam4.5698>
- Wang, Q.J., Yu, Z., Griffith, K., Hanada, K., Restifo, N.P., Yang, J.C., 2016. Identification of T-cell Receptors Targeting KRAS-Mutated Human Tumors. *Cancer Immunol. Res.* 4, 204–214. <https://doi.org/10.1158/2326-6066.CIR-15-0188>
- Wang, S., You, L., Dai, M., Zhao, Y., 2020. Mucins in pancreatic cancer: A well-established but promising family for diagnosis, prognosis and therapy. *J. Cell. Mol. Med.* 24, 10279–10289. <https://doi.org/10.1111/jcmm.15684>
- Wang, S., Zheng, Y., Yang, F., Zhu, L., Zhu, X.-Q., Wang, Z.-F., Wu, X.-L., Zhou, C.-H., Yan, J.-Y., Hu, B.-Y., Kong, B., Fu, D.-L., Bruns, C., Zhao, Y., Qin, L.-X., Dong, Q.-Z., 2021. The molecular biology of pancreatic adenocarcinoma: translational challenges and clinical perspectives. *Signal Transduct. Target. Ther.* 6, 1–23. <https://doi.org/10.1038/s41392-021-00659-4>

- Wang, T., Peng, Y., Li, R., Li, X., Zuo, C., 2021. Preliminary study on SPECT/CT imaging of pancreatic cancer xenografts by targeting integrin $\alpha 5$ in pancreatic stellate cells. *J. Cancer* 12, 1729–1733. <https://doi.org/10.7150/jca.51190>
- Wang, Y., Qi, J., Chen, X., 2022. Accurate Prediction of Epigenetic Multi-Targets with Graph Neural Network-Based Feature Extraction. *Int. J. Mol. Sci.* 23, 13347. <https://doi.org/10.3390/ijms232113347>
- Wang, Y., Sun, S.-N., Liu, Q., Yu, Y.-Y., Guo, J., Wang, K., Xing, B.-C., Zheng, Q.-F., Campa, M.J., Patz, E.F., Li, S.-Y., He, Y.-W., 2016. Autocrine Complement Inhibits IL10-Dependent T-Cell Mediated Antitumor Immunity to Promote Tumor Progression. *Cancer Discov.* 6, 1022–1035. <https://doi.org/10.1158/2159-8290.CD-15-1412>
- Wang, Y., Zhang, H., He, Y.-W., 2019. The Complement Receptors C3aR and C5aR Are a New Class of Immune Checkpoint Receptor in Cancer Immunotherapy. *Front. Immunol.* 10.
- Warburg, O., 1956. On the origin of cancer cells. *Science* 123, 309–314.
- Waters, A.M., Der, C.J., 2018. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb. Perspect. Med.* 8, a031435. <https://doi.org/10.1101/cshperspect.a031435>
- Winstead, E., 2022. Treating Cancer by Reducing Tumor-Related Inflammation - NCI [WWW Document]. *Natl. Cancer Inst.* URL <https://www.cancer.gov/news-events/cancer-currents-blog/2022/reducing-inflammation-to-treat-cancer> (accessed 1.17.23).
- Xiao, Z., Yeung, C.L.S., Yam, J.W.P., Mao, X., 2022. An update on the role of complement in hepatocellular carcinoma. *Front. Immunol.* 13.
- Xulu, K.R., Nweke, E.E., Augustine, T.N., 2023. Delineating intra-tumoral heterogeneity and tumor evolution in breast cancer using precision-based approaches. *Front. Genet.* 14, 1087432. <https://doi.org/10.3389/fgene.2023.1087432>
- Yagyu, T., Saito, H., Sakamoto, T., Uchinaka, E., Morimoto, M., Amisaki, M., Watanabe, J., Tokuyasu, N., Honjo, S., Ashida, K., Fujiwara, Y., 2019. Preoperative Albumin-Bilirubin Grade as a Useful Prognostic Indicator in Patients With Pancreatic Cancer. *Anticancer Res.* 39, 1441–1446. <https://doi.org/10.21873/anticancer.13260>
- Yamakawa, K., Ye, J., Nakano-Narusawa, Y., Matsuda, Y., 2021. Pathological Changes in Pancreatic Carcinogenesis: A Review. *Cancers* 13, 686. <https://doi.org/10.3390/cancers13040686>
- Yamamoto, T., Yanagimoto, H., Satoi, S., Toyokawa, H., Hirooka, S., Yamaki, S., Yui, R., Yamao, J., Kim, S., Kwon, A.-H., 2012. Circulating CD4+CD25+ Regulatory T Cells in Patients With Pancreatic Cancer: *Pancreas* 41, 409–415. <https://doi.org/10.1097/MPA.0b013e3182373a66>
- Yang, J., Nie, J., Ma, X., Wei, Y., Peng, Y., Wei, X., 2019. Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Mol. Cancer* 18, 26. <https://doi.org/10.1186/s12943-019-0954-x>

- Yang, S., Liu, Q., Liao, Q., 2021. Tumor-Associated Macrophages in Pancreatic Ductal Adenocarcinoma: Origin, Polarization, Function, and Reprogramming. *Front. Cell Dev. Biol.* 8, 607209. <https://doi.org/10.3389/fcell.2020.607209>
- Yano, S., Miwa, S., Mii, S., Hiroshima, Y., Uehara, F., Yamamoto, M., Kishimoto, H., Tazawa, H., Bouvet, M., Fujiwara, T., Hoffman, R.M., 2014. Invading cancer cells are predominantly in G0/G1 resulting in chemoresistance demonstrated by real-time FUCCI imaging. *Cell Cycle Georget. Tex* 13, 953–960. <https://doi.org/10.4161/cc.27818>
- Yarmoska, S.K., Alawieh, A.M., Tomlinson, S., Hoang, K.B., 2021. Modulation of the Complement System by Neoplastic Disease of the Central Nervous System. *Front. Immunol.* 12.
- Yin, M., O'Neill, L.A.J., 2021. The role of the electron transport chain in immunity. *FASEB J.* 35, e21974. <https://doi.org/10.1096/fj.202101161R>
- Yuan, Y., Ju, Y.S., Kim, Y., Li, J., Wang, Y., Yoon, C.J., Yang, Y., Martincorena, I., Creighton, C.J., Weinstein, J.N., Xu, Y., Han, L., Kim, H.-L., Nakagawa, H., Park, K., Campbell, P.J., Liang, H., 2020. Comprehensive molecular characterization of mitochondrial genomes in human cancers. *Nat. Genet.* 52, 342–352. <https://doi.org/10.1038/s41588-019-0557-x>
- Yunna, C., Mengru, H., Lei, W., Weidong, C., 2020. Macrophage M1/M2 polarization. *Eur. J. Pharmacol.* 877, 173090. <https://doi.org/10.1016/j.ejphar.2020.173090>
- Zelek, W.M., Morgan, B.P., 2020. Monoclonal Antibodies Capable of Inhibiting Complement Downstream of C5 in Multiple Species. *Front. Immunol.* 11.
- Zhang, H., Cui, B., Zhou, Y., Wang, X., Wu, W., Wang, Z., Dai, Z., Cheng, Q., Yang, K., 2021. B2M overexpression correlates with malignancy and immune signatures in human gliomas. *Sci. Rep.* 11, 5045. <https://doi.org/10.1038/s41598-021-84465-6>
- Zhang, L., Sanagapalli, S., Stoita, A., 2018. Challenges in diagnosis of pancreatic cancer. *World J. Gastroenterol.* 24, 2047–2060. <https://doi.org/10.3748/wjg.v24.i19.2047>
- Zhang, R., Liu, Q., Li, T., Liao, Q., Zhao, Y., 2019a. Role of the complement system in the tumor microenvironment. *Cancer Cell Int.* 19, 300. <https://doi.org/10.1186/s12935-019-1027-3>
- Zhang, R., Liu, Q., Li, T., Liao, Q., Zhao, Y., 2019b. Role of the complement system in the tumor microenvironment. *Cancer Cell Int.* 19, 300. <https://doi.org/10.1186/s12935-019-1027-3>
- Zhang, T., Ren, Y., Yang, P., Wang, J., Zhou, H., 2022. Cancer-associated fibroblasts in pancreatic ductal adenocarcinoma. *Cell Death Dis.* 13, 897. <https://doi.org/10.1038/s41419-022-05351-1>
- Zhang, T.-N., Yin, R.-H., Wang, L.-W., 2020. The prognostic and predictive value of the albumin-bilirubin score in advanced pancreatic cancer. *Medicine (Baltimore)* 99. <https://doi.org/10.1097/MD.00000000000020654>
- Zhang, Y., Lazarus, J., Steele, N.G., Yan, W., Lee, H.-J., Nwosu, Z.C., Halbrook, C.J., Menjivar, R.E., Kemp, S.B., Sirihorachai, V.R., Velez-Delgado, A., Donahue, K., Carpenter, E.S., Brown, K.L., Irizarry-Negron, V., Nevison, A.C., Vinta, A., Anderson,

- M.A., Crawford, H.C., Lyssiotis, C.A., Frankel, T.L., Bednar, F., Pasca di Magliano, M., 2020. Regulatory T-cell Depletion Alters the Tumor Microenvironment and Accelerates Pancreatic Carcinogenesis. *Cancer Discov.* 10, 422–439. <https://doi.org/10.1158/2159-8290.CD-19-0958>
- Zhang, Y., Yang, W., Li, W., Zhao, Y., 2021. NLRP3 Inflammasome: Checkpoint Connecting Innate and Adaptive Immunity in Autoimmune Diseases. *Front. Immunol.* 12.
- Zhang, Z., Kurashima, Y., 2021. Two Sides of the Coin: Mast Cells as a Key Regulator of Allergy and Acute/Chronic Inflammation. *Cells* 10, 1615. <https://doi.org/10.3390/cells10071615>
- Zhao, H., Wu, L., Yan, G., Chen, Y., Zhou, M., Wu, Y., Li, Y., 2021. Inflammation and tumor progression: signaling pathways and targeted intervention. *Signal Transduct. Target. Ther.* 6, 1–46. <https://doi.org/10.1038/s41392-021-00658-5>
- Zhao, T., Zhan, Z., Jiang, D., 2019. Histone modifications and their regulatory roles in plant development and environmental memory. *J. Genet. Genomics* 46, 467–476. <https://doi.org/10.1016/j.jgg.2019.09.005>
- Zhao, X., Liu, J., Ge, S., Chen, C., Li, S., Wu, X., Feng, X., Wang, Y., Cai, D., 2019. Saikosaponin A Inhibits Breast Cancer by Regulating Th1/Th2 Balance. *Front. Pharmacol.* 10.
- Zhou, L., Wu, Z., Jiang, C., Dai, S., 2022. Efficacy, Safety, and Impact on Patient Survival of PDL1/PD-1 Inhibitors versus FOLFIRINOX Regimens for Advanced Pancreatic Cancer. *Comput. Math. Methods Med.* 2022, 5430720. <https://doi.org/10.1155/2022/5430720>
- Zhou, L., Zhang, Z., Huang, Z., Nice, E., Zou, B., Huang, C., 2020. Revisiting cancer hallmarks: insights from the interplay between oxidative stress and non-coding RNAs. *Mol. Biomed.* 1, 4. <https://doi.org/10.1186/s43556-020-00004-1>
- Zhou, X., Yan, Y., Xu, M., 2022. Immune cell responses in pancreatic cancer and their clinical application. *Eur. J. Inflamm.* 20, 205873922110443. <https://doi.org/10.1177/20587392211044381>
- Zhu, H., Yu, X., Zhang, S., Shu, K., 2021. Targeting the Complement Pathway in Malignant Glioma Microenvironments. *Front. Cell Dev. Biol.* 9.
- Zhu, M., Ma, Z., Zhang, X., Hang, D., Yin, R., Feng, J., Xu, L., Shen, H., 2022. C-reactive protein and cancer risk: a pan-cancer study of prospective cohort and Mendelian randomization analysis. *BMC Med.* 20, 301. <https://doi.org/10.1186/s12916-022-02506-x>
- Zipfel, P.F., Skerka, C., 2009. Complement regulators and inhibitory proteins. *Nat. Rev. Immunol.* 9, 729–740. <https://doi.org/10.1038/nri2620>
- Żorniak, M., Sirtl, S., Mayerle, J., Beyer, G., 2020. What Do We Currently Know about the Pathophysiology of Alcoholic Pancreatitis: A Brief Review. *Visc. Med.* 36, 182–190. <https://doi.org/10.1159/000508173>

SUPPLEMENTARY TABLES

Table S1: List of dysregulated genes with their respective fold regulation.

Downregulated genes		Upregulated genes	
Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation
<i>CCR4</i>	-83.19	<i>C3</i>	562.22
<i>CD40LG</i>	-44.48	<i>B2M</i>	27.30
<i>IL2</i>	-28.49	<i>HLA-A</i>	7.84
<i>TLR7</i>	-22.75	<i>PRLP0</i>	6.64
<i>TLR6</i>	-22.48	<i>HLA-E</i>	5.98
<i>TLR9</i>	-21.85	<i>STAT3</i>	3.98
<i>IL6</i>	-20.88	<i>IRF3</i>	3.56
<i>IRAG1</i>	-18.69	<i>MYD88</i>	3.45
<i>IL10</i>	-17.71	<i>LYZ</i>	3.22
<i>NLRP3</i>	-17.18	<i>MAPK1</i>	3.09
<i>IL1B</i>	-15.97	<i>ACTB</i>	2.96
<i>CSF2</i>	-15.71	<i>STAT1</i>	2.49
<i>IL17A</i>	-14.80	<i>NFKBIA</i>	2.25
<i>TLR8</i>	-14.26		
<i>CCR8</i>	-11.52		
<i>CXCL10</i>	-11.47		
<i>IFNG</i>	-10.89		
<i>IL23A</i>	-10.65		
<i>CASP1</i>	-10.09		
<i>MX1</i>	-9.32		
<i>CCR6</i>	-9.24		

<i>MBL2</i>	-8.91
<i>HGDC</i>	-8.89
<i>GATA3</i>	-8.79
<i>NOD2</i>	-8.74
<i>TBX21</i>	-8.23
<i>IFNA1</i>	-7.99
<i>CCR5I</i>	-7.95
<i>IL13</i>	-7.18
<i>CXCR3</i>	-6.95
<i>TLR3</i>	-6.66
<i>TLR2</i>	-6.59
<i>IL4</i>	-6.34
<i>STAT4</i>	-6.03
<i>FOXP3</i>	-5.23
<i>IL5</i>	-5.21
<i>TLR5</i>	-5.09
<i>FASLG</i>	-4.62
<i>CXCL8</i>	-4.36
<i>IFNB1</i>	-4.34
<i>CD4</i>	-4.31
<i>CD80</i>	-4.14
<i>IL1A</i>	-4.06
<i>NOD1</i>	-3.74
<i>TLR1</i>	-3.50
<i>MPO</i>	-3.50
<i>APCS</i>	-3.50

<i>HPRT1</i>	-3.50
<i>TLR4</i>	-3.48
<i>MAPK8</i>	-3.44
<i>CD86</i>	-3.15
<i>CRP</i>	-2.90
<i>IRF7</i>	-2.90
<i>IRAK1</i>	-2.84
<i>IL18</i>	-2.79
<i>CD14</i>	-2.70
<i>STAT6</i>	-2.65
<i>TRAF6</i>	-2.64
<i>TYK2</i>	-2.57
<i>LY96</i>	-2.50
<i>CD8A</i>	-2.13
<i>IFNAR1</i>	-2.08

Table S2: Other clinicopathological characteristics of patients.

Parameters	Chronic Pancreatitis (n=6)	Resectable (n=13)	Locally Advanced (n=8)	Metastasis (n=4)	p-value
Hepatitis A					-
DISEASE					
Positive n (%)	0 (0)	0 (0)	0 (0)	0 (0)	
Negative n (%)	6 (100)	13 (100)	8 (100)	4 (100)	
Hepatitis B					-
Positive n (%)	0 (0)	0 (0)	0 (0)	0 (0)	
Negative n (%)	6 (100)	13 (100)	8 (100)	4 (100)	
Hepatitis C					-
Positive n (%)	0 (0)	0 (0)	0 (0)	0 (0)	
Negative n (%)	6 (100)	13 (100)	8 (100)	4 (100)	
Syphilis					-
Positive n (%)	0 (0)	0 (0)	0 (0)	0 (0)	
Negative n (%)	6 (100)	13 (100)	8 (100)	4 (100)	
INDICES IN SERUM					
Hb Index					0.332
1+ n (%)	1 (16.7)	1 (7.7)	0 (0)	1 (25)	
None n (%)	3 (50)	11 (84.6)	7 (87.5)	2 (50)	
Trace n (%)	2 (33.3)	1 (7.7)	1 (12.5)	1 (25)	
Bilirubin Index					0.163
1+ n (%)	0 (0)	10 (76.9)	6 (75)	3 (75)	
None n (%)	4 (66.7)	1 (7.7)	1 (12.5)	0 (0)	
Trace n (%)	2 (33.3)	2 (15.4)	1 (12.5)	1 (25)	
Lipaemia Index					0.445
1+ n (%)	0 (0)	0 (0)	0 (0)	0 (0)	
None n (%)	6 (100)	11(84.6)	8 (100)	3 (75)	
Trace n (%)	0 (0)	2 (15.4)	0 (0)	1 (25)	

Table S3: Blood chemistry, complement and CRP measurements between all PDAC patients who lived and died. The data is shown as Mean±SD and median [range].

Parameter	All (n=25)	Alive (n=8)	Died (n=17)	Significance (p=)
Age (years)	58±13	54±17	60±10	0.680
Haematocrit (L/L %)	0.32±0.06	0.35±0.06	0.30±0.06	0.076
WC count ^9/L	10.76±5.50 10.62 [3.34-26.14]	12.09±6.84 10.80 [3.78-26.14]	10.21±4.98 8.89 [3.34-23.37]	0.530
Haemoglobin ^9/L	10.35±1.93	11.29±2.00	9.92±1.80	0.120
PLT count (/L)^9	402±152 388 [154-810]	397±210 352 [167-810]	406±124 406 [154-721]	0.350
Red CC (/L)^12	3.75±2.60	3.78±0.71	3.75±1.90	0.470
CRP mg/L	91±93 46 [2-318]	62±91 36 [2-278]	105±94 82 [9-318]	0.097
C3 µg/ml	0.84±0.47 0.81 [0.34-2.41]	0.76±0.39 0.71 [0.34-1.32]	0.88±0.51 0.81 [0.34-2.41]	0.660
C5 ng/ml	21.02±7.49 23.73 [4.78-28.88]	22.36±5.29 23.06 [12.34-27.63]	20.39±8.40 24.16 [4.78-28.88]	0.700
C5/C3 ratio	31±17 28 [2-78]	37±21 27 [15-78]	31±17 28 [2-59]	0.430

Table S4: Cell proliferation statistics.

24 hours		48 hours	
Tukey's multiple comparisons test	Adjusted p-value	Tukey's multiple comparisons test	Adjusted p-value
Column A vs. Column B	0,0084	Column A vs. Column B	0,0084
Column A vs. Column C	0,0011	Column A vs. Column C	0,0011
Column A vs. Column D	0,0011	Column A vs. Column D	0,0011
Column A vs. Column E	0,0011	Column A vs. Column E	0,0011
Column A vs. Column F	0,0011	Column A vs. Column F	0,0011
Column A vs. Column G	0,0011	Column A vs. Column G	0,0011
Column A vs. Column H	0,0011	Column A vs. Column H	0,0011
Column A vs. Column I	0,0011	Column A vs. Column I	0,0011
Column A vs. Column J	0,0011	Column A vs. Column J	0,0011
Column B vs. Column C	>0.9999	Column B vs. Column C	>0.9999
Column B vs. Column D	>0.9999	Column B vs. Column D	>0.9999
Column B vs. Column E	>0.9999	Column B vs. Column E	>0.9999
Column B vs. Column F	>0.9999	Column B vs. Column F	>0.9999
Column B vs. Column G	>0.9999	Column B vs. Column G	>0.9999
Column B vs. Column H	>0.9999	Column B vs. Column H	>0.9999
Column B vs. Column I	>0.9999	Column B vs. Column I	>0.9999
Column B vs. Column J	>0.9999	Column B vs. Column J	>0.9999
Column C vs. Column D	>0.9999	Column C vs. Column D	>0.9999
Column C vs. Column E	>0.9999	Column C vs. Column E	>0.9999
Column C vs. Column F	>0.9999	Column C vs. Column F	>0.9999
Column C vs. Column G	>0.9999	Column C vs. Column G	>0.9999
Column C vs. Column H	>0.9999	Column C vs. Column H	>0.9999

Column C vs. Column I	>0.9999	Column C vs. Column I	>0.9999
Column C vs. Column J	>0.9999	Column C vs. Column J	>0.9999
Column D vs. Column E	>0.9999	Column D vs. Column E	>0.9999
Column D vs. Column F	>0.9999	Column D vs. Column F	>0.9999
Column D vs. Column G	>0.9999	Column D vs. Column G	>0.9999
Column D vs. Column H	>0.9999	Column D vs. Column H	>0.9999
Column D vs. Column I	>0.9999	Column D vs. Column I	>0.9999
Column D vs. Column J	>0.9999	Column D vs. Column J	>0.9999
Column E vs. Column F	>0.9999	Column E vs. Column F	>0.9999
Column E vs. Column G	>0.9999	Column E vs. Column G	>0.9999
Column E vs. Column H	>0.9999	Column E vs. Column H	>0.9999
Column E vs. Column I	>0.9999	Column E vs. Column I	>0.9999
Column E vs. Column J	>0.9999	Column E vs. Column J	>0.9999
Column F vs. Column G	>0.9999	Column F vs. Column G	>0.9999
Column F vs. Column H	>0.9999	Column F vs. Column H	>0.9999
Column F vs. Column I	>0.9999	Column F vs. Column I	>0.9999
Column F vs. Column J	>0.9999	Column F vs. Column J	>0.9999
Column G vs. Column H	>0.9999	Column G vs. Column H	>0.9999
Column G vs. Column I	>0.9999	Column G vs. Column I	>0.9999
Column G vs. Column J	>0.9999	Column G vs. Column J	>0.9999
Column H vs. Column I	>0.9999	Column H vs. Column I	>0.9999
Column H vs. Column J	>0.9999	Column H vs. Column J	>0.9999
Column I vs. Column J	>0.9999	Column I vs. Column J	>0.9999

Column A: Untreated cells; Column B, C, D, E, F, G, H, I, J: Concentration of Compstatin at 0, 1, 2, 3, 6, 13, 25, 50, 100 μ M, respectively.

Table S5: Cell cycle statistics.

Cell cycle phase	Untreated p-value	Compstatin p-value	Dimethyl Sulphide p-value
G1% vs. S%	<0.0001	0.011	0.0986
G1% vs. G2%	<0.0001	0.0044	0.0502
G1% vs. G0%	<0.0001	0.0053	0.0901
S% vs. G2%	0.9989	0.4086	0.8488
S% vs. G0%	0.0016	0.5755	0.9995
G2% vs. G0%	0.0017	0.9785	0.8906

Table S6: The list of up- and down-regulated proteins

Upregulated proteins		Downregulated proteins	
Protein ID	Name	Protein ID	Name
Q9UIL1	SCOC	P02760	AMBP
Q96KQ7	EHMT2	Q9Y5Y9	SCN10A
Q92896	GLG1	P0DTE7	AMY1B;AMY1C;AMY1A
P15531	NME1	P01857	IGHG1;
Q06323	PSME1	P01876	IGHA1
Q7Z5H4	VN1R5	P05109	S100A8
Q9HD33	MRPL47	P19823	ITIH2
Q15024	EXOSC7	P01036	CST4
Q9H1Z4	WDR13	P04350	TUBB4A
P0DP23	CALM1;CALM2;CALM3	P06702	S100A9
Q9NRX3	NDUFA4L2	P19013	KRT4
Q9BYD3	MRPL4	P58107	EPPK1
Q9H1E3	NUCKS1	P0C0L4	C4A;C4B_2
Q93045	STMN2	Q9UPN6	SCAF8
Q8IWS0	PHF6	P02788	LTF;
Q9BPZ3	PAIP2	P02538	KRT6A
Q9H3P7	ACBD3	P29508	SERPINB3;SERPINB4
P49366	DHPS	Q16594	TAF9
O95573	ACSL3	Q16647	PTGIS
Q14534	SQLE	Q9HBH5	RDH14
P62256	UBE2H	Q9Y4G6	TLN2
Q14696	MESD	P20674	COX5A
P48506	GCLC	P02768	ALB

P33316	DUT	P68032	ACTC1
Q9P260	RELCH	Q15208	STK38
O60231	DHX16	P0C0S8	H2AC17;H2AC7;H2AC20;H2AC19;H2AC12;H2AC14; H2AJ
Q9Y3P9	RABGAP1	P04004	VTN
Q99447	PCYT2	Q9NVI7	ATAD3A
Q9NVA1	UQCC1	O43464	HTRA2
Q641Q2	WASHC2A;WASHC2C	P61599	NAA20
P0DPB5	POLR1D	P69905	;HBA2
Q96E11	MRRF	Q9H147	DNTTIP1
Q9Y2G5	POFUT2	Q9H6T3	RPAP3
P84085	ARF5	Q5HYJ3	FAM76B
Q8IV38	ANKMY2	Q99570	PIK3R4
P11234	RALB	P51608	MECP2
P21291	CSRP1	Q06033	ITIH3
Q15723	ELF2	P55081	MFAP1
O94916	NFAT5	Q5GLZ8	HERC4
P29966	MARCKS	Q92667	AKAP1
Q9NP77	SSU72	Q15758	SLC1A5
Q9Y587	AP4S1	Q15067	ACOX1
O95292	VAPB	P30040	ERP29
P16989	YBX3	Q8NI27	THOC2
Q13523	PRPF4B	Q9P0I2	EMC3
Q0VDF9	HSPA14	Q96K17	BTF3L4
Q8TD55	PLEKHO2	Q07817	BCL2L1
Q8NI22	MCFD2	Q3KQU3	MAP7D1

P54259	ATN1	Q8WWI5	SLC44A1
Q9NWB6	ARGLU1	Q9Y4Z0	LSM4
Q969Z3	MTARC2	P46013	MKI67
O43181	NDUFS4	Q9H773	DCTPP1
Q9P035	HACD3	O95249	GOSR1
		P17612	PRKACA
		Q86UE4	MTDH
		Q8TCJ2	STT3B
		P56211	ARPP19
		P41208	CETN2;CETN1
		Q9NVT9	ARMC1
		P08134	RHOC

Table S7: List of equipment used with their manufacturer and catalogue numbers.

Instrument/Equipment	Manufacturer and catalogue number
TissueRuptor	Qiagen 9001272
Nanodrop Spectrophotometer	ND-1000 UV
Spectrophotometer	Anthos 2010
SimpliAmp™ Thermal cycler	ThermoFisher A24812
Real-time PCR Machine	ThermoFisher QuantStudio 1
High-Speed Refrigerated Centrifuge	Heal Force Neofuge 13R
96 well centrifuge 5810R	Eppendorf 5811DL473204
The Muse® cell analyzer	Luminex 0500-3115
Dry bath	Techne Dri-Block DB-3
Multiskan Sky Spectrophotometer	ThermoFisher Scientific 51119500
Desktop Computer Monitor 19.5'	Philips 203VSLHSB2
SimplyAmp™ Thermal cycler	ThermoFisher Scientific A24812
Gel documentation system G-BOX	SYNGENE 103609
Deluxe Vortex Mixer	Chiltern MT19
Electrophoresis Power Supply – EPS 301	Amersham Pharmacia Biotech 01024552
Explorer Weighing scale	Ohaus E12140
Rotifix 32A Centrifuge	LABOTEC D-78532
Airstream Laminar Flow	LABOTEC ESCO Class II BSC
Autoclave Machine	Sturdy Apex Group SA-300VF
Microscope	OLYMPUS IX2-ILL30 6K16339
TC20™ Automated Cell counter	BIO-RAD 508BR1331

Microwave Oven	DEFY DMO 350
Heracell 150i CO ₂ Incubator	ThermoFisher Scientific 50116047
Ice Machine	SCOTSMAN CM 2933 5
Fridge	BOSCH KGN49A122
Glacier -86°C Ultralow temperature freezer	NUAIRE NU-9668E
PlateFuge Microcentrifuge	Benchmark Scientific E1019292
Tacta [®] mechanical 1- channel pipette 0,5-10 µL, 20-200 µL, 100-1000 µL	Sartorius 16032096/16032404/16032
Tacta [®] mechanical 8-channel pipette 30-300 µL	Sartorius 16032603
Gel electrophoresis system	Owl Scientific plastics, inc. 048589
Contrast microscope	OLYMPUS IX51

Table S8: List of reagents or chemicals used with their manufacturer and catalogue numbers.

Reagents/chemicals/compounds	Manufacturer and catalogue number
RNAlater	Qiagen 76106
Nuclease free water	Qiagen 129317
Trizol	Sigma T9424
Ethanol	Sigma 34870-2.5L
Chloroform	MERCK 65873-2.5L
Iso-propanol	Sigma I9516-500ml
FBS	Biowest S007430204
Penicillin-Streptomycin	Sigma P4333-100ml
DMEM	Sigma D6429-500ml
Trypsin-EDTA	Sigma T3924-500ml
TaqMan gene expression mastermix	ThermoFisher Scientific 4369016
Compstatin	WhiteSci 2585-1mg
Gemcitabine Hydrochloride CRS	MERCK Y0000675-50mg
PD-1/PD-L1 Inhibitor 1, small molecule	ABCAM Ab230369-10mg
Trypan Blue Dye 0.40%	BIO-RAD #1450013 15ml
Deionized water	Sigma BCCB7111
Phosphate Buffered Saline (PBS)	Sigma 806552-500ml
Dimethyl Sulfoxide (DMSO)	Sigma D8418-250ml
Propidium Iodide	Sigma 25535-16-4
GAPDH F Primers Sequence: 5'GGTATCGTGGAAGGACTCAT3'	Inqaba biotech S06B0

GAPDH R Primers Sequence: 5'ACCACCTGGTGCTCAGTGTA3'	Inqaba biotech S06B1
C3 F primers Sequence: 5'CTGCCCAGTTTCGAGGTCAT3'	Inqaba biotech S2178
C3 R primers Sequence: 5'CAATCGGAATGCGCTTGAGG3'	Inqaba biotech S2179
C5 F primers Sequence: 5'CTCCAACCATGGGCCTTTTG3'	Inqaba biotech S1187
C5 R primers Sequence: 5'TTCAGATGCTCCAACACGGA3'	Inqaba biotech S1188
MRPL-19 F primers Sequence: 5'CCTAGGCCGGAGTTTCCAA3'	Inqaba Biotech S2176
MRPL-19 R primers Sequence: 5'GACTCAAGAACCTGCGTTCC3'	Inqaba Biotech S2177
Agarose	VWR Amnesco 0710 – 100g
TBE Buffer	Sigma T3202 1L
100bp DNA ladder	New England BioLabs N3231S
6x loading dye, no SDS	New England BioLabs B7025S
Ethidium bromide	VWR Amnesco LIFE SCIENCE 0710 – 100g X328 -10mg/ml

Table S9: List of kits used with their manufacturer and catalogue numbers.

Kits	Manufacturer and catalogue number
RT ² First Strand	Qiagen 330401
RT ² SYBR® Green qPCR mastermix	Qiagen 330500
RT ² Profiler PCR Array (96-well format) Human Innate & Adaptive Immune Responses	Qiagen PAHS-052ZA 330231
Human Complement C3 ELISA	Abcam ab108822
Human Complement C5 ELISA	Abcam ab125963
Super Script™ VILO™ cDNA synthesis	ThermoFisher Scientific 11754050
ProtoScript® II First Strand cDNA synthesis	New England Biolabs E6560S
C3 primer assay	Thermo Fisher 4331182
C5 primer assay	Thermo Fisher 4331182
MRPL-19 assay	Thermo Fisher 4331182
XTT assay for cell viability and proliferation	MERCK 11465015001

Table S10: List of softwares used with the link to their websites.

Softwares	Website
Qiagen RT ² Profiler PCR Array	https://www.qiagen.com/us/product-categories/instruments-andautomation/analytics-software
Reactome	https://reactome.org/
Cytoscape	https://cytoscape.org/
Human protein Atlas	https://www.proteinatlas.org/ENSG00000125730-C3
ShinyGo	http://bioinformatics.sdstate.edu/go/
ELISA analysis	https://www.elisaanalysis.com/app
Real-Time PCR analysis	https://apps.thermofisher.com/apps/spa/#/
STATISTICA	https://www.statista.com/
SPSS	https://www.ibm.com/products/spss-statistics/
GraphPad Prism	https://www.graphpad.com/scientific-software/prism/

Table S11: List of materials/consumables used with their manufacturer and catalogue numbers.

Materials/Consumables	Manufacturer and catalogue number
Pipette Tips (sizes 10 µL, 200 µL, 1000 µL)	ThermoFisher Scientific AM12635, AM12650 AM12660
Eppendorf tubes/ disposable microcentrifuge tube	Sigma BR780400
Corning® PCR tubes	Sigma CLS6571
Disposable Reagent Reservoir	Cat #097803 50ml
Four-way microtube rack	Sigma R5651
Glass Beaker	LASEC
Measuring cylinder	LASEC
MicoAmp Optical 96-well Reaction plate	Applied biosystems 4306737
Nunc™ Delta Surface 96-well culture plates	ThermoFisher Scientific 167425
Nunc Multidish 6-well culture plates	ThermoFisher Scientific 163251
T-75 Cyto-One® Flask, Green Vented Filter	Starlab 2472004
Corning® 25 cm ² cell culture flask	Sigma CLS430372
TC10™ System Counting Slides, Dual Chamber	BIO-RAD Cat. #145-0011
Plate PCR Seal Adhesive Optical	LASEC B0140116
50 ml Polypropylene Conical Tube	Greiner bio-one LASEC 352070
15 ml Polypropylene Conical Tube	Greiner bio-one LASEC 188261
Sterile Surgical Blade	PARAMOUNT® BS EN 27740
Glass Petri dish	LASEC
Parafilm® M, roll size 4 in. × 125 ft	Sigma P7793-1EA
Orange handle scissors 210 mm	Treeline TSCO210

Surgical Tweezers	Transact® TI-03-2034
Corning® Cryogenic vials	Sigma CLS430659
TissueRuptor® Disposable probes	Qiagen 990890 (25)
Syringe PP/PE without needle 20 mL	Sigma Z116882
Syringe Filter	Sartorius Minisart® 16534
Autoclave Tape	Sigma BR61750
Aluminium Foil	Sigma Z185159
Kimwipes Jumbo paper towel	Kimberly-Clark 066716
Funnel	LASEC
Latex Examination Gloves	LASEC RLAS1GL014-0000S
Lab Coat	TANC XS
EDTA-treated vacutainer collection tubes (Purple top)	BD VP1082
Permanent Marker	Artline® EK70

SUPPLEMENTARY FIGURES

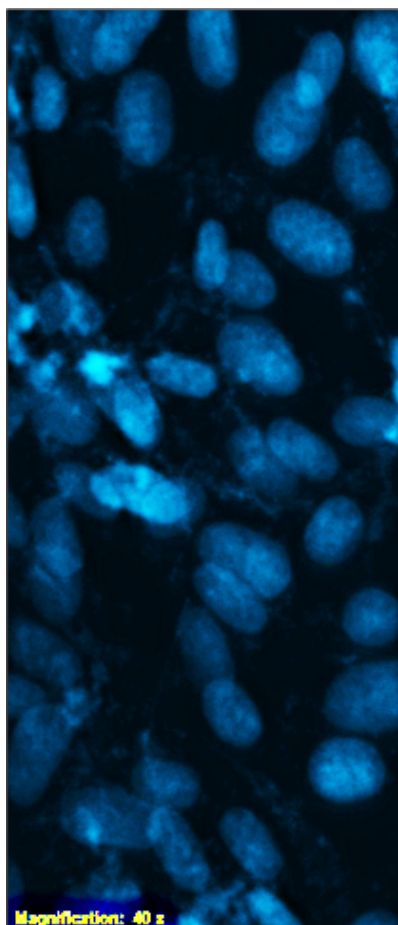


Figure S1: Mycoplasma detection by DAPI staining. MIA PaCA-2 stained cells showing mycoplasma negative result.

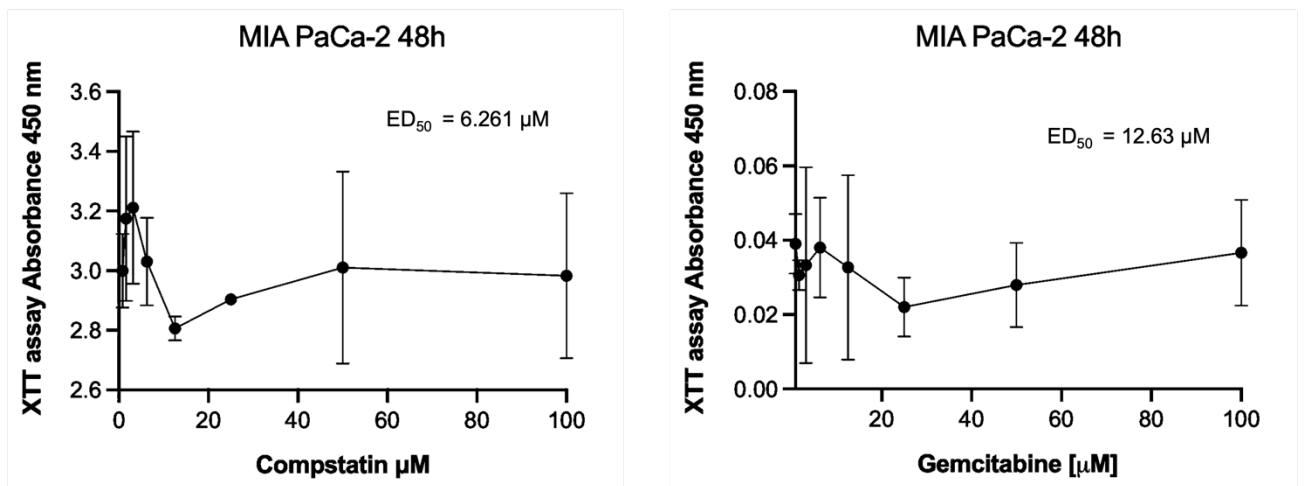


Figure S2: Dose response curve showing effective dose of compstatin and gemcitabine on MIA PaCA-2 cells. The effect of pharmacological inhibition of the complement pathway in pancreatic cancer was investigated using compstatin and gemcitabine as a positive control. Inhibition of the complement pathway induced cell proliferation in MIA PaCA-2 cells whereas gemcitabine was toxic to MIA PaCA-2 cells.

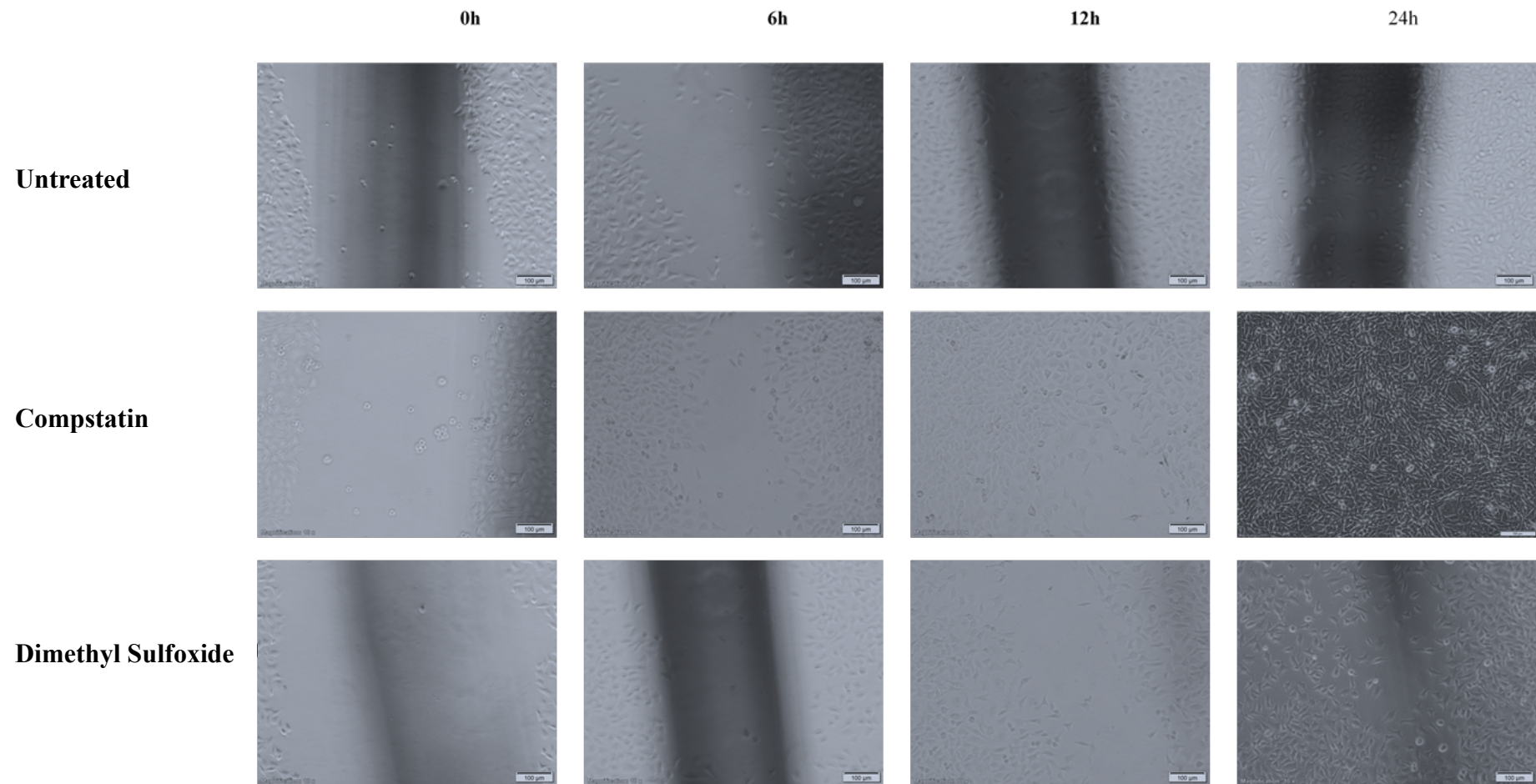


Figure S3: Cell migration of MIA PaCA-2 cell line during treatment with Compstatin. Cells show increased migration when complement is pharmacologically inhibited.

APPENDIX A: TURNITIN REPORT

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ORIGINALITY REPORT

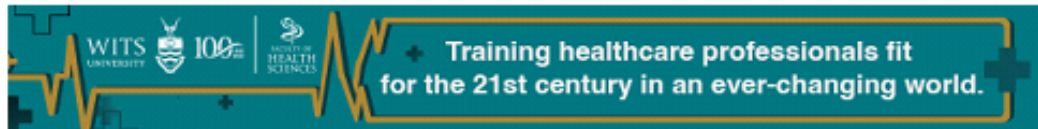
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Zanele Nsingwane

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APPENDIX B: STUDY INFORMATION SHEET (PDAC & CHRONIC PANCREATITIS)



Study title: Identification of (Novel) Immune Targets with Potential Roles in the Progression of Pancreatic Ductal Adenocarcinoma (PDAC).

Greetings Sir/Madam:

Introduction:

Good day, we, Prof. Geoffrey Candy, Dr Emmanuel Ekene Nweke and Zanele Nsingwane, are doing research on Pancreatic cancer. Research is a process used in seeking new knowledge. In this study we want to Identify potential immune targets in Pancreatic Ductal Adenocarcinoma cancer progression. This will be done by getting a tissue biopsy during the whipple procedure and drawing blood samples from patients for the three stages of PDAC which are resectable, non-metastatic and metastatic patients. This will allow us to study determine the role of potential targets in each stage and compare it with that of the control which is chronic pancreatitis.

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 immunopathogenesis 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 stage (resectable, non-metastatic and metastatic patients) and in this study your blood samples and tissue would be required and it will be taken once. About 10 ml of your blood will be extracted in a purple cap tube (10 ml). The blood sample collected will be processed into, plasma. Various techniques will be used to investigate the expression of these potential immune

targets present in the blood at each stage of the cancer and then compared with each other as well as with the control which is the chronic pancreatitis.

Risks of being involved in the study: We are extracting the blood through your veins and this could cause some discomfort and pains but there is no risk to you. Furthermore, for patients undergoing the Whipple procedure, this will be done by your personal surgeon that specializes in this field.

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 to 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 parties. In order to keep your identify 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 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 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: August 2019

APPENDIX C: STUDY INFORMATION DOCUMENT (HEALTHY CONTROLS)



Study title: Identification of (Novel) Immune Targets with Potential Roles in the Progression of Pancreatic Ductal Adenocarcinoma (PDAC).

Greetings Sir/Madam:

Introduction:

Good day, we, Prof. Geoffrey Candy, Dr. Ekene Nweke and Zanele Nsingwane are carrying out a research study on Pancreatic cancer. Research is a process used in seeking new knowledge. In this study, we want to learn the role of metabolites in Pancreatic Ductal Adenocarcinoma cancer progression. This will be done by drawing blood samples from patients for the three stages of PDAC which are resectable, non-metastatic, and metastatic as well as patients with chronic pancreatitis and healthy volunteers. This will allow us to identify (novel) immune targets involved in PDAC progression and these will be compared to the controls (chronic pancreatitis and healthy controls).

Invitation to Participate: For us to study PDAC, we are asking you to take part in this research study.

What is involved in the study: You are among the healthy volunteers we would like to recruit for this study. We are asking for your permission to draw a blood sample, and this will be carried out by qualified persons.

Risks of being involved in the study: There is no risk to you directly, but you might feel some pains during the blood extraction.

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 several 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 patients in the future. However, should there be a discovery that may benefit you we would contact your treating doctor who would contact you through your clinic.

Participation is voluntary: If you decide to 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 will 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 parties. 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 link 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 cancer and diagnosis, not in names and personal information. It is the intention to publish the data, showing proteins that 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 the intention to publish the data, showing proteins that may be of interest for all the patients in the group – not that of individuals participating in the study.

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 concerns 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 at 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: August 2019

APPENDIX D: INFORMED CONSENT SHEET



Consent Form: Identification of (Novel) Immune Targets with Potential Roles in the Progression of Pancreatic Ductal Adenocarcinoma (PDAC).

Dear Patient,

You are currently admitted to our hospital and have been diagnosed with Pancreatic Ductal Adenocarcinoma (PDAC). The Hospital not only renders treatment but is also actively involved in conducting research aimed at improving the quality of care that we deliver. From time to time such research involves the use of patient records from which information is extracted. We will also be using blood sample taken through your vein for this research study. This sample extraction will not jeopardize your safety and there will be no risk at all. The use of such information and sample is subject to the following:

1. Approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand.
2. 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 parties. In order to keep your identify 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 for this study we are only interested in the cancer and diagnosis, not in names and personal information. It is the intention to publish the data, showing potential immune targets which may be of interest for all the patients in the group – not that of individuals participating in the study.

We would like to obtain your consent to use information from your file for the purpose of this research, subject to the aforementioned conditions. If you choose not to give consent, this will

not compromise your treatment in any way. If at any time you choose to withdraw consent you are free to do so and will not be prejudiced in any way.

Should you wish to contact us at any stage regarding consent, contact Dr Jones Omoshoro at (011) 9339267 or Dr John Devar (011) 7171000 or Professor Geoffrey Candy 011-7172574

A. Consent Given

I _____ hereby give consent for my records to be used as per the above mentioned conditions for the purposes of research:

PATIENT: _____

Name	Signature	Date
------	-----------	------

RESEARCHER: _____

Name	Signature	Date
------	-----------	------

WITNESS: _____

Name	Signature	Date
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APPENDIX E: ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE
WITWATERSRAND.
JOHANNESBURG



R14/49 Miss Zanele Nsingwane

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190734

NAME: Miss Zanele Nsingwane
(Principal Investigator)
DEPARTMENT: Surgery
Chris Hani Baragwanath Academic Hospital
PROJECT TITLE: Investigating the immune response in pancreatic
ductal adenocarcinoma progression
DATE CONSIDERED: 26/07/2019
DECISION: Approved unconditionally
CONDITIONS:
SUPERVISOR: Prof Geoffrey Candy, Dr Ekene Nweke and Dr John Devar
APPROVED BY: 
Dr C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL: 29/11/2019

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

DECLARATION OF INVESTIGATORS

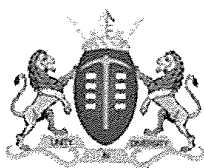
To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we 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 resubmit the application to the Committee. I agree to submit a yearly progress report. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed July and will therefore be due in the month of July each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).


Principal Investigator Signature

06-12-2019
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX F: PERMISSION TO CONDUCT RESEARCH



GAUTENG PROVINCE

HEALTH
REPUBLIC OF SOUTH AFRICA

MEDICAL ADVISORY COMMITTEE

CHRIS HANI BARAGWANATH ACADEMIC HOSPITAL

PERMISSION TO CONDUCT RESEARCH

Date: 8th October 2019

TITLE OF PROJECT:

Investigating the Immune Response in Pancreatic Ductal Adenocarcinoma Progression.

UNIVERSITY: Witswatersrand

Principal Investigator: Zanele Nsingwane

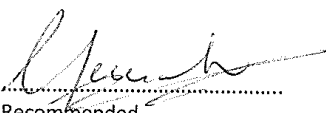
Department: Surgery

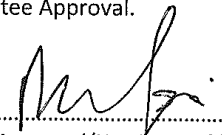
Supervisor : Prof Geoffrey Candy

Permission Head Department (where research conducted): Yes

The Medical Advisory Committee recommends that the said research be conducted at Chris Hani Baragwanath Academic Hospital. The CEO / management of Chris Hani Baragwanath Academic Hospital is accordingly informed and the study is subject to:-

- **Permission having been granted by the Committee for Research on Human Subjects of the University of the Witwatersrand.**
- The Hospital will not incur extra costs as a result of the research being conducted on its patients within the hospital
- The MAC will be informed of any serious adverse events as soon as they occur
- Permission is granted for the duration of the Ethics Committee Approval.


Recommended
(On behalf of the MAC)
Date: 8/10/2019


Approved/Not Approved
Hospital Management
Date: 08/10/2019