EARLY IMMUNE RESPONSES IN ACUTE PANCREATITIS AND THEIR ROLE IN PREDICTING DISEASE SEVERITY

Mwangala Nalisa

Student No: 1742389

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Supervisor: Dr. Pascaline N Fonteh Fru

Co-supervisors: Professor Martin D Smith

Dr. Tanya N Augustine

DECLARATION

I, Mwangala Nalisa declare that this Thesis is my own, unaided work. It is being submitted for the 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)_____15th_____day of___November_____2021____in___JOHANNESBURG

ABSTRACT

The 2012 Atlanta classification of acute pancreatitis (AP), which stratifies the severity as one of three risk categories, namely mild (MAP), moderately severe (MSAP), and severe (SAP), does not predict severity. Identifying immune parameters for use as prognostic markers that may be more accurate in stratifying patients' risk profiles at the early phase of acute pancreatitis is likely to improve treatment strategies. This study aimed to identify and assess the potential of early immune markers that could be used to stratify the three patient groups of AP. Forty patients diagnosed with AP (21 MAP, 14 MSAP, and 5 SAP) were recruited from the Chris Hani Baragwanath and the Charlotte Maxeke Johannesburg Academic Hospitals in Johannesburg, South Africa. Twelve millilitres of blood was sampled from the patients at Day (D) 1, 3, 5, and 7 post-epigastric pain and assayed together with a once-off sample from six age and sex-matched healthy controls (HC). For cytokine analysis, multiplex assay kits were used. An RT^2 profiler array and real-time polymerase chain reaction, as well as multiparametric flow cytometry assays, were used to profile innate and adaptive immune response-related genes and leucocytes, respectively. Of the 40 patients, biliary and alcohol-induced pancreatitis were equally common (n=18, 45% each) and were the most prevalent aetiologies. The IL-6 cytokine increased with AP severity and over time, peaking on Day 7 with significant differences between Day 3 and 7 in MAP (p=0.001) and MSAP group (p=0.013). Genes that were highly expressed included chemokine (C-C motif) receptor 8 (*CCR8*) and myeloperoxidase (*MPO*). These two genes were identified as potential severity markers with a fold change of 1172.45 and 91.77, respectively. While IL-6 increases with severity are not novel, the link to *CCR8* and *MPO* gene expression was established. Further linkages were made with increasing frequency of classical monocytes ($CD14^+CD16^-$) and the depletion of $CD14^+HLADR^-$ monocyte subset to severity. In conclusion, differences in *CCR8 and MPO* gene expression and elevation of IL-6 and their linkage to different innate and adaptive immune cell frequencies suggest that these markers have a role in disease severity and should be explored further.

DEDICATION

To my mother and father.

Thank you for the gift of perseverance

PROJECT OUTPUTS

Presentations arising from this study

1. **Nalisa, M.**, Nweke, E.E., Smith M.D, Augustine, T., Metzger, R., Devar, J.W., Jones, J.O. Fru, P. *CCR8* expression may be linked to disease severity and elevated IL-6 secretion in acute pancreatitis. Bert Myburgh Research Forum, Department of Surgery, 25 November 2020, Johannesburg, South Africa. **First Prize Winner**.
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LIST OF ABBREVIATIONS AND SYMBOLS

AP	Acute Pancreatitis
APACHE II	Acute Physiology and Chronic Evaluation II
APC	Allophycocyanin
Apcs	Antigen Presenting Cells
ARDS	Acute Respiratory Distress Syndrome
ART	Antiretroviral Therapy
ATV/R	Atazanavir/Ritonavir
BD	Becton Dickinson
BISAP	Bedside Index of Severity in Acute Pancreatitis
BMI	Body Mass Index
BP	Bandpass
BUV	BD Horizon Brilliant™ Ultraviolet
BV	Brilliant Violet™
CBA	Cytometric Bead Array
CCL1	Chemokine ligand 1
CCL5	Chemokine ligand 5
CCR7	C-C Chemokine Receptor Type 7
CCR8	Chemokine (C-C motif) receptor 8
CD	Cluster Of Differentiation
Cdna	Complementary Deoxyribonucleic Acid
CHBAH	Chris Hani Baragwaneth Academic Hospital
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CRP	C - Reactive Protein
CT	Computed Tomography
CXCL-8	Chemokine (C-X-C Motif) Ligand-
Cy	Cyanine
DAMPs	Damage Associated Molecular Patterns
DBC	Determinant Based Classification
DRV/R	Darunavir/Ritonavir
EDTA	Ethylenediamine Tetraacetic Acid

EFV	Efavirenz
ERCP	Endoscopic Retrograde Cholangiopancreatography
FACS	Fluorescence-Activated Cell Sorting
FACSDiva	Fluorescence-Activated Cell Sorting Diva Software
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HAART	Highly Activated Antiretroviral Therapy
HAPS	Harmless Acute Pancreatitis Score
HMGB1	High-Mobility Group Box Protein 1
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen – Antigen D Related
HPB	Hepatopancreaticobiliary
HTG	Hypertriglyceridemia
ICU	Intensive Care Unit
IFN-γ	Interferon-Gamma
IL	Interleukin
IL-6R	IL-6 receptor
ILC	Innate Lymphoid Cells
IPC	Inter Plate Calibrator
LOS	Length of Hospital Stay
LPV/R	Lopinavir/Ritonavir
MAP	Mild Acute Pancreatitis
MAPK	mitogen-activated protein kinases
MCP-1	Monocyte Chemoattractant Protein 1
MFI	Median Fluorescence Intensity
MIP-1	Macrophage Inflammatory Protein
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
MSAP	Moderate Severe Acute Pancreatitis
MODS	Multiple Organ Dysfunction Syndrome

NF-κB	Nuclear Factor Kappa B
NOD1	Nucleotide-binding oligomerisation domain-containing protein 1
NRTIS	Nucleoside Reverse Transcriptase Inhibitors
NTC	No Template Control
OF	Organ Failure
PAMPS	Pathogen Associated Molecular Patterns
PBMCS	Peripheral Blood Mononuclear Cells
PE	Phycoerythrin
PE-CF594	Phycoerythrin Cyanine based dyes
PerCP	Peridinin-Chlorophyll-Protein
PIs	Protease Inhibitors
PMT	Photomultiplier Tubes
PPC	Positive Pcr Control
PRR	Pattern Recognition Receptors
QC	Quality Control
QRT PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RAC	Revised Atlanta Classification
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
R_S	Spearman's Coefficient Or Spearman's Correlation
RSD	Robust Standard Deviation
RVD	Retroviral Disease
SAHCS	The Southern African HIV Clinicians Society
SAP	Severe Acute Pancreatitis
SD_{EN}	Standard Deviation of The Electronic Noise
SPINK1	Serine Protease Inhibitor Kazal Type 1
SIRS	Systemic Inflammatory Response Syndrome
TNF-α	Tumour Necrosis Factor-Alpha
TLR1	Toll-like receptor 1

UK	United Kingdom
USA	United States of America
V:V	Volume Per Volume
WHO	World Health Organisation

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Acute pancreatitis (AP) is an inflammatory disease that leads to the autodigestion of the pancreas (Afghani, 2014). Depending on its severity, it initiates complex systemic immune responses that dysregulate immune cells and inflammatory molecules such as cytokines (Fonteh, Smith and Brand, 2018). Biochemical tests such as C-reactive protein are used for monitoring severity, but these are not always sensitive and are time-dependent. Existing classification systems for AP, such as the Revised Atlanta Classification (RAC) and the Determinant Based Classification (DBC) stratify patients for improved care and are not meant to predict severity (Talukdar and Vege, 2015). In the South African setting, distinguishing between the three risk categories of AP is done using the 2012 Revised Atlanta Classification guidelines. The risk categories are mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), and severe acute pancreatitis (SAP). These guidelines assist clinicians in diagnosing AP and distinguishing the higher risk categories, MSAP, and SAP using multiple assessments over 48 hours (about 2 days) after admission (Banks et al., 2013). Current regimens focus on assessing risk factors such as age, comorbidities, alcohol abuse, and obesity (Wu and Banks, 2013).

Treatments for AP are supportive and time-sensitive (Wu and Banks, 2013). Ideally, prognostic markers and accuracy of stratification of patients should be performed between 12 to 24 hours upon hospitalisation (Wu and Banks, 2013; Tenner et al., 2013). Interventions are highly dependent on results of biochemical tests and, depending on the discretion of the clinician's assessment of the patient, may include fluid resuscitation; pain management; nutritional support through parenteral nutrition or enteral feeding; and in the case of organ failure (OF), it may be necessary to transfer patients to an intensive care unit (Wu and Banks, 2013). However, organ support at admission remains the most critical form of intervention.

Consequently, the need arises for understanding the pathogenesis of systemic inflammation and the development of organ dysfunction as a tool for treatment strategies (Kylänpää, Rakonczay and O'Reilly, 2012). Therefore, identifying immune parameters for use as prognostic markers that may be more accurate in stratifying patients' risk profiles at the early phase of acute pancreatitis

is likely to improve treatment strategies. Predicting severity will assist in streamlining not only care but also better resource management. This study focused on assessing biomarkers that can predict severity in the early stages of the disease.

1.2 Literature Review

1.2.1 Disease incidence and aetiology of acute pancreatitis

The annual incidence of AP is estimated to be between 5 and 80 people per 100,000 worldwide (Nesvaderani et al. 2015). The United States, Finland, and Australia have the highest incidence rates on record worldwide, with approximately 73 cases per 100,000 (Nesvaderani et al., 2015). The AP incidence rates in high-income countries are well described, while those in low to middle-income and upper-middle-income countries, except India and China, are not well known (Talukdar and Vege, 2015; Roberts et al., 2017).

A 2012 AP study that investigated retrospective data in selected European countries and the USA found the incidence of AP to be at a 30-year high (Roberts et al., 2013). The study looked at 10,589 cases of AP between 1999 and 2010 (Roberts et al., 2013). The authors revealed an incidence rate increase of approximately 9%, which translated to an annual increase rate of 3% (Roberts et al., 2013). The reported yearly increase worldwide is between 2 to 4% (Pang et al. 2018), with mortalities in necrotising pancreatitis reported at up to 4.2% in high-income countries (Nesvaderani et al., 2015).

The two most common causes of AP are gallstones and alcohol (Pang et al., 2018). Other aetiologies include endoscopic retrograde cholangiopancreatography (ERCP), hypertriglyceridemia, nucleoside reverse transcriptase inhibitors (NRTIs), and idiopathic or unknown causes (Afghani, 2014). Less common causes of AP include trauma and scorpion bites (Afghani, 2014; Nesvaderani et al., 2015).

Risk factors associated with AP are highly dependent on the lifestyle of a given population (Nesvaderani et al., 2015). These risk factors include excessive alcohol use, obesity, and drug addiction, amongst others. Individuals with a body mass index (BMI) of more than 30 kg/m^2 are at a higher risk of AP (Yadav and Lowenfels, 2013). Demographic risk factors that impact the severity of AP include age, with patients older than 60 years in the high-risk category and genetic predisposition (Wu and Banks, 2013; Leal and Almeida, 2019).

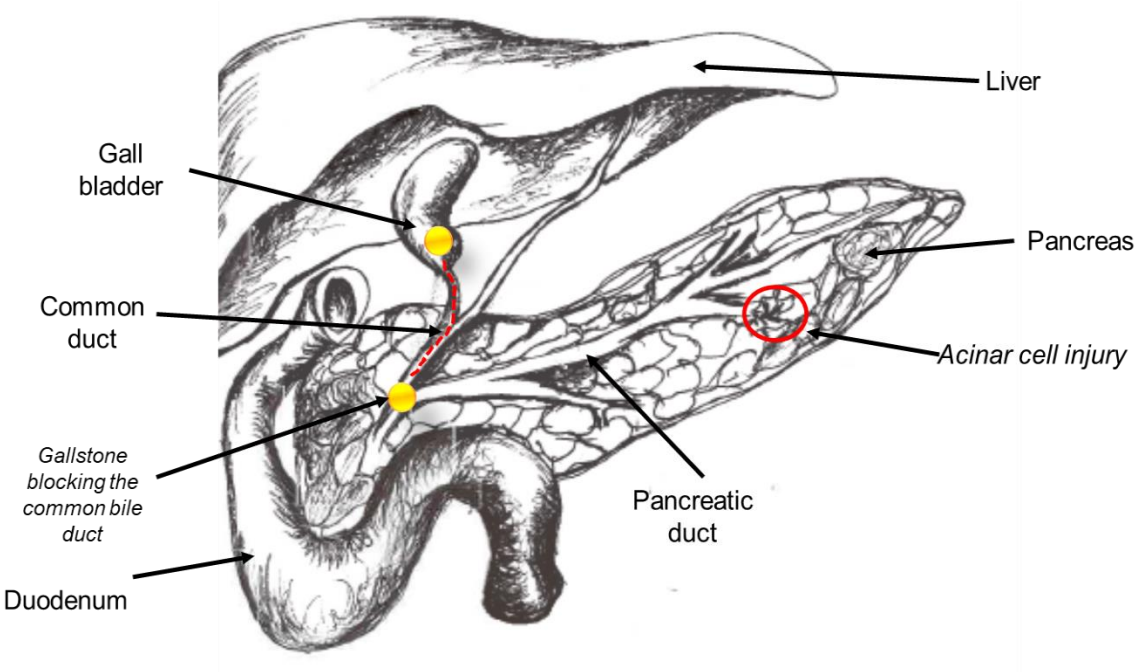
Within the South African population, statistics on AP are scarce and insufficient (Anderson et al., 2008). South African studies done in the 1990s show that the most common cause of AP was alcohol (John et al., 1997). This was common in 83% of the sampled population of 186 patients (John et al. 1997). In another AP study done between 2001 and 2006 at Addington Hospital in Durban, South Africa, alcohol too was the primary cause of AP (Anderson et al., 2008). The study included 282 patients, with 62% of AP cases being alcohol-related AP and 14% attributed to gallstone disease, and mortality recorded at 9% (Anderson et al. 2008). The study also revealed that AP was a common comorbidity in human immunodeficiency virus (HIV)-positive patients with an incidence of 5.7% (Anderson et al. 2008). Six of the 282 patients were on antiretroviral therapy (ART) specifically, didanosine and stavudine (Anderson et al. 2008). Less common causes of AP identified in these South African studies include dyslipidemia and acute idiopathic pancreatitis (Anderson et al., 2008).

Additionally, in a population with a high HIV rate, such as South Africa, it is essential to consider antiretrovirals (ARVs) as risk factors in diagnosing AP. A 2014 systematic review on the association of ARVs and AP showed that the annual incidence might be up to 40% in the HIV-positive population (Oliveira et al., 2014). Similar to the Anderson 2008 study in South Africa, this study confirmed that didanosine had the highest correlation to AP amongst all ARV drugs assessed (Anderson et al. 2008; Oliveira et al. 2014). Other ARVs used in South Africa, such as stavudine, zidovudine, and efavirenz, were implicated in more than 10 cases of AP. Antiretroviral therapy (ART) as an independent risk factor is not well elucidated and was secondary to hyperlipidemia and alcohol abuse in some cases (Meintjes et al., 2012; Oliveira et al. 2014).

Apart from South African studies, there has not been much research in sub-Saharan Africa on AP. Using the search words; "the incidence of AP in sub-Saharan Africa," the studies in Durban in 2008 and Johannesburg in 1997, described above, make up most results, either as stand-alone articles or as references. The exception is a 1997 study at Jos University Teaching Hospital in Jos, Nigeria, where the incidence of AP was 1.4% in a population of 13,000 patients (Ugwu, Obekpa, and Kidmas, 1997). The results from the latter study were from a total of 15 patients, with alcohol-related AP accounting for 10 of the cases, ART-related in two cases, and gallstone disease in one case (Ugwu, Obekpa, and Kidmas, 1997).

1.2.2 Diagnosis and severity classification in acute pancreatitis

Acute pancreatitis can occur as an isolated attack or recurrent episodes, with the potential risk ranging from a mild to a severe form (Banks et al., 2013). The latter may result in pancreatic necrosis (Littner et al., 2011; Banks et al., 2013) and or OF. The consensus document, known as the RAC is frequently used to diagnose AP. The onset of AP is defined as pain in the abdominal area that radiates to the back (Banks et al., 2013). There are two types of AP, interstitial oedematous pancreatitis and necrotising pancreatitis (Banks et al., 2013). Interstitial oedematous pancreatitis is the milder form occurring in most patients, and necrotising pancreatitis is the more severe form, and approximately 20% of AP patients fall within this group (Banks et al. 2013). Figure 1.1 shows a pictorial description of an inflamed pancreas caused by gallstones or biliary pancreatitis.



Drawing by Mwangala Nalisa

Figure 1.1 Diagram of a pancreas demonstrating bile duct obstruction, resulting in acinar cell injury causing acute pancreatitis.

Clinical assessment and diagnosis of AP consist of pain assessment (Wu and Banks, 2013). After that, a combination of biochemical tests such as serum amylase, lipase, and CRP, which is a commonly accepted indicator of severity, are conducted (Banks et al., 2013). Serum amylase and lipase levels equal to or greater than three times the upper limit of normal (Banks et al., 2013) are used for the diagnosis. The normal amylase levels are 23 to 85 U/L, and normal lipase levels range from 0 to 160U/L) (NHLS, 2021). In addition to these, a computerised tomography (CT) scan can be used for detection (Banks et al., 2013; Ferreira et al., 2015).

The severity of AP is classified as mild, moderate, or severe (Banks et al., 2013). MAP presents with no OF and no local complications, and MSAP presents with transient OF within 48 hours of admission (Dellinger et al. 2012; Banks et al. 2013; Campos et al., 2013). If OF is present for more than 48 hours after admission, the patient is diagnosed as SAP. Moderately severe AP is further defined by specified local complications exacerbating the comorbid disease. Local complications could come in the form of peripancreatic fluid collections, pancreatic and peripancreatic necrosis (sterile or infected), pseudocyst, and walled-off necrosis, which could either be sterile or infected (Banks et al. 2013). Table 1.1 below shows the different categories and criteria for the classification of AP.

Table 1.1. Revised Atlanta Classification of severity in acute pancreatitis (Banks et al. 2013; Campos et al. 2013).

Severity	**Patient Profile**	**Classification of severity**	
		pancreatic necrosis	**Organ Failure (OF)**
Mild acute pancreatitis	No local or systemic complications	No	No
Moderately severe acute pancreatitis	*Local or systemic complications without persistent OF	Yes, sterile	Yes, Transient in less than 48 hours
Severe acute pancreatitis	Single or multiple OF	Yes, infected	Yes, Persistent for more than 48 hours

**Local: acute (peri) pancreatic fluid collection, pancreatic pseudocyst, acute necrotic collection, pleural effusion*.
OF: organ failure

A patient with SAP will present with local complications and will likely develop systemic inflammatory response syndrome (SIRS). If SIRS persists, it increases the risk of mortality to an estimated 30% (Banks et al. 2013; Campos et al. 2013; Yadav, 2014; Cho et al. 2015). The SIRS is exacerbated by a complex cytokine cascade with dysregulated pro-inflammatory cytokines,

leading to a compromised immune system (Oiva et al., 2010; Kylänpää, Rakonczay and O'Reilly, 2012; Fonteh, Smith and Brand, 2018).

Apart from the RAC, the DBC is also widely used. The DBC guidelines advocate including an additional subgroup termed 'critical' (Dellinger et al., 2012). Therefore, in the DBC, the SAP group will have either infected pancreatic necrosis or persistent OF, and the critical group will have infected pancreatic necrosis and persistent OF (Dellinger et al., 2012). A systematic review of both classification systems found that the DBC was more accurate in predicting the need for clinical intervention than the RAC (Talukdar and Vege, 2015). At the same time, RAC was more accurate in predicting the length of hospital stay (Talukdar and Vege, 2015). The DBC emphasises that patients with both infected peripancreatic necrosis and persistent OF cannot be classified as severe as their mortality is higher than patients with either sterile or infected necrosis, hence classifying them as "critical" is more appropriate. Talukdar and Vege (2015) found that a patient with both peripancreatic necrosis and persistent OF had an increased mortality rate compared to a patient with either one of these complications.

In the first week of AP onset, the resultant inflammation of the pancreas may result in SIRS. If this condition persists, it may lead to OF, which is one of two critical determinants of severity of AP for both classifications (Banks et al., 2013; Dellinger et al., 2012). However, these classifications are meant to stratify patients for improved care and not predict severity at presentation (Talukdar and Vege, 2015). Therefore, there is a need to find methods to predict AP disease severity. A method that incorporates the analysis of key innate and adaptive immune responses is an option that could be used to complement the classification systems and, consequently, refine potential interventions that may inhibit the pro-inflammatory pathway and improve outcomes in AP disease management.

1.2.3 Current clinical management strategies in acute pancreatitis

Treatments for AP are mainly supportive and time-sensitive (Wu and Banks, 2013), with estimated annual costs reaching more than US$2 billion in the United States (Trikudanathan et al., 2019). Stratification of patients is crucial for patient survival and needs to be done accurately from the onset of disease. Wu and Banks found that patient management in the first 24 hours after admission is essential to mitigate OF's adverse effects (Wu and Banks, 2013). In South Africa, particularly at

Chris Hani Baragwanath Academic Hospital (CHBAH), most patients come to the hospital on Day 3 (72 hours) post onset of pain, as observed during the recruitment period in this study. This delay in the presentation may impact timely intervention that is highly dependent on biochemical tests, which require observation at admission, at 24 hours, 48 hours, 72 hours, and after seven (7) days if necessary (Banks et al., 2013). At these time points, the evaluation of these patients is highly dependent on the discretion and experience of the clinicians diagnosing the AP patients (Wu and Banks, 2013; Leal and Almeida, 2019).

The modified Marshall score is used to determine OF in three organ systems - the renal, cardiovascular, and respiratory systems (Banks et al., 2013). The sepsis-related organ failure assessment (SOFA) score is similar to the Marshall score and is commonly used in the DBC guideline (Banks et al., 2013; Dellinger et al., 2012). Moreover, if SAP is suspected at admission and prediction of risk of mortality is required, the Bedside Index of Severity in Acute Pancreatitis (BISAP) is used (Wu and Banks, 2013; Chandra et al. 2017; Leal and Almeida, 2019). The Harmless Acute Pancreatitis Score (HAPS) uses a "reverse" system compared to BISAP (Wu and Banks, 2013). HAPS can predict which patients are least likely to be at risk of developing SAP by evaluating blood tests including haematocrit, creatinine, and physically examining the patient for symptoms of guarding on the abdomen (Wu and Banks, 2013). If patients are admitted to the intensive care unit (ICU), the Acute Physiology and Chronic Health Evaluation II (APACHE II) scoring, designed to predict mortality, is used. This scoring system can be performed at any time during the stay in ICU. However, clinicians could see it as cumbersome for use due to the multiple variables involved (Leppäniemi et al., 2019).

1.2.4 Mechanisms for the pathophysiology of acute pancreatitis

It is generally accepted that the premature release or activation by trypsin of proenzymes (including trypsinogen) from the pancreas is the initial trigger of pancreatitis. What remains unknown is stratifying patients effectively into the severity risk categories early at disease onset (Leal and Almeida, 2019). Knowledge of mechanisms involved in disease pathogenesis is essential to predict patient outcomes.

Under normal circumstances, trypsin and other proteolytic enzymes will be inhibited from being activated by serine protease inhibitor, Kazal type 1 (SPINK1), secreted by acinar cells. Initiating events of AP pathology causes early activation of trypsin which blocks the transport of trypsin to

the small intestine (Do, 2015; Frandy and Wirawan, 2019). The activation of trypsin leads to premature activation of lipase and elastase, causing intracellular damage of cells and subsequently inflammation and thrombosis. Damaged acinar cells cannot regulate trypsin activity leading to further tissue damage through excessive amounts of activated enzymes within pancreatic tissue. Lipase, in particular, will cause necrosis to adipocytes within the pancreas leading to local recruitment of pro-inflammatory markers, including cytokines (Lerch and Gorelick, 2013; Frandy and Wirawan, 2019).

The damaged tissue at the injury site activates innate immunity, including neutrophil infiltration and macrophage recruitment. This delicate association between neutrophils and macrophages determines whether the damaged tissue will be healed or further inflamed to cause a dysregulation that determines the severity of the AP (Habtezion, 2015). If homeostasis is not achieved, neutrophils will produce IFN-γ that initiates the recruitment of pro-inflammatory macrophages (Habtezion, 2015). Continued stimulation of the innate immune system results in a systemic response that causes OF (Yang, Meng and Xu, 2015).

1.2.5 Innate and adaptive immunity

In AP, when the pancreatic tissue is damaged, monocytes and macrophages are responsible for maintaining inflammation (Machado and Coelho, 2012; Endo et al., 2014). These cells are part of innate immunity. Understanding the pathophysiology of AP requires understanding the immune system. Beyond structural and chemical barriers to pathogens, the immune system has two fundamental lines of defence: innate immunity and adaptive immunity (Marshall et al., 2018). Innate immunity is the first immunological mechanism for fighting against an intruding pathogen (Marshall et al., 2018). It is a rapid immune response initiated within minutes or hours after detection. It has no immunologic memory. The first responders of innate immunity are neutrophils and macrophages, responsible for phagocytosis (Nicholson, 2016). If the infection persists, T lymphocytes are recruited to the scene. These are unlike the innate immune cells as they have long-term memory and form part of the adaptive immunity (Nicholson, 2016).

Adaptive immunity is antigen-dependent and antigen-specific (Marshall et al., 2018). It has the capacity for memory, which enables the host to mount a more rapid and efficient immune response upon subsequent exposure to antigens (Marshall et al., 2018). There is a great deal of synergy between the adaptive immune system and its innate counterpart. Defects in either system could

provoke inappropriate inflammation, autoimmune diseases, immunodeficiency disorders, and hypersensitivity reactions.

The diverse responses are dependent on specific genes expressed by both innate and adaptive immune cells that can read an environment through different receptors. These include receptors on the innate immune system such as pattern recognition receptors (PRRs), Toll-like receptors (TLRs), killer activated receptors (KARs), and killer inhibitor receptors (KIRs) (Amarante-Mendes et al. 2018). The adaptive immune system includes complement receptors, Fc receptors, B cell receptors, and T cell receptors (Biassoni et al., 2001; Nicholson, 2016; Fonteh, Smith and Brand, 2018). These receptors assist cells in reading their microenvironment by switching genes on and off depending on the required stimuli (Nicholson, 2016). These genetic changes also determine which cytokines are released by the cells (Holdsworth and Gan, 2015).

1.2.5.1 Cytokines and the immune system

Cytokines are small protein molecules released by cells to act as immune system messengers (Zhang and An, 2007; Gulati et al., 2016). They work in both the innate and adaptive immune systems and are mainly produced by macrophages and helper T cells (Gulati et al., 2016). The mode of action of cytokines are autocrine (action on the cell that produces it), paracrine (action nearby the cell), or endocrine (action on a distant cell) (Zhang and An, 2007). Cytokines can produce various stimuli through receptors on the surface of target cells (Gulati et al., 2016). Once the cytokine binds to the complementary receptor, this signal initiates gene expression in the cell (Gulati et al., 2016). This expression may result in a cellular and humoral immune response, initiation of inflammation response, hematopoiesis, proliferation, and differentiation (Gulati et al., 2016).

The differentiation and activation of $CD4^+$ T cells into either Th1, Th2, Th17, Treg1, or Tfh cells, require a specific set of cytokines (Zhu, Yamane and Paul, 2010; Zhu and Zhu, 2020). The presence of IL-12 and IFN-γ determine Th1 differentiation (Liu et al., 2007). Th2 subsets require IL-4, IL-7, IL-2, and thymic stromal lymphopoietin (TSLP), and Th17 will differentiate in the presence of 1L-6, IL-21, IL-23 and is induced by TGF-β (Liu et al., 2007; Zhu and Zhu, 2020). The type of cytokine produced is also specific to each subset. Th1 cells mainly produce IFN-γ and protect the body from intracellular pathogens, particularly viruses and bacteria (Liu et al., 2007; Zhu and Zhu, 2020). Th2 cells produce IL-4, IL-5, IL-9, IL-13, and IL-25, which act on ectoparasites (Zhu,

Yamane and Paul, 2010; Raphael et al., 2015). The Th17 cell subsets use IL-17A, IL-17F, and IL-22, to control bacteria and fungi (Zhu, Yamane and Paul, 2010). T_{regs} that are $CD4^{+}CD25^{+}$ maintain homeostasis through the production of IL-10 and TGF-β and prevent autoimmune diseases (Zhu and Zhu, 2020). This differentiation process is specific, and one T helper cell subset may suppress the differentiation of another subset (Liu et al., 2007).

In AP pathophysiology, TNF-α and IL-1β, which are mediators of IL-6, have been identified as essential regulators of the pro-inflammatory phase of the disease (Sternby et al., 2021). IL-10 has been described as a critical anti-inflammatory molecule (Sharma et al., 2017; Sternby et al., 2021). Whereas IL-8, encoded by the chemokine (C-X-C motif) ligand 8 (*CXCL-8)* gene, drives a dysregulation of immune cells, which results in an overactivation of neutrophils which drives the "cytokine storm" and the ensuing systemic response that may result in OF (Sternby et al., 2021). Previous research on a South African cohort of patients demonstrated that AP follows a Th17 response because of the elevation of Th17A and IL-6 (Kay, Smith, and Brand, 2017). Sternby and colleagues (2021) add to this body of knowledge by describing IL-1β, IL-6, IL-8, and IL-10 as the most relevant predictive cytokines for early stratification of the three risk categories of patients and attributed disease severity to IL-1β and IL-6 (Sternby et al. 2021). Despite all the efforts to find early predictive markers, no study has confirmed that AP pathology is driven by a Th1, Th2, or Th17 response.

1.2.5.2 Th1, Th2 and Th17 Cells

The T-lymphocytes are divided into two major types, CD4 and CD8 T cells. The CD4 T cells are the primary producers of cytokines. The cells are subdivided into five types Th1, Th2, Th17, T regulatory (T_{regs}), and follicular T helper (Tfh) cells (Zhu and Zhu, 2020).

The CD4 or T helper (Th) cells are white blood cells produced in the thymus and regulate the body's immune response to pathogens (Zhu, Yamane, and Paul, 2010; Zhu and Zhu, 2020; Routy and Isnard, 2021). The primary function of CD4 cells is helping B cells produce antibodies, regulating the function of macrophages, generating CD8+ T cytotoxic and memory cells, and controlling autoimmunity (Swain, McKinstry and Strutt, 2012; Zhu and Zhu, 2020). When CD4 cells are depleted, the body becomes susceptible to pathogens, a good example of this is HIV infection, which depletes the cells giving room to opportunistic infections (Routy and Isnard, 2021).

The five major CD4 cells have diverse functions.

Th1 cells: These cells as responsible for Type 1 responses against intracellular pathogens through secretion of IL-12. When the naïve cells are converted to a Th1 cell, IFN-γ activates macrophages that phagocytose invading pathogens and initiate the production of antibodies by B-lymphocytes (Luckheeram et al., 2012; Swain, McKinstry and Strutt, 2012; Zhu and Zhu, 2020).

Th2 cells: Th2 cells are activated by IL-4 released from macrophages. Once activated, they release IL-4, IL-5, and IL-13, resulting in a Th2 response against extracellular parasites (Luckheeram et al., 2012; Swain, McKinstry and Strutt, 2012; Zhu and Zhu, 2020).

Th17 cells: IL-6 and Transforming Growth Factor Beta (TGF-β) convert naïve $CD4^+$ cells to Th17 cells. These cells target extracellular microbes such as fungi and bacteria, and if the cells are overproduced, it may cause a dysregulation that results in autoimmune disease (Luckheeram et al., 2012; Swain, McKinstry and Strutt, 2012; Zhu and Zhu, 2020).

T_{regs}: TGF-β and IL-2 convert naïve CD4+ cells into T_{regs} in the presence of a Foxp3 transcription factor. The cells produce IL-10 and TGF-β to counter the pro-inflammatory response and suppress the immune system, preventing autoimmunity (Zhu and Zhu, 2020).

Tfh: Follicular helper T cells: this differentiation is caused by IL-6 and IL-21 in the presence of the Bcl6 transcription factor. These cells assist B cells to produce antibodies (Zhu and Zhu, 2020).

A set of innate cells known as innate lymphoid cells (ILCs) exhibit similar responses as cells of Th1, Th2, and Th17 polarisation (Vivier et al., 2018). These lymphocyte cells reside in tissue and lack the CD3 antigen that is found on CD4 T and CD 8 cells (Vivier et al., 2018). They are divided into three groups, ILC1, ILC2, and ILC3 (Marshall et al., 2018; Vivier et al., 2018). The cytokines produced by these cells and Th1, Th2 and Th17 cells are highlighted in Figure 1.2.

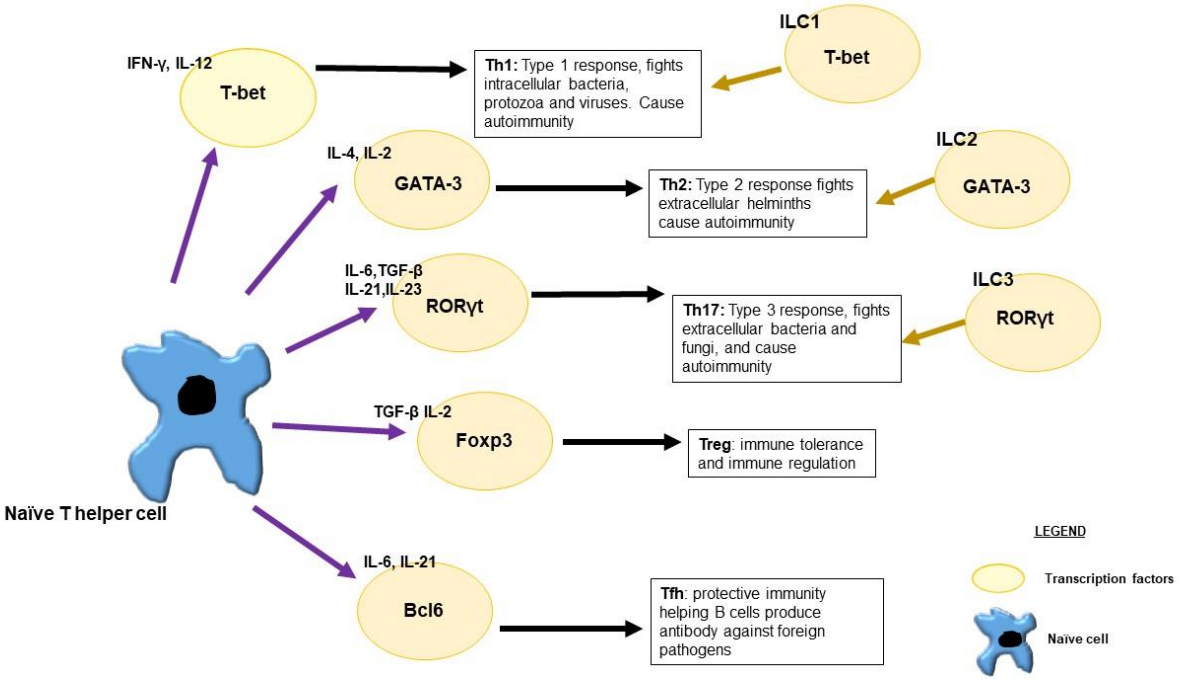


Figure 1.2 T-helper (Th)1, Th2, and Th17 differentiation of CD4 naïve cell and its innate lymphoid cell (CD3-) counterpart. Th1 cells express cytokine interferon (IFN)-γ and transcription factor T-bet, resulting in type 1 immune response to intracellular pathogens; Th2 cells expresses interleukin (IL)-4/IL-5/IL-13 and transcription factor GATA3, to induce a type 2 immune response towards extracellular pathogens, for example, worms; Th17 cells expresses IL-17/IL-22 and transcription factor RORγt, resulting in type 3 immune responses to extracellular bacteria and fungi. Tfh (follicular T helper) cells assist B cells to produce antibodies by expressing Bcl6 and producing IL-21. T regulatory (Treg) cells express Foxp3 to regulate responses that are responsible for homeostasis. The diagram also depicts a group of innate immune cells known as ILCs, which mimic T helper cells. They produce the same cytokines as Th1, Th2, and Th17 cells and are activated by the same transcription factor. These cells are ILC1, ILC2, and ILC3. *Adapted from Swain, McKinstry and Strutt, (2012) and Zhu and Zhu (2020).* **Notes:** Bcl6: B cell lymphoma 6; CD: cluster of differentiation; Foxp3: Forkhead box protein P3; GATA-3: GATA3 (GATA binding protein 3); IFN-γ: interferon-gamma; IL: interleukin; ILC: innate lymphoid cells; Th: T-helper; Tfh: follicular T helper; Treg: T regulatory cells; RORγt: Retinoic-acid receptor-related orphan nuclear receptor gamma t.

1.2.6 Innate and adaptive cells in acute pancreatitis pathophysiology

1.2.6.1 Macrophages and monocytes in acute pancreatitis

Macrophages are innate immune system cells involved in antigen presentation and phagocytosis and are significant producers of cytokines (Hu et al. 2020; Peng, Li and Yu, 2021). Macrophages reside in lymph nodes, in tissues of the liver, lungs, and spleen, as well as the pleural and peritoneal cavity (Machado and Coelho, 2012). Macrophages found in organs have two lineages. Some were once circulating monocytes that differentiated into macrophages in tissue, and others are derived

from early development stages from progenitor cells in the embryo, particularly in the yolk sac (Italiani and Boraschi, 2014; Hu et al. 2020).

Circulating monocytes will differentiate into macrophages in inflamed tissue (Coillard and Segura, 2019). These cells have a life span of approximately 20 hours and are divided into three classes, namely classical ($CD14^+CD16^-$), non-classical ($CD14^-CD16^+$), and intermediate ($CD14^+CD16^+$) (Yang et al., 2014; Peng, Li and Yu, 2021). The $CD14^+CD16^-$ and $CD14^+CD16^+$ monocyte subsets are acute inflammation (Coillard and Segura, 2019). These subsets are most likely to penetrate the tissue and differentiate into macrophages at the injury site in AP. When these monocytes enter the tissue, they will differentiate into two phenotypes of macrophages - either proinflammatory M1 macrophages or anti-inflammatory M2 macrophages (Orekhov et al., 2019; Shrivastava and Bhatia, 2010; Peng, Li and Yu, 2021).

The M1 macrophages induce what can be described as a Th1 response after stimulation by IFN-γ, lipopolysaccharide, and granulocyte/macrophage colony-stimulating factor (GM-CSF) and will produce reactive oxygen species (ROS), nitric oxide, various chemokines as well as TNF-α, IL-1β, and IL-6, which are pro-inflammatory cytokines (Machado and Coelho, 2012; Peng, Li and Yu, 2021; Hu et al. 2021). The M2 macrophages initiate responses similar to Th2 responses and can be further divided into M2a, b, c, and d subtypes (Hu et al., 2020). These cell types are induced in the presence of IL-4 and IL-13 (M2a); TLR, and IL-1 receptor agonists (M2b); TGF-β, IL-10, and glucocorticoid (M2c); M2d, are mainly found in tumours and are known as tumour-associated macrophages (Peng, Li and Yu, 2021).

These macrophage subsets produce diverse responses. M2a macrophages increase the expression of several chemokines, IL-10 and TGF-β (Hu et al. 2020). The M2b macrophages release the chemokine CCL1, which activates $T_{regs,}$ making them an essential cell subset in homeostasis. The M2c macrophages express CD86 and HLA-DR at very low levels (Hu et al. 2020). CD86 is involved in T cell activation and downregulation of HLA-DR on CD14 positive monocytes (Shay and Kang, 2013). Downregulation of HLA-DR on monocytes has been implicated in organ dysfunction in AP (Dabrowski et al., 2008).

Studies on AP animal disease models have shown that high levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 are directly linked to macrophage activation resulting from acinar cell injury due to trypsin (Lundberg et al., 2000; Machado and Coelho, 2012; Peng, Li and

Yu, 2021). A particular type of tissue-resident macrophage known as peritoneal macrophages is implicated in the initial production of TNF-α as a result of trypsin activation in pancreatic tissue and the peritoneal cavity (Lundberg et al., 2000; Machado and Coelho, 2012). An experimental mouse model of AP by Machado and Coelho (2012) demonstrated that peritoneal lavage leads to a decrease in TNF-α and IL-6 in serum and an increase in the anti-inflammatory cytokine, IL-10.

Other disease models that look at the role of peritoneal macrophages in pathological disorders have singled out CCR8 as the primary molecule inducing cytokine activity in this set of macrophages. CCR8 and its ligand, CCL1, recruit and activate macrophages in type 1 diabetes (Cantor and Haskins, 2007). CCR8, a chemokine receptor, is highly expressed on peritoneal macrophages, monocytes, and cells of Th2 lineage, including ILC2 and ILC3 (Cantor and Haskins, 2007; Oshio et al. 2014; Uhlen et al. 2019). Oshio and colleagues (2014) demonstrated that NF-κB is suppressed in CCR8 deficient mice and macrophage chemotaxis in the peritoneal cavity is CCL1 dependent.

1.2.6.2 Neutrophils in acute pancreatitis

Like monocytes and macrophages, Neutrophils originate from the common myeloid progenitor and account for 70% of white blood cells in circulation (Peng, Li and Yu, 2021). During pancreatic acinar cell injury, these cells are recruited by tissue-resident macrophages through the production of neutrophil chemoattractants, such as CXCL-1, CXCL-2, IL-1α, and monocyte chemoattractant protein-1 (MCP-1) (Prame Kumar, Nicholls and Wong, 2018). At this point, macrophages will extend the lifespan of neutrophils by producing GM-SCF, TNF-α, and granulocyte colony-stimulating factor (G-CSF), initiating inflammation (Prame Kumar, Nicholls and Wong, 2018). As part of their antimicrobial activity, neutrophils contain defensins, MPO, acid phosphatase, lysozymes, alkaline phosphatase, and lysozymes that act against pathogens (Peng, Li and Yu, 2019). The mode of action is through the formation of neutrophil extracellular traps (NETs) (Carestia, Kaufman and Schattner, 2016). These NETs eliminate invasive pathogens by producing netlike structures containing nuclear DNA and histones (Merza et al., 2015). Merza and colleagues demonstrated that the MPO concentration in plasma, a component of NETs, increased with disease severity (Merza et al., 2015).

Additionally, activated neutrophils within pancreatic tissue can affect the surrounding environment and produce molecules such as cathepsin G and azurocidin, which recruit monocytes and macrophages to the site of inflammation (Prame Kumar, Nicholls and Wong, 2018). Damaged

tissue will produce chemokines such as CXCL-1, CXCL-2, and CXCL-8, which recruit more neutrophils to the site of injury (Peng, Li and Yu, 2021). Monocytes, macrophages, and neutrophils essentially work together to maintain this inflammatory process (Prame Kumar, Nicholls and Wong, 2018). In AP mice models where neutrophils were depleted, there was a decrease in concentrations of monocytes (Machado and Coelho, 2012). Another mouse study showed that depletion of neutrophils in the periphery through an anti-rat neutrophil antibody treatment prevented acute lung injury in SAP (Yang, Meng and Yu, 2015).

1.2.6.3 Natural killer cells and acute pancreatitis

NK cells are cytotoxic lymphocytes that are part of innate immunity (Willinger, 2019). NK cells are divided into subgroups based on their expression of CD16 and CD56, i.e., $CD16^{-}CD56^{+}$ and $CD16^{+}CD56^{-}$ cells (Poli et al., 2009). In AP, Dabrowski and colleagues (2008) found excessive depletion of NK cells in peripheral blood in SAP patients compared to MAP and healthy controls, proposing a correlation between NK cells depletion and severity. Other studies have also demonstrated this link by showing that the depletion of NK cells is associated with severity in AP (Wang et al., 2017). These studies show promise in the use of NK cells as predictive markers of AP. Considering the relationships or possible linkages between these cell subsets and monocyte/macrophage and neutrophil elevation in the early stages of AP shows potential for predicting severity and was explored as part of this PhD project (Chapter 4).

1.2.6.4 Lymphocytes in acute pancreatitis: B and T cells

The T cells and B cells originate from the common lymphoid progenitor in the bone marrow (Zhu and Zhu, 2020). T cells, including $CD4^{+}$ and $CD8^{+}$ cells, develop in the thymus and are activated in the periphery of multiple tissues and organs (Ding et al., 2020; Zhou et al., 2020). B lymphocyte cells develop in the foetus's liver, mature in the bone marrow, and are primarily responsible for antibody production (Ding et al. 2020; Zhu and Zhu, 2020). CD4 T cells are essential in defending the body against infections by eliciting specific immune responses through differentiation into Th1, Th2, Th17, T_{regs}, or Tfh type cells (Zhu and Zhu, 2020). CD4 T cells will differentiate into effector T cells when naïve T cells receive a signal from APCs (Ding et al., 2020; Zhou et al., 2020). The APCs include dendritic cells, macrophages, Langerhans cells, and B cells. These cells display antigens on their surface and present them to naïve T cells and B cells, leading to their activation (Stagg and Knight, 2001). Once activated, these cells become effector cells of CD4

lineage. They, in turn, activate CD8 T cells, macrophages to kill infected cells and assist B cells in antibody production resulting in a humoral immune response (Zhu and Zhu, 2020).

In the pathophysiology of AP, cytokines produced by T cells and B cells contribute to dysregulation of the immune systems and compensatory anti-inflammatory response (CARS) and OF (Singh and Garg, 2016; Fonteh, Smith and Brand, 2018). CARS, a condition meant to counter the pro-inflammatory response, can result in an immunocompromised system if homeostasis is not reached (Singh and Garg, 2016). In AP patients with this condition, $CD14^{+}HLA\text{-}DR^{-}$ monocytes are upregulated, resulting in depletion of T and B cells, which undergo programmed cell death or apoptosis (Singh and Garg, 2016; Fonteh, Smith and Brand, 2018).

Furthermore, Fonteh and colleagues (2018) postulate that $CD4^{+}$ cells may contribute to the inflammatory process of AP and that Th1, Th2, and Th17 responses may result in different severities in AP. A review of several studies by Fonteh, Smith and Brand (2018) showed that IL-22 produced by Th17 cells offers protection against damage of acinar cells, whereas IL-17 may contribute to the damage of the pancreas by activation of inflammatory molecules. Moreover, findings from our laboratory show that IL-6 drives the development of AP by activation of Th17 cells, although studies in the late phase of SAP show that the immune imbalance in AP may be independent of the Th17 pathway (Kay, Smith and Brand, 2017; Thomson et al. 2019). From reviewed work on AP studies, there is no consensus on the role of $CD8^{+}$ cells in AP pathogenesis (Fonteh, Smith and Brand, 2018).

1.2.7 Predictive markers of severity in acute pancreatitis

Many of the current tools used in the clinical diagnosis of AP cannot effectively predict severity, as alluded to in the above sections. The challenge for AP is that its pathophysiology consists of multiple poorly defined theories (Singh and Garg, 2016; Frandy and Wirawan, 2019). Therefore, identifying a set of predictive markers using one routine test will provide an ideal, reliable, and more accurate stratification of patients into different risk categories. Several studies have identified possible markers and assessed their sensitivity and specificity in predicting AP disease outcomes, as depicted in Table 1.2.

Table 1.2 Various predictive markers for acute pancreatitis and their potential in the disease diagnosis

Marker	Time of assessment	Cut off value	Sensitivity (%)	Specificity (%)
C-reactive Protein	At admission, 48 hours, 72 hours	150 mg/dL	80–86	61–84
Procalcitonin (PCT)	At admission and if infection is suspected	0.5 ng/mL	73	87
growth differentiation factor-15	At admission	3290 pg/mL	90	86
pentraxin 3	At admission	1.05 pg/mL	85	88
Reduced lymphocyte count	At 48 hours onset of pain	0.66×10^9 /L	83.7	66.7
Neutrophil lymphocyte ratio (NLR)	At 48 hours onset of pain	NLR >8.5	n/a	n/a
IL-6	At admission and 72 hours	50 pg/mL	91.3 - 100	89.7- 96.8
IL-8	At admission	n/a*	n/a	n/a
IL-10	At admission	n/a	n/a	n/a
IL-17A	At admission	n/a	n/a	n/a
IL21	At admission	n/a	n/a	n/a
IL-23	At admission	n/a	n/a	n/a
IFN family	At admission	n/a	n/a	n/a
Nuclear factor	At admission	n/a	n/a	n/a
TNF- α	At 24 hours after admission	12 pg/mL	56	81
ICAM-1	At admission, 24hours	25 ng/mL	61.1	71.4
Amylase	At admission	1000 U/L	55–84	95
Lipase	At admission, can be detected up to 14 days onset of disease	n/a	80	60

Notes: Adapted from Meher et al. 2015; Shen et al., 2015; Deng et al. 2017; Li et al. 2017; Bhanou, Balachandran and Jain, 2018. n/a: not available; ICAM: intercellular Adhesion Molecule 1; IL: interleukin; IFN: interferon; TNF: Tumour necrosis factor.

In multiple studies on inflammatory molecules that can act as predictive markers in AP, CRP, nuclear factor kappa B (NF-κB) and interleukin (IL)-6 are the most common (Mayerle et al. 2012; Vasseur et al. 2014; Jakkampudi et al. 2016; Sharma et al. 2017). CRP is an acute-phase reactant produced by the liver and induced by IL-6 (Meher et al., 2015). This molecule is well described as an inflammatory marker for disease and is an effective predictive marker of severity at 48 hours after admission (Leal and Almeida, 2019). Some studies found that its strength as a predictive

marker is at 72 hours after admission (Leal and Almeida, 2019; Meher et al., 2015; Stirling et al., 2017).

NF-κB is involved in the excess production of calcium within acinar cells, which results in premature activation of trypsinogen (Lerch and Gorelick, 2013). This molecule is also responsible for inducing ROS, and its activation leads to the cytokine cascade that manifests as SIRS (Lerch and Gorelick, 2013; Jakkampudi et al., 2016; Sharma et al. 2017). NF-κB is a transcription factor involved in cell proliferation, cellular responses to free radicals (reactive oxygen species, ROS), and production of inflammatory cytokines, such as IL-2, IL-6, tumour necrosis factor (TNF)-α, IL-1β, and IL-8 (Lerch and Gorelick, 2013).

The cytokine TNF-α is an early mediator of inflammation and is secreted by acinar cells, followed by IL-6 and IL-8. Although TNF-α has been demonstrated as a marker to distinguish severity, in the first 24 hours, it is said to be inferior to IL-6 over time (Staubli, Oertli and Nebiker, 2015). IL-6, in particular, is a better predictor of MSAP and SAP than CRP and APACHE II (van den Berg et al., 2020). In vitro studies support IL-6 linkage to the severity of AP. Activation of STAT-3 in acinar cells can be suppressed by using IL-6 antibody therapy; this neutralises the production of IL-6 and consequently reduces severity (Manohar et al., 2017). Another critical inflammatory molecule in AP pathophysiology is IL-8 or CXCL-8. This molecule is a neutrophil chemoattractant and an early predictor of severity. A correlation between multiple organ dysfunction syndrome (MODS) in AP and IL-8 increase has been reported (Manohar et al., 2017; Staubli, Oertli and Nebiker, 2015). Epithelial cells, monocytes and macrophages predominantly produce IL-8. Although it performs better than TNF-α as an early marker, it is less superior to IL-6 as an early marker as it decreases over time (Manohar et al., 2017; Staubli, Oertli and Nebiker, 2015).

Multiple studies in AP continue to focus on understanding the immune system and the ensuing cytokine cascade associated with it (Oiva et al. 2010; Azab et al. 2011; Meher et al. 2015; Shen et al. 2015). Shen and colleagues demonstrated that multiple OF caused by infected pancreatic necrosis is linked to down-regulation of human leukocyte antigen (HLA-DR) and a decline in lymphocyte activation (Shen et al. 2015). Many of the scoring systems used to monitor patients require total WBC (NHLS, 2021). Azab and colleagues found that the ratio of neutrophils to lymphocytes can serve as a better indicator for severity than WBC, with higher frequencies of neutrophils indicating an increase in severity of AP (Azab et al., 2011). This theory is justified in

that neutrophil activation is responsible for the initial inflammatory response of the innate immune system through the production of TNF-α, IL-1, and IL-6 (Meher et al., 2015). Hence, the authors suggest that instead of using WBC in the scoring systems for AP, the neutrophil-lymphocyte ratio, of which values greater than 4.7 predicts SAP, should be used.

On the other hand, Shen and colleagues dispute the neutrophil-lymphocyte ratio as a predictor of severity and instead advocate using HLA-DR to monitor T-lymphocyte populations (Shen et al., 2015). This theory is supported by the work of Dabrowski and colleagues (2008), who found that HLA-DR downregulation on monocyte subsets was associated with OF in SAP patients (Dabrowski et al., 2008).

Considering that different cells and biomolecules are involved in AP initiation and progress, an integrated approach in understanding the innate and adaptive immune responses and their respective inflammatory mediators in the pathophysiology of AP is critical. Predicting disease severity and identifying predictive markers for novel targets in AP treatments is therefore essential. As explained in the following sections, this study attempts to unravel the immune perturbations involved in the various AP categories in an African population.

1.3 Study Rationale

The severity of the inflammatory response in AP determines which patients will suffer organ failure and thus severe acute pancreatitis (SAP). Existing grading systems are based on clinical assessment, and no clear markers of immune perturbations have been determined. A holistic approach to understanding innate and adaptive immune responses and their associated inflammatory mediators in the pathogenesis of AP is, therefore, an essential part of predicting severity. An example is the case of CD8+ cells, where there is currently no consensus on their role in the pathogenesis of AP (Fonteh, Smith and Brand 2018). This study sought to identify prognostic markers in the early phase of AP to identify at-risk patients and simultaneously characterise and quantify innate and adaptive immune cells. The findings were subsequently correlated to clinical outcomes to potentially identify immune cells, genes and or cytokines that could be used as biomarkers for early prediction of severity in AP. Its addition to the existing body of knowledge, findings from this study will contribute to better clinical management and improved patient outcomes, especially in the black South African population where data on AP is scarce, and HIV, which is a known comorbidity of AP, is high.

1.4 Objectives

The main objectives of the study were:

1.4.1. To determine if secreted cytokines exhibit a Th1, Th2, or Th17 polarisation.

1.4.2. To characterise phenotypic changes of innate and adaptive immune cells.

1.4.3. To correlate immune status data with clinical outcomes of patients.

1.5 Overall methodology

Figure 1.3 provides the overall methodology that was followed to answer the study objectives. Following informed consent, 12 mL of blood was sampled from the study participants. From this sample, whole, fresh blood was used for the immunophenotyping studies. Plasma samples were isolated and stored at -80 °C until needed for the cytokine analysis studies. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood and used in the analysis of innate and adaptive immune response using an RT2 profiler array assay, which screens for the effect of AP on 96 genes. Following the screening assay, selected genes were further validated using polymerase chain reaction arrays. A record of patient clinical parameters was also collected during sample collection or retrospectively to assist in interpreting the laboratory-based findings.

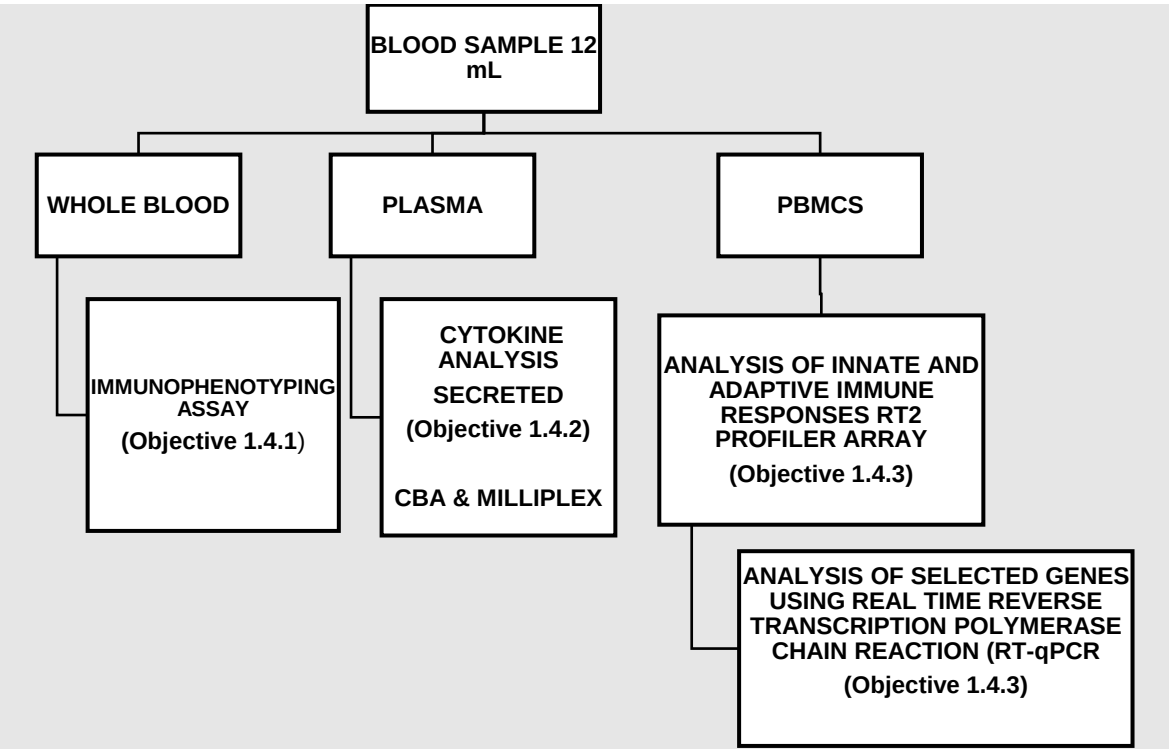


Figure 1.3 Flow diagram and summary of methods. **Notes:** CBA: cytometric bead array; PBMCs: peripheral blood mononuclear cells; RT-qPCR: Real-Time Reverse Transcription Polymerase Chain Reaction.

The chapters that follow focus on patient demographics and clinical outcomes (Chapter 2), an analysis of the various cytokine markers (cytokines – Chapter 3 and 4), and cytokines genes immune cells (Chapter 4). Findings are reported for both the control group and the different patient categories. The clinical outcomes reported in Chapter 2 are discussed in Chapter 2 and the lab-based work in Chapters 3 and 4. Chapter 5 consists of the study limitations, and future directions are discussed in the context of its translational potential.

CHAPTER 2

DEMOGRAPHIC DATA, PATIENT CHARACTERISTICS, AND CLINICAL OUTCOMES

2.1 Introduction

In 2017 acute pancreatitis was the most common gastrointestinal cause of hospital admission in the western world, with an estimated cost of over US$2 billion of taxpayer's money (Schepers et al. 2013; Ting et al. 2017) and numbers continue to increase. There is little data regarding the epidemiology of this disease within the South African setting, making it difficult to estimate its financial cost. Local estimates of the prevalence of AP (John et al. 1997; Anderson et al. 2008; Anderson and Thomson, 2017) reveal that the occurrence of AP is within that of international estimates of approximately 80 in 100,000 (Nesvaderani et al. 2015). The changing dynamic in deaths from communicable diseases in Africa compared to non-communicable diseases (NCDs) makes it imperative that life science research in Africa adapts to address this potential disease burden. Approximately 70% of deaths worldwide are due to NCDs (WHO, 2020). In South Africa, this statistic is at 51% (WHO, 2020), suggesting that NCDs such as diabetes, cancers, and heart diseases outnumber deaths from complications due to HIV/AIDS. This lifestyle-related change in trends is inevitably true for AP. Trends already show an increase in biliary pancreatitis worldwide, due to poor diets and obesity, with the leading causes being gallstone-related and alcohol-related AP (Roberts et al., 2013).

The current study focused on AP patients who identified as of African descent at Chris Hani Baragwanath Academic Hospital (CHBAH) and Charlotte Maxeke Johannesburg Academic hospital (CMAJH) referral hospitals Johannesburg, situated in South Africa's most populous province, Gauteng. This chapter reports patient demographics, including age, severity, aetiology, BMI, comorbidities, and primary outcomes. In this document, primary outcomes are referred to as clinical outcomes and include ICU admission, length of hospital stay (LOS), and in-hospital death. In the next section, some important risk factors of AP are explained first.

2.1.1 Excessive alcohol consumption and acute pancreatitis

South Africa's annual alcohol consumption is approximately 16.6L per drinker, which is the highest globally (van Walbeek, and Blecher, 2014). In South Africa, the impact of alcohol on human health includes dependence (substance abuse), liver cirrhosis, and hypertension (van Walbeek and Blecher, 2014). Data on alcohol patterns and AP incidence in South Africa is scanty. However, a 2017 report showed that alcohol-related AP was more frequent in HIV negative populations than in the HIV-positive population (Anderson and Thomson, 2017). In other regions of the world, trends of alcohol-related AP and its prevalence are well documented. A European study reviewed trends in the aetiology of AP from 1999 to 2010 and revealed that women under the age of 35 and men between 35 and 54 were more likely to have alcohol-related AP (Yadav and Lowenfels, 2006; Roberts et al. 2013). Disease incidence was further linked to social status and coincided with annual holidays such as Christmas, New Year, and in the case of Germany, Oktoberfest (Roberts et al. 2013).

2.1.2 Age as a risk factor of acute pancreatitis

Multiple studies have demonstrated a correlation between severity and age in AP (Yadav and Lowenfels, 2006; Roberts et al., 2013; Roberts et al., 2017; Koziel et al., 2018). In Europe, the incidence of AP in the population above 60 years is estimated to be between 85 to 150 per 100,000 (Yadav and Lowenfels, 2006; Roberts et al., 2017). The main aetiology in older patients was cholelithiasis with a high likelihood of MSAP or SAP, especially those above 80 years old (Koziel et al., 2018). The authors highlighted the difficulty in stratification and diagnosis in older patients due to comorbid disease (Koziel et al., 2018).

2.1.3 Obesity as a risk factor for acute pancreatitis

In the South African population, the number of overweight, obese, and severely obese people has increased since 1998 (Stats SA, 2016). Black African women in South Africa have the highest rate of obesity (Stats SA, 2016). One in five black South African women is likely to be overweight, obese, or severely obese ($BMI \geq 35 kg/m^2$) (Stats SA, 2016). Obesity is not a cause of AP, although it is linked to poor outcomes in AP patients (Solanki, Barreto and Saccone, 2012). This condition causes fat to infiltrate the pancreas and compound inflammation (Solanki, Barreto and Saccone, 2012; Li et al., 2019). Obesity in AP has been linked to renal failure, respiratory failure, and

prolonged LOS (Li et al., 2019). In South Africa, there are currently no statistics on obesity as a risk factor for AP.

2.1.4 Human immunodeficiency virus (HIV) and acute pancreatitis

The incidence of AP in the HIV population is 40% more than in the general population (Oliveira et al., 2014). The introduction of HAART (highly active antiretroviral therapy) in the mid-90s saw an upsurge of AP patients among HIV-positive individuals (Bush and Kosmiski, 2003; Dragovic, 2013; Oliveira et al., 2014; Bhurwal et al., 2018). This treatment introduced new conditions affecting the pancreas, including lactic acidosis and metabolic abnormalities (Oliveira et al., 2014). Hypertriglyceridemia, in particular, became common in HIV-positive patients after the introduction of HAART (Bush and Kosmiski, 2003). Change in body fat distribution caused by protease inhibitors (PIs), which are part of HAART, cause insulin resistance and HTG associated AP (Bush and Kosmiski, 2003). The Southern African HIV Clinicians Society (SAHCS) guidelines for antiretroviral therapy in adults for 2020 prescribes atazanavir/ritonavir (ATV/r), darunavir/ritonavir (DRV/r), efavirenz (EFV), and lopinavir/ritonavir (LPV/r) (Nel et al. 2020).

Additionally, the use of NRTIs, including didanosine and stavudine, have been implicated as common causes of AP in patients on HAART (Dragovic, 2013). Other authors cite Zidovudine and efavirenz (a non-nucleoside reverse transcriptase inhibitor) and protease inhibitors as major causes of AP in the HIV-positive population (Oliveira et al. 2014). These NRTIs, non-NRTIs, and PIs named here are part of prescribed HAART treatment in Southern Africa (Nel et al. 2020). A South African study carried out between 2001 and 2008 demonstrated that HIV-associated AP was prevalent in 17% of the sampled population of 627 AP patients (Anderson and Thomson, 2017).

2.1.5 Diabetes as a comorbidity of acute pancreatitis

Diabetes Mellitus is a common metabolic disease affecting approximately 422 million adults globally (WHO, 2016). Type II diabetes is a lifestyle disease that is preventable through exercise and avoiding a sedentary lifestyle (WHO, 2016). A systematic review by Li and colleagues showed that the risk of severity and poor outcomes in diabetic patients with AP was 30% more than those without diabetes (Li et al., 2019). Additionally, diabetes, secondary to AP, occurs in 15% of AP patients and will develop within a year of AP diagnosis (Richardson and Park, 2021). This type of diabetes has been identified as type 3c diabetes mellitus or T3cDM, or pancreatogenic diabetes (Richardson and Park, 2021). Although Li and colleagues reviewed the occurrence of diabetes in

AP patients, the authors do not clarify the type of diabetes which was common (Li et al., 2019). In another study, Richardson and Park attempt to associate T3cDM to AP even though this type of diabetes is more commonly associated with pancreatic cancer and chronic pancreatitis (Richardson and Park, 2021). Most South African studies do not report diabetes as a comorbidity in AP.

2.2 Objectives

The objective of this chapter was to assess the demographic characteristics of patients recruited and highlight vital clinical outcomes.

2.3 Materials and Methods

2.3.1 Site of study

Participants were recruited from the Hepatopancreaticobiliary (HPB) units of the CHBAH and the CMJAH in Johannesburg, Gauteng Province, South Africa. The laboratory analysis of the blood samples discussed in this Chapter and in Chapters 3 and 4 were conducted at the Department of Surgery laboratories, Faculty of Health Sciences at the University of Witwatersrand. Patients were recruited according to the revised Atlanta classification (Banks et al. 2013).

2.3.2 Sampling Strategy

Convenience sampling was used to recruit patients presenting with AP at CHBAH's HPB unit, which met the study's inclusion criteria described in section 2.3.2.1. This study was a pilot study, and hence, the sample size was not calculated. Generally, the unit noted 38 AP patients per annum in this study and previous studies (Thomson et al., 2018).

Healthy volunteers within the age range and gender of the sampled patient population were recruited. Patients and healthy control groups self-identified as of African descent. On average, patients presented at the hospital approximately 72 hours after onset of epigastric pain (Day 3 of pain). All patients included in the study were duly informed, and written consent was received before blood samples were taken. Recruiting clinicians were not part of the project; instead, they formed part of the respective hospitals' staff complement. Study participants were included or excluded according to the following criteria:

2.3.2.1 Patients were included if they met the following requirements:

a. Patients whose age was greater or equal to 18 years old and who self-identified as being of African descent.
b. Patients with AP diagnosed according to the Revised Atlanta guidelines shown in Table 1.1 (Chapter 1).
c. Patients who had started to experience AP related symptoms within 72 hours and up to 7 days before admission.

2.3.2.2 Patients were excluded for the following reasons:

a. Patients who had been diagnosed with a chronic medical condition associated with inflammation, including autoimmune diseases.
b. Patients who were undergoing recent (within one month of current admission) therapy with immunomodulatory medications.
c. Patients with comorbidities requiring the use of antibiotics within a month before admission.

2.4 Ethics approval and consent

Ethics approval was obtained from the medical Human Research Ethics Committee (HREC) of the University of the Witwatersrand, clearance certificate No M180133. Before the recruitment of subjects, a participant information sheet (Appendix A**)** detailing the research was provided to the patient and explained in a language they understood and in the presence of a registered health practitioner**.** Written informed consent was then obtained before blood sampling using the form in Appendix B. A healthy control group consisting of age and gender-matched volunteers from the Faculty of Health Sciences was sampled. Upon recruitment, volunteers, received an information sheet explaining the study (see control information sheet in Appendix C) and thereafter were required to sign consent forms (Appendix D after explaining what the study entailed Clinical outcomes data, including CRP, ICU admission and HIV status, were collected using a data sheet (Appendix E). This group consisted of individuals with no known history of pancreatitis, pancreatic-related diseases, or other diseases that could render them immunocompromised. Volunteers were staff and students from the department of surgery. The healthy control group was recruited after patients with AP had been recruited. These volunteers were then age and gender-matched to recruited AP patients. Blood sampling is described in detail in section 3.2.2.

2.5 Data Analysis

The analysis reported in this chapter is on the LOS, BMI, ICU admission, and age of patients. LOS and BMI were calculated in Microsoft Excel version 7 (Washington, USA). LOS was the difference between the date of admission and the date of discharge; ICU admission, denoted by a "yes" or "no," to affirm whether or not the patient was admitted to ICU. Interventions included any surgery performed on patients including prophylactic surgery such as ERCP. The study used the BMI classification according to the Strategy for preventing and controlling obesity in South Africa (DoH, 2016). Four BMI categories, namely; underweight (BMI <18.5 kg/m^2); normal weight (BMI = 18.5 to 24.9 kg/m^2); overweight (BMI = 25 to 29.9 kg/m^2); obese (BMI ≥30 kg/m^2), were considered. The BMI was calculated as weight divided by height squared.

Statistical analysis was performed in GraphPad Prism™ version 8 (California, USA). The data was non-parametric, and a Kruskal-Wallis test was used to determine differences in age and LOS between the MAP, MSAP, and SAP groups. Analysis of correlation of the age of the patients and gender; the age of patients and severity; the age of patients and aetiology were performed using Eta Coefficient test statistic, η. The data was interpreted as explained in Appendix G. The age of patients was the independent variable, and gender, severity, and aetiology were dependent variables. Eta coefficient was used for correlation between nominal and interval scale variables. Spearman's correlation was used to correlate BMI to the severity of patients as a risk factor of AP.

2.6 Results

2.5.1 Demographics, aetiology and risk factors in acute pancreatitis patients

Patients were recruited from August 2018 until September 2019. A total of 51 patients with AP were reported to the study. Of these patients, 40 were recruited for this study according to the prescribed inclusion criteria. The remaining 11 were excluded based on: refusal to consent (n=3), recruitment period was beyond the first week of disease onset (n=7), and one who did not identify with African descent. The sampling was done on day 1 to 7 post-onset of pain, and blood tubes were collected as long as the patients were in hospital and starting from the day they presented. Table 2.1 shows which blood samples were collected for each patient on respective days.

Table 2.1 Number of samples collected and respective days as well as gender, age, height, weight, and severity category

Patient Sample No	Gender	Age	Height (m)	Weight (kg)	Severity	Day 1	Day 3	Day 5	Day 7
CHAP001	F	32	1.72	115	MAP	✓			
CHAP002	F	52	1.57	100	MAP	✓	✓		
CHAP003	M	53	1.67	63	MAP		✓		
CMAP001	F	31	1.50	55	MAP	✓	✓	✓	✓
CHAP005	M	37	1.71	70.5	MAP	✓			
CHAP006	F	46	1.58	62	MAP	✓	✓	✓	✓
CMAP002	F	28	n/a	53	MAP	✓	✓		
CMAP003	F	61	n/a	n/a	MAP	✓	✓	✓	✓
CHAP011	F	33	1.69	155	MAP	✓			
CHAP013	F	34	n/a	81	MAP			✓	✓
CHAP024	M	44	1.78	72	MAP	✓	✓	✓	✓
CHAP026	M	54	1.65	53	MAP		✓	✓	✓
CHAP028	F	23	1.71	81	MAP		✓		
CHAP029	M	34	1.73	60	MAP		✓		
CHAP030	M	37	1.79	79	MAP			✓	✓
CHAP032	M	49	1.8	58	MAP		✓		
CHAP034	F	39	1.6	80	MAP			✓	✓
CHAP036	F	59	1.3	70	MAP			✓	✓
CHAP037	F	66	1.31	93	MAP			✓	✓
CHAP038	F	57	1.38	93	MAP		✓	✓	✓
CHAP022	M	23	1.70	48	MAP		✓	✓	
CHAP027	M	35	1.61	96	MAP			✓	
CHAP015	M	46	1.86	98	MSAP			✓	✓
CHAP004	F	n/a	1.70	50	MSAP	✓	✓		
CHAP007	F	40	n/a	50	MSAP	✓	✓	✓	✓
CHAP008	M	38	n/a	63	MSAP	✓	✓	✓	✓
CHAP010	M	36	1.56	57	MSAP	✓			
CHAP014	M	35	1.58	65.5	MSAP			✓	✓
CHAP016	M	38	1.69	74.5	MSAP	✓			
CHAP018	F	25	1.56	79	MSAP			✓	✓
CHAP019	F	76	1.40	89	MSAP			✓	✓

CHAP021	F	42	1.59	57	MSAP		✓	✓
CHAP025	M	35	1.74	35	MSAP	✓	✓	✓
CHAP033	M	29	1.43	61	MSAP	✓	✓	
CHAP035	M	26	1.65	84	MSAP	✓	✓	
CHAP012	F	40	n/a	58	SAP	✓		
CHAP017	F	58	n/a	n/a	SAP	✓		
CHAP020	M	69	n/a	81	SAP	✓	✓	✓
CHAP023	M	33	1.82	88	SAP	✓	✓	✓
CHAP031	F	56	1.53	97	SAP	✓	✓	✓

Notes: The table shows (✓) days on which blood samples were collected from patients. F: female; kg: kilograms; M: male; m: metres; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

From the sampled population, the severity risk distribution was 21 (52%) in the MAP group, 14 (35%) in the MSAP group, and 5 (13%) in the SAP group (refer to Figure 2.1). The gender distribution was 21 (52.5%) females and 19 males (47.5%). The median age was 38 years and age range of 26 to 76, as stipulated in Table 2.2.

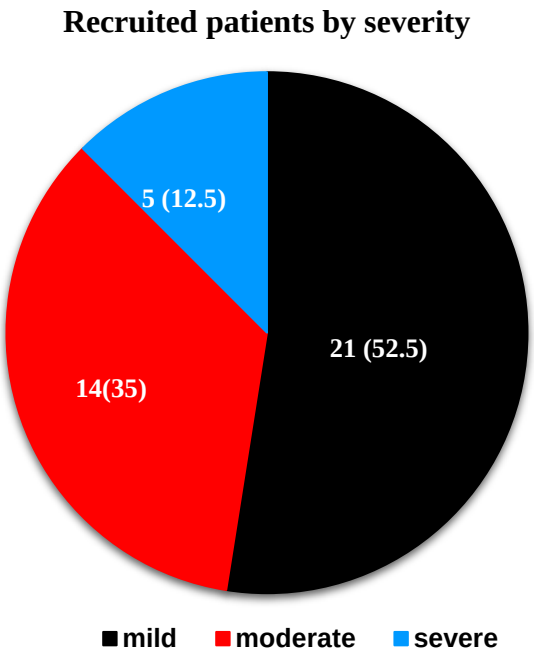


Figure 2.1 The percentage of patients in different risk categories of acute pancreatitis out of the 40 patients recruited for this study. **Notes:** *n* (%): number and percentage; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

Table 2.2 Patient characteristics and variables assessed

Parameter	Value (*n*=40)
Demographics	
Age in years [median (IQR)]	38 (26-76)
Male [*n*, (%)]	19 (47.5)
Female [*n*, (%)]	21 (52.5)
BMI kg/m^2 [median, (IQR)], all patients	26.4 (22.7-37.7)
MAP [median, (IQR)]	*24.8 (22.7-39.7)*
MSAP [median, (IQR)]	*26.2 (23.0-30.3)*
SAP [median, (IQR)]∞	*34.0 (30.3-37.7)*
Aetiology	
Biliary [*n*, (%)]	18 (45.0)
Alcohol [*n*, (%)]	18 (45.0)
Idiopathic [*n*, (%)]	1(2.5)
ARVs [*n*, (%)]	2(5.0)
ERCP [*n*, (%)]	1(2.5)
Severity	
Mild [*n*, (%)]	22 (55.0)
Moderate [*n*, (%)]	13 (32.5)
Severe [*n*, (%)]	5 (12.5)
Clinical outcomes∞	
Median length of Hospital Stay (LOS) MAP [median; IQR]	6 (2-24)
Median LOS MSAP [median; IQR]	11 (5-20)
Median LOS SAP [median; IQR]	31 (13-40)
Total Number of ICU admissions [*n*,%]	8 (20)
MAP group (n)	*1*
MSAP group (n)	*3*
SAP group (n)	*4*
Death in hospital [*n*, (%)], Total	4 (10)
MAP group (n)	*0*
MSAP group (n)	*1*
SAP group (n, %)	*3*
Surgical procedures*∞	
Total number of patients that had procedures	11 (28)
Total laparoscopic cholecystectomy[*n*,%],	7(18)
MAP group (n)	*6*
MSAP group (n)	*1*
SAP group (n)	*0*
Endoscopic Retrograde Cholangiopancreatography[*n*,%],	4(10)
MAP group (n)	*2*
MSAP group (n)	*1*
SAP group (n)	*1*
Comorbidities§ ∞	
Total number that had comorbidity [*n*, %]	14 (35)
Total number that had two or more comorbidities [*n*, %]	2 (5)
HIV positive [n, %]	8 (20)
Hypertension [n, %]	1(2.5)
Diabetes [n, %]	1 (2.5)
Non-diabetic hyperglycaemia [n, %]	2 (5)
Other [n, %]	1(2.5)

§Some patients may have more than one comorbidity; ∞Consists of missing data within the group *All patients had gallstone related acute pancreatitis. BMI: Body Mass Index; ICU: Intensive Care Unit; IQR: interquartile range; LOS: length of stay; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

The main aetiologies were alcohol-related AP and gallstone-related (cholecystolithiasis) with 18 patients (45%) each. Two patients (5%) had pathologies associated with ARVs, and one (2.5%) had idiopathic AP. Another had ERCP-induced AP (refer to Table 2.2 and Figure 2.2).

2.5.2 Characteristics of patients in different severity categories

Comparisons of aetiology across groups showed that in the MAP group, the primary cause was biliary related AP 55% (12 out of 22 patients) and 41% (9 out of 22) with alcohol-induced AP. In the MSAP group, 62% (8 out of 13) had biliary-related AP, and 31% (4 out of 13) patients had alcohol-induced AP. ARVs were listed in one patient as a risk factor. In the SAP group, two patients had biliary-related AP. Another two patients were speculated to have ARVs as a cause of AP, and one patient was listed as having idiopathic AP. The latter patient had acute renal failure and acute respiratory distress syndrome (ARDS) and was one of the in-hospital mortalities described in section 2.6.3, which detailed the clinical outcomes of the sampled population. The fifth patient had alcohol-induced AP. Post-ERCP pancreatitis was reported only in one case from a total of 40 patients (refer to Figure 2.2). The patient was classified in the MAP group.

2.5.2.1 Human Immunodeficiency Virus (HIV) positive patients on antiretroviral (ARVs)

For the 8 patients with HIV, 7 were on ARVs, of whom presented with 2 MAP, 3 MSAP, and 2 SAP. The patients' age range was between 40 to 54 years old, and the gender distribution was 57% (*n*=4) females and 43% (*n*=3) males. Five (5) of the patients were on the Efavirenz / Emtricitabine / Tenofovir (TDF/EFV/FTC), a combination of Non-NRTI (efavirenz) and NRTI (emtricitabine, tenofovir DF) (Nel et al. 2020). One patient was on a combination of zidovudine (AZT) plus lamivudine (3TC) and alluvia (AZT/3TC/alluvia). AZT and 3TC are both NRTIs and alluvia is a combination of the PIs, Lopinavir, and ritonavir. One patient was reported to be on Rifafour for the treatment of Tuberculosis.

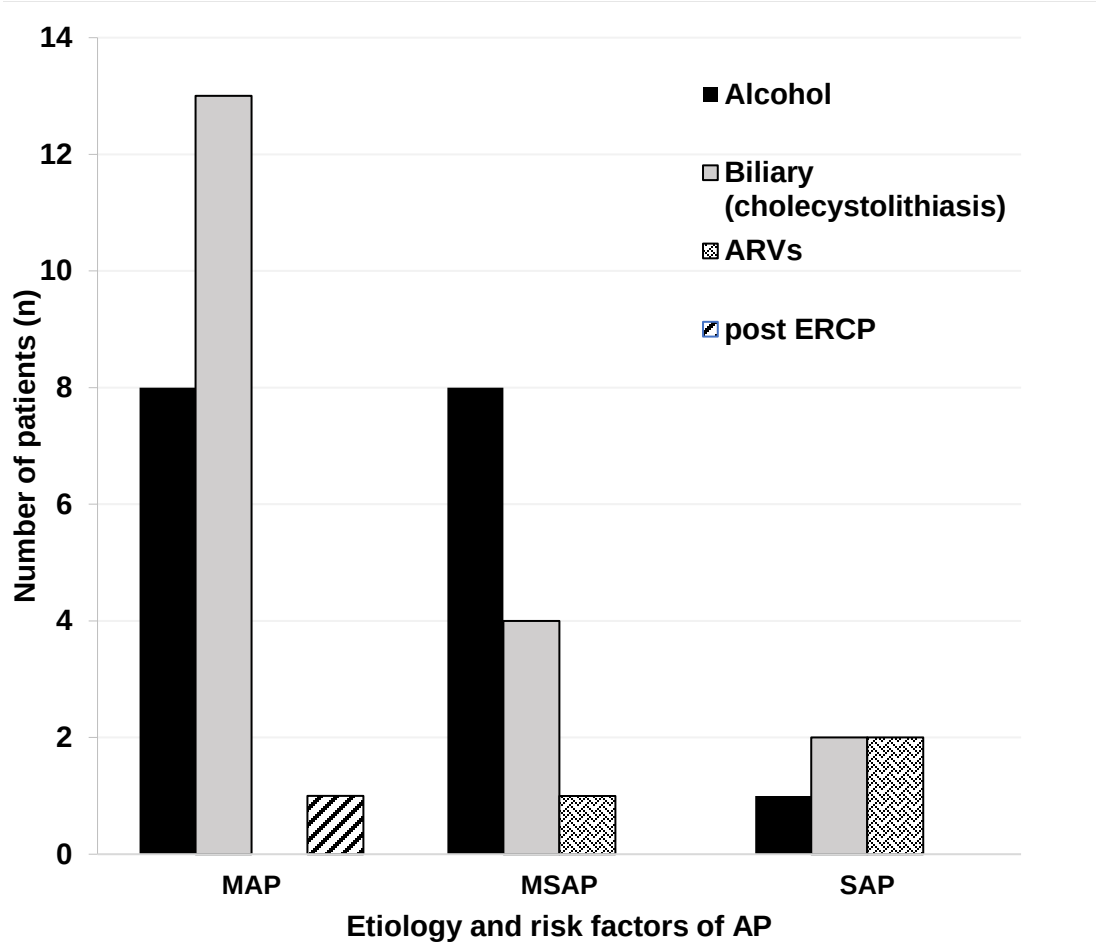


Figure 2.2 Aetiology and risk factors for AP in respective severities. **Notes**: AP: acute pancreatitis; ARVs: antiretroviral drugs; ERCP: Endoscopic retrograde cholangiopancreatography; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis

The median age for both MAP and MSAP groups was 38 years, and that of the SAP was 56 years (refer to Figure 2.3).

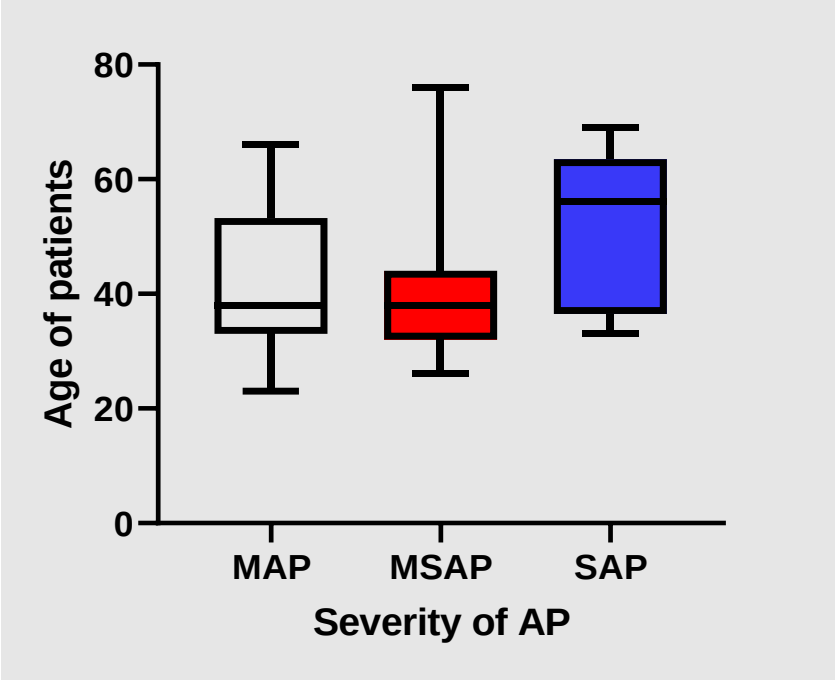


Figure 2.3 Age of AP patients by severity group. Data takes into consideration 18 out of 21 patients (86%) for the MAP group as data sets for three (3) patients were missing; for the MSAP group, 13 out of 14 (93%) were included. The SAP group had no missing data for age and severity. AP: acute pancreatitis; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis

2.5.2.2 Correlation of age, aetiology, and severity

The eta coefficient, denoted as η, is used when one variable is nominal and another is an interval scale—using age as an independent variable and aetiology as a dependent variable, $\eta = 0.916$. Hence, age was strongly associated with aetiology. The association of severity with age showed that $\eta = 0.878$. The findings established a strong association ($\eta = 0.878$) between severity and age in patients.

2.5.2.3 Trends of Body Mass Index and aetiology of acute pancreatitis

In the SAP group, the overall median BMI was 34.0 kg/m^2 (30.0-37.7), the highest being 54.3 kg/m^2. Data from 33 patients were collated. Seven of the total sampled population data on BMI was missing. Patients with biliary-related AP with BMI $\geq$ 30 made up 39% (13/33) of the patients (refer to Figure 2.4). Of these obese patients, 85% (11 out of 13) were female, and 15% (2 out of

13) were male. The male patients were more likely to have alcohol-related AP, with 89 % of the 18 alcohol-related patients identifying as male.

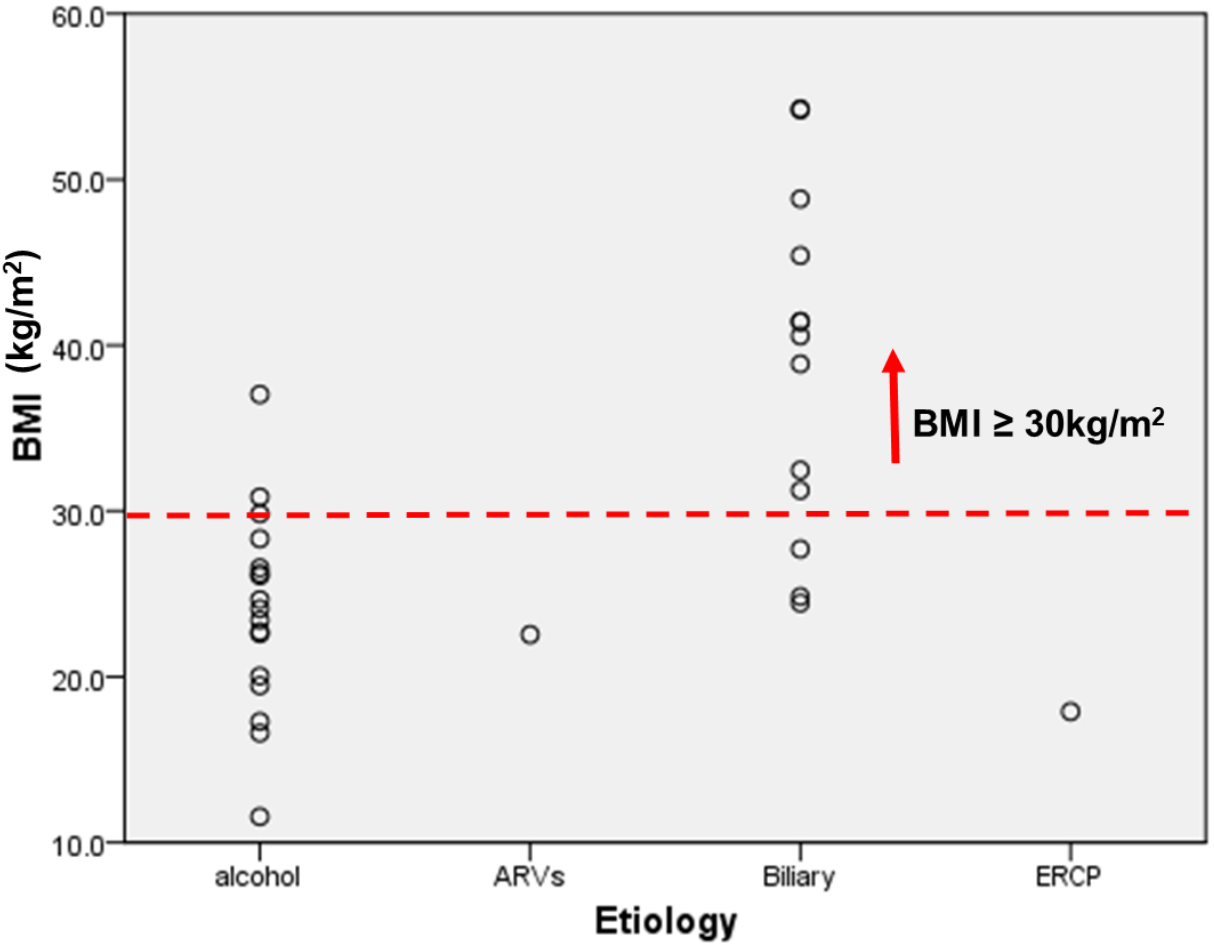


Figure 2.4 A scatter plot of body mass index (BMI) and aetiology of acute pancreatitis of individual patients. Most patients with biliary-related AP fell under this obese category (BMI ≥30kg/m^2). Notes: ARVs: antiretrovirals; BMI: body mass index; ERCP: endoscopic retrograde cholangiopancreatography.

2.5.3 Clinical outcomes, interventions, and comorbidities in the patient sample

In this study, the clinical outcomes included LOS, ICU admission (whether the patient was admitted to ICU or not), in-hospital death, and the need for intervention. Common comorbidities were HIV. The LOS of patients was assessed by severity group. The SAP group had a median LOS of 31 days ranging from 14 to 40 days. The MSAP group was 11 days with a range of 5 to 20 days, and the MAP group LOS median was six days (Figure 2.5 and Table 2.2). The LOS for

the SAP group was significantly different from the MAP group with a p=0.03, but not significantly different from the MSAP group (p=0.47).

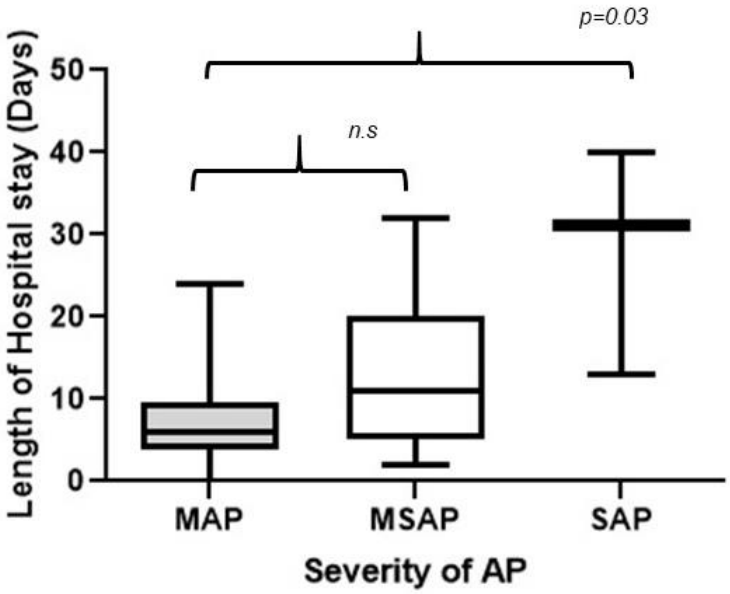


Figure 2.5 A box and whisker plot of LOS per severity group. Data in the above graph represents the medians and interquartile ranges of the three risk categories. AP: acute pancreatitis; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

Seven of the patients were admitted to the ICU (18 %, out of 39 patients, the ICU data for one patient was missing). Four patients died in the hospital (3 SAP and 1 MSAP, refer to Table 2.2). Only one had a comorbid disease, specifically type II diabetes, from the patients who died in the hospital.

The most common comorbidity was HIV, occurring in 20% (8/40) of patients. One patient in the SAP group had diabetes and was HIV positive. This patient subsequently died, having had multiple OF. Other patients who died did not have comorbidities but developed local complications.

2.6 Discussion

The major challenge for the global health sector today is NCDs. Even more so for low and middle-income countries, with a prediction of five times more deaths in these countries being attributed to NCDs by 2030 (Hofman, 2014). Like many Sub-Saharan African countries, South Africa's economy is burdened by the increase in the incidence of NCDs coupled with an existing burden of communicable diseases, including HIV/AIDS (Hofman, 2014). This burden places pressure on health care systems and the compromised health of the country's workforce, ultimately affecting the country's economic development (Hlafa, Sibanda and Hompashe, 2019). AP is an NCD that is not well understood in African populations. Statistics on the prevalence of AP are insufficient for decision-making. Understanding the prevalence of disease in specific populations could assist government planning and management guidelines in health care systems.

Currently, the international incidence rate of AP is 80 per 100,000 (Anderson and Thomson, 2017). In this study, the recruitment rate of AP patients was at least three patients per month between August 2018 and September 2019. This figure considers the final sample of 40 patients. During the recruitment period, risk factors and aetiology in the sampled population were recorded. An important risk factor for AP severity is the patient's age (Roberts et al., 2017). Studies focusing on older patients in Switzerland (Koziel et al. 2018) and Poland (He et al. 2020) found that patients 60 years and older are likely to have more adverse outcomes. In this study, the median age for both the MAP and MSAP groups was 38 and 56 years old, respectively. In these European countries, people above the age of 60 make up approximately 30% of the population (Poland, 32%) and Switzerland, 31%), whilst in South Africa, this population makes up only 9% of the total population (Index Mundi, 2020; Stats SA, 2019). Koziel and colleagues concluded that patients above 65 years of age were more prone to have SAP and experience more adverse outcomes than MAP and MSAP groups (Koziel et al., 2019). In the sampled population in this study, only 13% (n=5) had SAP; of these, 60% died (n=3), and these patients were on average significantly older (p=0.03) than those in the MAP group.

The results showed that the age of sampled patients had a strong correlation with aetiology. Older patients were more likely to have biliary AP. Of the five (5) SAP patients in the sampled population, only one (1) had alcohol-related AP. A systematic review of studies in 17 European countries by Roberts et al. (2017) reports trends towards a smaller ratio between alcohol and biliary-related AP among younger patients (less than 60 years old). This observation is in line with

the results of this study. A 1:1 ratio of alcohol-related pancreatitis and biliary-related pancreatitis (*n*=18, for both aetiologies) was reported. The levels of alcohol consumption in the cohort of patients for the current study were not included as a variable, and it may be beneficial to include them in future studies of AP.

Research evidence and clinical practice dictate that elderly AP patients (above 60 years of age), especially those with preexisting conditions, should be placed in ICU at admission (He et al., 2020). Studies on AP prevalence are required to make clinical management decisions with financial implications such as ICU admissions. In the case of the sampled population, 20 % (*n*=8) were admitted to ICU. Considering that South Africa has a younger population, it is recommended that factors such as preexisting conditions and patient history be used to make this decision.

Another demographic variable directly correlated to poor prognosis for AP patients is obesity (Khatua, El-Kurdi, and Singh, 2017). Khatua and colleagues report an increased trend of biliary AP patients falling into the obese category, i.e. patients with BMI $\geq$ 30 kg/m^2 made up 39 % (*13*/33) for those with available datasets (Khatua, El-Kurdi, and Singh, 2017). Within the SAP group, the median BMI was 34 kg/m^2, suggesting that patients were mainly falling in the category of obese. In the MAP and MSAP group, the median BMI was within the overweight category (24.8 kg/m^2 for MAP and 26.2 kg/m^2 for MSAP).

Obesity in AP has several effects. It may lead to the formation of stones in the gall bladder, a causal agent of AP, and act as a risk factor in AP severity (Khatua, El-Kurdi, and Singh, 2017). The results showed that obesity was found in 33% of patients (13 out of 40 patients). Obesity is a risk factor for AP and can lead to more severe outcomes in patients with diabetes (Khatua, El-Kurdi, and Singh, 2017). Obesity may subsequently lead to type II diabetes (DOH, 2016; Pang et al., 2018). These results show possible linkages of obesity and severity in this South African cohort of patients. Studies from Germany, the UK, and the USA, showed that obesity as a risk factor and diabetes as a comorbidity of AP were common in these populations (Afghani, 2014; Weitz et al., 2016; Shah, Mourad and Bramhall, 2018). Longitudinal studies of AP in the South African population are recommended to establish trends of obesity as a risk factor and diabetes as a comorbidity in AP patients.

The most common comorbidity in this cohort was HIV. These findings are not surprising given that there were 7.2 million HIV seropositive individuals in South Africa in 2019, the largest HIV

population in the world (Satoh and Boyer, 2019). Approximately 20% (*n*=8) of the sampled population from this study was HIV positive and on ARVs. These results align with previous findings, highlighting ARVs and alcohol abuse as risk factors for AP in the South African population (John et al., 1997; Anderson and Thomson, 2017). Complications in AP may be compounded by using ARVs (Oliveira et al., 2014; Anderson and Thomson, 2017). After HIV, type II diabetes and non-diabetic hyperglycaemia were the most common comorbidities. From the sampled patients, it was not possible to ascertain whether the use of ARVs preceded the onset of AP. It was only in two cases where the clinician speculated that the cause of AP might be related to the use of ARVs. However, the patient's specific ARV treatment is amongst those listed in studies that highlight HAART as a risk factor for AP (Oliveira et al., 2014; Anderson and Thomson, 2017). The treatment regimen for the majority of the patients included NRTIs and PIs. These medications are part of those prescribed per the SAHCS guidelines for antiretroviral therapy in adults (Nel et al., 2020). It is proposed that further research be carried out to evaluate the relationship between the current prescription of ARVs and the prevalence of AP.

Patients in the MAP group, as expected, did not develop local complications or OF as defined by the RAC (Banks et al., 2013). However, surgical interventions were common in this group. Six laparoscopic cholecystectomies out of seven were performed in this group. The laparoscopic cholecystectomy was also performed on patients with cholecystolithiasis. laparoscopic cholecystectomy is a common medical intervention to prevent disease recurrence (Jee et al., 2018). ERCP is a standard procedure for managing and diagnosing digestive system diseases (van Geenen et al., 2009; Nesvaderani, Eslick, and Cox, 2015). Post ERCP AP is the most common disease or complication associated with this procedure (Thaker, Mosko and Berzin, 2015). Post-ERCP pancreatitis was reported in 2.5% (*n*=1) of the recruited patients. This statistic is similar to the reported international statistics of ERCP-related AP, ranging between 2 to 10 % (Thaker, Mosko and Berzin, 2015).

The LOS of AP patients has long been established as an outcome that correlates with severity (Bollen, 2016). The average length of stay in the USA for AP patients is estimated between 5 to 8 days for all severity risk groups (Wu and Banks, 2013; Singh et al., 2017). Patients in the SAP group had the most prolonged LOS, and understandably so, due to the clinical management required for this group of patients (Bollen, 2016). The discrepancy of LOS between the USA and South African studies may be due to physician to patient ratios, which is 2.5 to 10,000 for South

Africa and 25 in 10,000 for the USA (Bateman, 2018). SAP patients require specialist care which may be limited in the South African setting. The SAP group also had the highest number of patients admitted to ICU. Within the MAP, the main factors that led to extended hospital stay were persistent AP symptoms with a potential for the patient requiring a laparoscopic cholecystectomy (Singh et al., 2017). In our cohort, patients in the MAP group had LOS ranging from 2 to 24 days. The most prolonged stay was a patient with non-diabetic hyperglycaemia and gout. Patients who underwent a laparoscopic cholecystectomy had a varied LOS of 5 to 11 days, which on average, is comparable to the American studies described above (Singh et al., 2017).

Besides LOS, another clinical outcome assessed was in-hospital mortality, which is directly linked to a more severe form of the disease (Banks et al., 2013; Afghani, 2014). The results show a 10% mortality (4 patients out of 40), which is higher compared to other South African studies, which report a 5.7%, albeit within the international rate of 10% (Yadav and Lowenfels, 2006; Anderson and Thomson, 2017). Both CHBAH and CMJAH are referral hospitals, so it may be that these deaths could also be attributed to delay in seeking specialist care. The demographics of this cohort of patients were distinct from other studies on AP as this is the first study that observed early immune responses in AP in a black African population with HIV as common comorbidity.

In conclusion, from the 40 patients sampled over the study period, it was found that biliary and alcohol-induced pancreatitis were equally common aetiologies. Biliary-related AP patients were obese as compared to alcohol-induced AP patients. AP severity increased with age, and the SAP group stayed longer in the hospital. Given the high HIV prevalence in South Africa, it was not surprising that the most common comorbidity was HIV (n=8) from a total of 12 patients with comorbidities. Overall, the findings align with international trends; however, specifics to our patient cohort such as HIV prevalence and treatment and a younger population should be considered during patient management. One of the limitations of this study is the overall number of recruited patients, which was relatively small, although trends observed provide additional knowledge that can be further validated with a longitudinal study. The following chapters will focus on assessing cytokines and innate and adaptive immune system genes that may be potential early immune markers in AP.

CHAPTER 3

SELECTED CYTOKINES AS POTENTIAL PROGNOSTIC MARKERS OF ACUTE PANCREATITIS

Introduction

Acute pancreatitis (AP) is an inflammatory disease that presents with epigastric pain, amylase, and lipase concentrations, three times the upper limit of normal (Banks et al., 2013). This condition is localised to the pancreas and is triggered by the premature release of digestive enzymes resulting from damaged pancreatic acinar cells (Leung and Ip, 2006; Zheng et al., 2013). Currently, no treatment can reverse the severity of patients upon presentation (Deng et al., 2017). Hence, the focus has been on early stratification and intervention to improve patient outcomes (Wu and Banks, 2013). The initial local injury of pancreatic acinar cells results in damage that triggers the production of cytokines and chemokines, causing inflammation (Szatmary, Gukovsky and Angeles, 2016; Fonteh, Smith, and Brand, 2018). This process is typical in severe cases and causes a systemic response known as a "cytokine storm," which is a dysregulation of the immune system (Kylänpää, Rakonczay, and O'Reilly, 2012; Fonteh, Smith, and Brand, 2018).

Cytokines are inflammatory molecules that recruit leucocytes to the injury site during AP progression (Nieminen et al., 2014). Organ failure distinguishes the two most severe forms of AP, namely MSAP and SAP from mild AP (Banks et al. 2013), and is directly correlated with proinflammatory cytokines such as TNF-α, IL-1β, IL-2, IL-6, and IL-10 (Fonteh, Smith and Brand, 2018; Sternby et al. 2021). Current research has focused on understanding this dysregulation (Oiva et al. 2010; Azab et al. 2011; Shen et al. 2015; Thomson, Brand, and Fonteh, 2018).

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The inflammatory process is marked by the migration of WBCs to the site of injury, known as an acute inflammatory response (Gulati et al., 2016). This response activates macrophages in tissue to produce TNF-α, IL-1, and IL-6 (Zhang and An, 2007; Fajgenbaum and June, 2020). These three cytokines induce coagulation and vascular permeability by activating cells of the endothelium and increasing the number of adhesion molecules such as ICAM-1 (Gulati et al., 2016). These adhesion molecules then bind to monocytes and lymphocytes, which further recruit neutrophils, lymphocytes, and monocytes in the bloodstream through the release of chemokines (Gulati et al., 2016). Macrophages also release IL-8 through induction by TNF-α and IL-1, facilitating the pro-inflammatory environment (Duque and Descoteaux, 2014; Gulati et al., 2016).

South African studies in AP, particularly those undertaken by our laboratory in the Department of Surgery at Wits University, have identified several other cytokines that play a role in the severity of AP. Kay, Smith and Brand (2017) found IL-17A and IL-10 at higher concentrations in MAP patients at Day 3 of admission, and IL-6 was significantly elevated from Day 1 to 5 post-admission. The authors hypothesised that a Th17 response was responsible for inflammation in AP. Subsequent studies found that IL-17A was not involved in disease progression in the late phase of SAP more than seven days onset of pain; IL-21 was elevated in SAP patients and may be the cause of immune perturbations observed, although transient (Thomson, Brand and Fonteh, 2018; Thomson et al., 2019). These studies attribute inflammation in AP to cytokines associated with the T helper lineage of cells. While similar cytokines were explored in the current study, the focus was on the early phase and Th17 related cytokines (IL-17A, IL-21, IL-6, IFN-γ, IL-23, IL-28λ, and TNF-β) that have not been explored extensively for AP. The sampling times were also based on the onset of epigastric pain and not on admission in the current study. The focus on Th17 cytokines was also based on two theoretical mechanisms associated with AP severity. Firstly, Th17 cells act as clearing agents for pathogens, and secondly that they incite tissue inflammation in autoimmune diseases (Korn et al., 2009).

This chapter thus focuses on cytokines that have been reported to have a role in the severity of AP. It seeks to demonstrate links between clinical admission data and specific cytokine expression to propose alternative parameters that may be helpful to clinicians in predicting severity in AP. The aim is to explore the link between the expression of plasma cytokines and the severity of AP patients to determine prognostic markers for effective and timely stratification. Given that the data here focuses on these biomarkers as proteins at the secreted/circulating level, the findings are also

subsequently discussed with respect to the intracellular markers at the gene level from the PCR studies and the associated cell types from immunophenotyping studies in Chapter 4.

3.1 Objectives

The objectives of this chapter were:

i. To determine if secreted cytokines exhibit a Th1, Th2, or Th17 polarisation.
ii. To correlate admission data to cytokine expression in patient samples

3.2 Materials and methods

3.2.1 Patient recruitment and sample collection

Patients were recruited and samples collected as described in section 2.3.

3.2.2 Blood sampling

Twelve millilitres (12 ml) of blood were drawn into three 4 mL blood tubes. Two of the blood tubes were purple top BD vacutainer® ethylenediaminetetraacetic acid (EDTA) tubes (BD Biosciences, New Jersey, USA) at four-time intervals, namely Day 1 (once clinically diagnosed); Day 3; Day 5, and Day 7 of onset of pain. The third 4 ml of blood was collected in one BD vacutainer® red top tubes with clot activator (BD Biosciences, New Jersey, USA) for separating serum from cells. The serum was collected for other ongoing studies of the department. All blood samples were processed within 6 hours of phlebotomy for use in immunophenotyping studies or for storage (plasma, serum and PBMCs) for subsequent use.

Clinicians within the HPB unit of the respective hospitals diagnosed and stratified the patient. The modified Marshall score was used to assess OF, and this information was recorded. The information on stratification was recorded from the patient file according to the clinician's notes into the datasheet in Appendix E. Other clinical data recorded from patient hospital files relevant for this chapter included CRP, creatinine, platelets, WBC levels at admission, and patients' outcomes, including LOS and ICU admission (refer to Appendix E).

Figure 3.1 is a flow diagram showing the overall sampling criteria and associated assays that were performed to answer the objectives associated with this chapter (listed in 3.2 above).

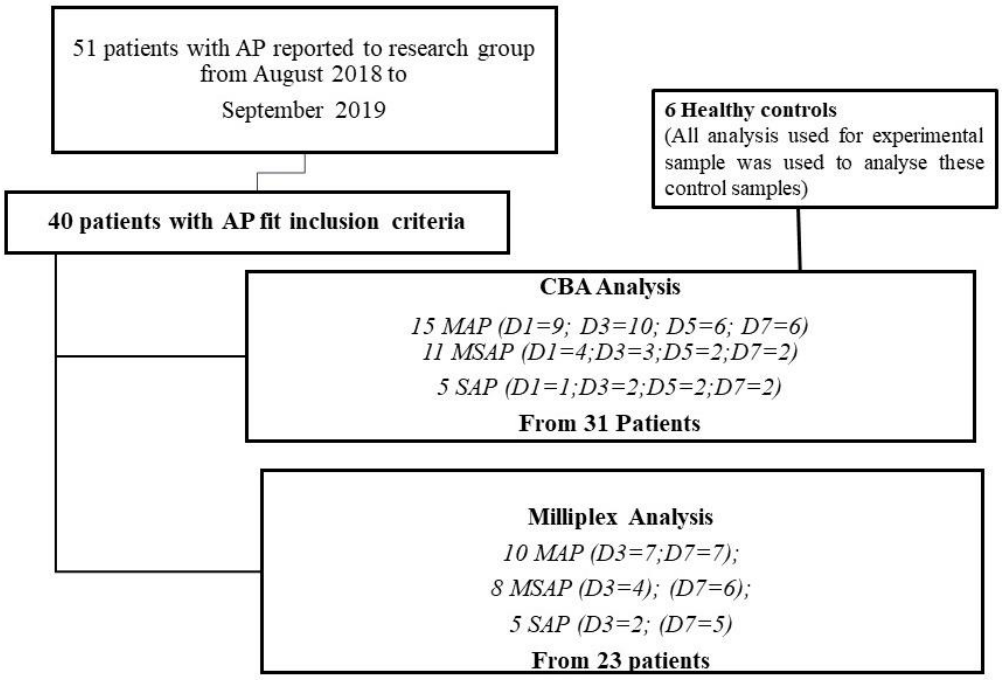


Figure 3.1 A flow diagram showing the overall sampling criteria and associated CBA and MILLIPLEX® assays. **Notes:** D: Day of the specific severity group; n: number; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.2.3 Sample processing

Samples were processed in the laboratory within 4 hours of phlebotomy. Plasma was isolated from whole blood samples by gravity separation for 45 minutes at room temperature, then centrifuged at 300 x *g* for 30 minutes. Plasma samples were aliquoted (200 µL in 1.5 mL microtubes) and stored at -80 °C until use.

3.2.4 Cytokine analysis

Two different methods were used for the analysis of cytokines within the plasma samples. The first was a BD BioSciences cytometric bead array Th1/Th2/Th17 kit that served as an exploratory step to determine the concentration of interleukin (IL-2), IL-4, IL-6, IL-10, tumour necrosis factor (TNF), IFN-γ, and IL-17A cytokines. The assay was done on 31 AP patients (15 MAP, 11 MSAP, and 5 SAP) and six (6) healthy control donor samples on days 1, 3, 5, and 7 post epigastric pain (Figure 3.1). The second analysis was done using a MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit (Catalogue No. HTH17MAG-14K-07, Millipore™, Massachusetts, USA). In the MILLIPLEX® assay, preselected cytokines, based on the performance of the CBA analysis and based on literature and previous work from the research group, were used (Kay, Brand and Smith, 2017). These cytokines were; IL-17A, IL-21, and IL-6, IFN-γ, IL-23, IL-28λ, and TNF-β measured from 23 AP patient samples (10 MAP, 8 MSAP, and 5 SAP) from the pool of 31 patient samples tested in the CBA assay on days 3 and 7 post epigastric pain. The six (6) healthy controls were also included. Other Th17 cytokines were selected according to their prescribed roles in the literature and their role in autoimmune diseases and AP (Zambrano-Zaragoza et al., 2014; Deng et al., 2017; Thomson et al., 2019).

3.2.4.1 Th1/Th2/Th17 Cytometric Bead Array Kit

A total of 31 patient samples were recruited at the time of analysis. Samples from Day 1, 3, 5, and 7 were analysed. At the time of the study, there were 15 MAP, 11 MSAP, and 5 SAP patients.

Preparation of serial dilutions: Human Th1/Th2/Th17 cytokine standards were reconstituted and serially diluted before mixing with the Capture Beads and the PE Detection Reagent. Ten (10), Falcon® 15-mL tubes (Corning Inc, New York, USA) were then labeled as follows "Top Standard"; 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 and negative control. The standards were transferred to the tubes, reconstituted with 2 mL of assay diluent, and left at room temperature for 15 minutes. The standard was mixed by pipetting, then 300 µL of assay diluent was added to each labelled tube. Serial dilutions were then prepared by taking 300 µL from the Top Standard and placing it in the 1:2 tube and after that following labelled tubes up to the 1:256 tube and mixed by pipetting each tube with a new tip. One tube was prepared, which contained the Assay Diluent only and served as the negative control (0 pg/mL).

Mixing Human Th1/Th2/Th17 Cytokine Capture Beads: At the start of the experiment, seven capture beads provided by the manufacturer were pooled into an empty container provided in the kit. The capture beads were vortexed for 3 to 5 seconds. The total number of assay tubes for the experiment was fifty unknowns (AP plasma samples), nine cytokine dilutions, and one negative control, 60 tubes. A 10-µL aliquot of each capture bead was added for each assay tube to be analyzed. All the assay tubes were then arranged into another tube labelled "Mixed Capture Beads and Vortexed". For example, 5,14 µL of IL-6 Capture Beads × 60 assay tubes = 308.4 µL of IL-6 capture beads required. The mixed beads were centrifuged at 200 x *g* for 5 minutes.

The supernatant was discarded, and beads were resuspended in Serum Enhancement Buffer. The capture beads were incubated for 30 minutes, away from light, and transferred to assay tubes.

Performing the Human Th1/Th2/Th17 Cytokine Assay: the preparation of the standards was performed according to the manufacturer's recommendation. A standard curve was used to determine concentrations of the various cytokines in the patient samples. The standards, respective dilutions, and concentrations are listed in Table 3.1.

Table 3.1 Table showing standard dilution factors and the final concentration of capture beads in respective dilutions

Tube number	Cytokine standard dilution	Concentration (pg/mL)
1	no standard dilution (Assay Diluent only)	0
2	1:256	20
3	1:128	40
4	1:64	80
5	1:32	156
6	1:16	312.5
7	1:8	62.5
8	1:4	1250
9	1:2	2500
10	Top standard	5000

Notes: pg/mL: picogram per millilitre.

Round bottom Falcon® 5 mL polystyrene tube (Corning Inc, New York, USA) were labelled with the sample numbers. The mixed capture beads from (ii) above were vortexed, and 50 µL of the mix was added to all assay tubes. This was followed by 50 µL of the Human Th1/Th2/Th17 Cytokine Standard dilutions to the control tubes (Table 3.1). A 50µL volume of each of the 31 plasma samples was then added to the corresponding labelled tubes.

After that, 50 µL of the Human Th1/Th2/Th17 Phycoerythrin (PE) Detection Reagent was added to all assay tubes. Wash buffer was used to clean each assay by centrifugation at 200 x *g* for 5 minutes. The samples were then resuspended in a 300 µL wash buffer solution.

The samples were then incubated in the dark for 3 hours. The performance and set-up for the flow cytometer were performed at this stage. The samples were acquired on an LSR Fortessa II (BD™ Biosciences, New Jersey, USA). FCAP Array version 2.0 Software (BD™ Biosciences, New Jersey, USA) was used as per the manufacturer's instructions to analyse and extract the cytokine concentrations.

3.3.5.2 Using the MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit

Using the MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit (Millipore™, Massachusetts, USA), plasma levels of seven preselected Th17-related cytokines, namely; IL-17A, IL-21, and IL-6, IFN-γ, IL-23, IL-28λ, and TNF-β were measured. Cytokine analysis was performed in duplicate from 23 patient samples, of whom 10 were MAP, 8 MSAP and 5 SAP. Six healthy controls were also included (Figure 3.1).

Preparation of reagents for MILLIPLEX® Assay: antibody-immobilized Beads were prepared on the same day as the experiment. The beads were not premixed, and an Ultrasonic cleaner (Eumax Corp., Tamil Nadu, India) was used to mix beads for 30 seconds each. The beads were vortexed for 1 minute. From each of the seven cytokines or analytes (IL-17A, IL-21, and IL-6, IFN-γ, IL-23, IL-28λ, and TNF-β) 60 µL was mixed with 2.58 mL of bead diluent in a mixing bottle provided in the kit. The manufacturer recommends that this bead mixture be 3.0 mL, so in this project, for 60 µL of seven cytokines, the total is 420 µL. Microcentrifuge tubes were labelled as Quality Control 1 and Quality Control 2 (QC1 and QC2) for the quality controls.

QC1 and QC2 were reconstituted with 250 µL deionized water and vortexed to ensure mixing. QC 1 and QC 2 were placed on the benchtop for 10minutes. The manufacturer provided wash buffer as a 10x dilution and was diluted to a 1x dilution with deionized water (60mL of wash buffer added to 540mL of deionized water). The serum matrix was dissolved in 500 µL of deionized water for 10 minutes. The human Th17 standard was reconstituted in 250 µL deionized water, vortexed for 10 seconds, and allowed to dissolve for 10 minutes. Six (6) microcentrifuge tubes were labelled as standards 6, 5, 4, 3, 2, and 1. A volume of 160 µL of assay buffer was added to each of the six tubes. A dilution of 1:5 was made by adding 40 µL of Standard 7 into Standard 6 until all tubes

were diluted (Standards 7 down to 1). An additional tube was labelled standard 0 and had negative control with assay buffer only. All reagents were left at room temperature for 30minutes before use.

Performing MILLIPLEX® assay on plasma samples: prepared plasma samples were allowed to thaw at room temperature. Two hundred microlitre assay buffer was added to each 96-well-plate provided in the MILLIPLEX® kit. The plate was then sealed and washed with a buffer for 10 minutes at medium speed on an Eppendorf® plate shaker (Sigma-Aldrich, Missouri, USA). The assay buffer was then decanted, and the plate was tapped face down on paper towels to remove the excess buffer. Then 25 µL of the Standards and controls were added to the plate.

A volume of 25 µL serum plasma matrix background was added before adding 25 µL neat patient plasma samples to respective wells. The same volume of mixed beads was added to each well using a multiplex pipette.

All samples were added in duplicates. The plate was sealed and covered with foil and placed on a plate shaker overnight for 18 hours at 4ºC. Then the sample was washed twice using 200 µL of wash buffer. Detection antibodies were warmed to room temperature, and 25 µL was pipetted into each well. The plate was incubated for 1 hour at room temperature.

After incubation, 25 µL of Streptavidin-Phycoerythrin was added to each well after it was poured into a reservoir. The plate was then incubated on a shaker for 30 minutes at room temperature. Then, the plate was washed twice, and the samples were resuspended using 150 µL of sheath fluid. The samples were placed on a plate shaker for 5 minutes. The 96-well plate was run on a BioPlex® 2200 system (BioRAD, California, USA). Data was collected and analysed using BioPlex® Manager 5.0 software (BioRad, California, USA).

3.3 Data analysis

Data censoring was performed on cytokine data for both CBA and MILLIPLEX analysis. In the case of CBA, most of the data set was designated as being out of range (OOR). For these data sets, the concentration (pg/mL) was designated as zero. Mean value imputation was used if the missing values were less than 50% of a data set for a particular cytokine (Augustine, Duarte and Candy, 2020). Values that were below the limit of detection were considered undetectable. The differences in expression of cytokines in the CBA analysis, MFI readings were used to compare different severity groups. The MILLIPLEX® analysis was then used to assess IL-6 and other cytokines selected from literature and those previously studied in our laboratory.

The cytokine data were analysed using GraphPad Prism™ version 8 (California, USA). A Shapiro-Wilk test was used to test for normality. Once the data was determined to be non-parametric, the Kruskal-Wallis test was performed to test for significant differences ($p < 0.05$) between the healthy control groups and between the MAP, MSAP, and SAP groups. A Dunn's multiple comparison test was used to perform a post hoc analysis.

For analyzing correlation, SPSS version 21 (IBM, Illinois, USA) was used. Spearman's correlation was used to assess the correlation between clinical test results at admission and selected outcomes. The degree of association between selected clinical tests at admission (CRP, WBC, creatinine, and platelets) and patient outcomes (LOS and ICU admission) was analysed.

3.4 Results

3.4.1 Patient demographics

The demographic parameters of the sampled population are described in Table 2.2 in Chapter 2.

3.4.2 Clinical admission data and patient outcomes

CRP, creatinine, WBC, and platelet count at admission were collected from patient hospital files and analysed. The correlation coefficient and respective *p* values are listed in Table 3.2.

Table 3.2 Correlation of selected clinical outcomes

Parameter	CRP (mg/mL)			WBC (x10^9/L)			Creatinine (µmol/L)			Platelets (x10^9/L)		
Severity	r_s =0.234	*n*=28	*p*=0.231	r_s =0.649**	*n*=35	*p*=0.649	r_s =-0.228	*n*=31	*p*= 0.217	r_s =-0.493	*n*=26	*p*=0.010*
Admission to ICU	r_s =0.242	*n*=20	*p*=0.155	r_s =0.288	*n*=26	*p*= 0.426	r_s = 0.362	*n*=19	*p*=0.235	r_s =0.362	*n*=19	*p*=0.714
LOS	r_s =0.928**	*n*=13	*p*=0.781	r_s =0.974**	*n*=21	*p*=0.156	r_s =0.653**	*n*=17	*p*=0.004**	r_s =0.397	*n*=13	*p*=0.127

The list of correlation coefficients using Spearman's coefficient is interpreted as explained in Appendix G. Where rs = 1 means a perfect positive correlation and rs = -1 a perfect negative correlation).

Notes: *n*= total number of sampled patients included from the total 40 patients (indicates missing data for example, if n=26 then 14 patients had missing data); **strong positive correlation, strong negative correlation. * significantly different as p≤0.05*;* CRP: C-reactive protein; ICU: intensive care unit; LOS: length of stay; WBC: white blood cells.

3.4.3 Cytokine analysis

An exploratory CBA assay was performed on 31 (15 MAP, 11 MSAP, and 5 SAP) patient samples from Day 1, 3, 5, and 7 post epigastric pain. The CBA was followed by a MILLIPLEX® analysis of 23 (10 MAP, 8 MSAP, and 5 SAP) Day 3 and 7 samples, selected from the pool of 31 samples used in the CBA studies. The choice of days used for the MILLIPLEX assay was based on the results from CBA analysis, which showed no significant changes on Day 5 and due to insufficient samples from the Day 1 group who were patients admitted at least 24 hours post-onset of pain. For the 31 patients included in this analysis, the etiologies and risk factors of AP were 43% (10 out of 31 patients) gallstone-related and 48% (11 out of 31 patients) alcohol-related, with one patient (4%) having had ERCP induced AP and another with ARV as a risk factor.

3.4.3.1 Cytometric Bead Array (CBA) assay

Data were expressed in Mean Fluorescent Intensity (MFI) in the exploratory CBA assay. In the analysis of the data, only the MFI of IL-6 revealed differences between patient plasma samples at Day 3 (Figure 3.2). On Day 1, the MAP group had a high expression of IL-6 at above 5,000 MFI, which was significantly different from healthy controls (p= 0.0149). A significant difference was reported on Day 3 in the MAP with p=0.0043 compared to the healthy control. In the MSAP group, there was a significant difference at Day 3 (p=0.035) and 7 (p=0.030). IL-6, MFI levels were in the region of 5000 for the SAP patient (Figure 3.2).

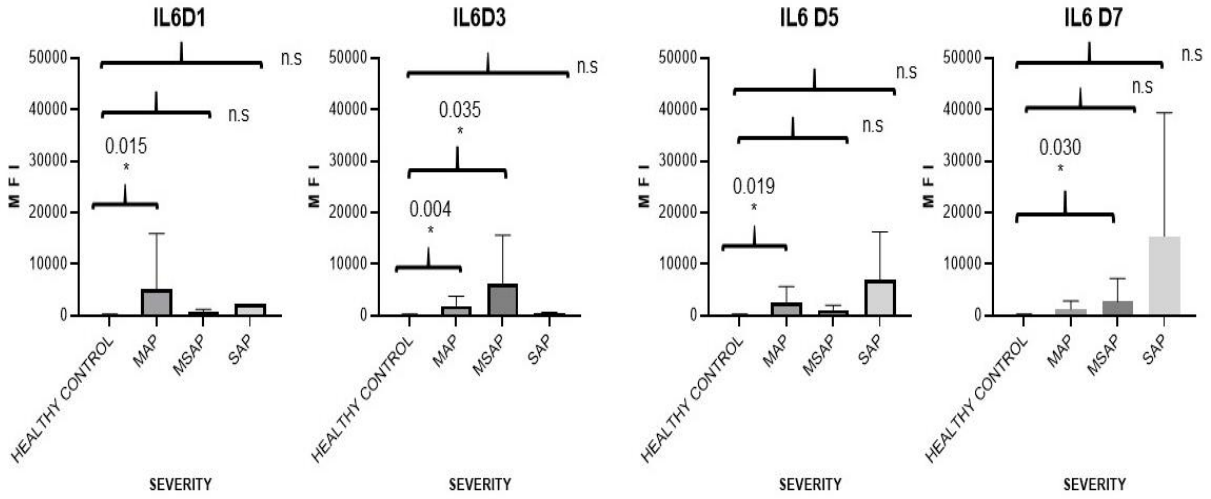


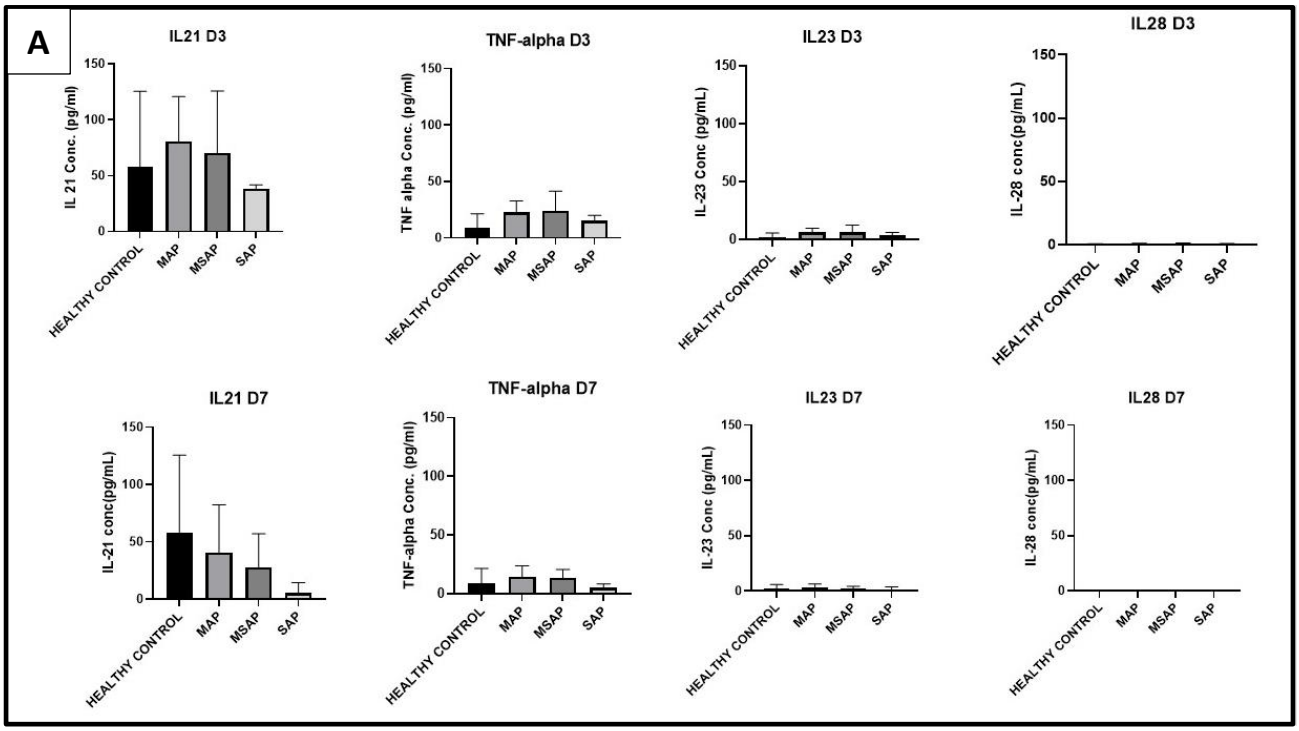
Figure 3.2 Protein analysis using the Th1/Th2/Th17 cytometric bead array (CBA) kit. In the MAP and MSAP groups, IL-6 showed significant differences on Day 3 compared to the healthy control group p=0.004 and p=0.035, respectively. On Day 5, the MAP showed significant differences with p= 0.019, and the MSAP group had a significant difference with p=0.030 compared to the healthy control group on Day 7. **Notes:** D: Day of the specific severity group; n: number; IL: interleukin; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis

3.4.3.2 MILLIPLEX® Human TH17 Magnetic Bead Panel

The results of the MILLIPLEX® data showed visible trends between severities over time and between groups. Analysis of concentration at Day3 and Day 7 for IL-21, IL-23, and IL-28λ showed no significant changes compared to the healthy controls and between groups (Figure 3.3). IL-17A, IFN-γ, and TNF-β were below the limit of detection and data were inputted as described in section 3.4. Only IL-6 showed significant differences compared to the healthy control; however, this was not the case between the different severity groups of AP. IL-21 showed an increasing concentration from healthy controls to the MSAP group; however, the SAP group on Day 3 and 7 had concentrations below 20 pg/mL (Figure 3.3).

The concentration of IL-6 in the MAP group was above 30 pg/mL on Day 3 and dropped to almost zero on Day 7. A similar trend was seen in the MSAP group (12 pg/mL to just above zero). The IL-6 concentration was significantly different at Day 3 for MAP and MSAP than healthy controls with p=0.001 and p=0.013, respectively (Figure 3.4A). The concentration of the SAP group was not significantly different at both Days 3 and 7 compared to healthy controls. However, the concentration of IL-6 in the SAP group, on the other hand, was much higher than the MAP and

MSAP groups and remained consistent at approximately 50 pg/mL over time (Day 3 and 7), as shown in Figure 3.4B. IL-21 concentration had a reverse trend with Day 3 MAP, SAP and healthy controls being above 50pg/mL and those of Day 7 showing a decrease especially in SAP group with median concentration dropping from 38pg/mL on Day 3 to 0.8pg/mL on Day 7.



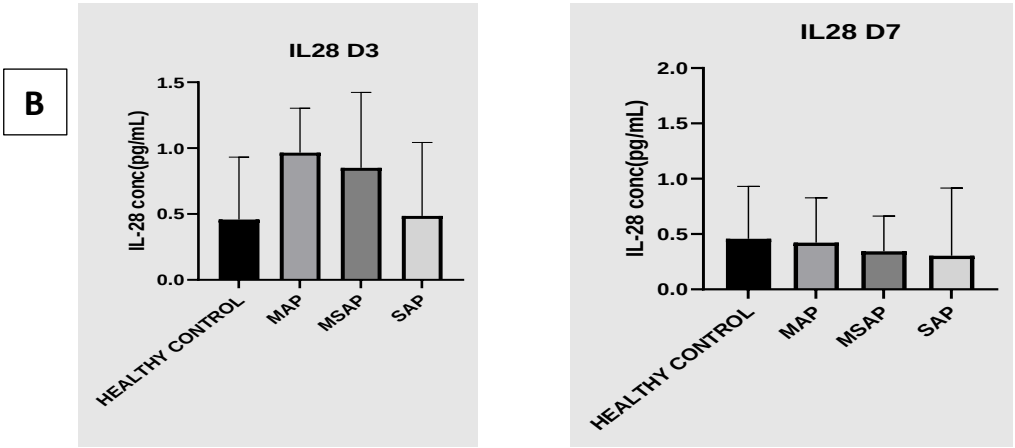


Figure 3.3 Protein analysis using MILLIPLEX® for the seven cytokines on Day and 7. **A**: Analysis was done for IL-17A, IL-21, and IL-6, IFN-γ, IL-23 and IL-28λ. The concentration of IL-21, IFN-γ, IL-23, and IL-28λ was not significant compared to the controls and amongst groups at Day 3 and 7. **B:** The concentration of IL-28λ had the lowest range from all cytokine, Day 3 concentration ranged from 0.1 to 1.49pg/mL, and Day 7 ranged from 0-1.22pg/mL. **Notes:** D: Day of the specific severity group; n: number; IL: interleukin; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

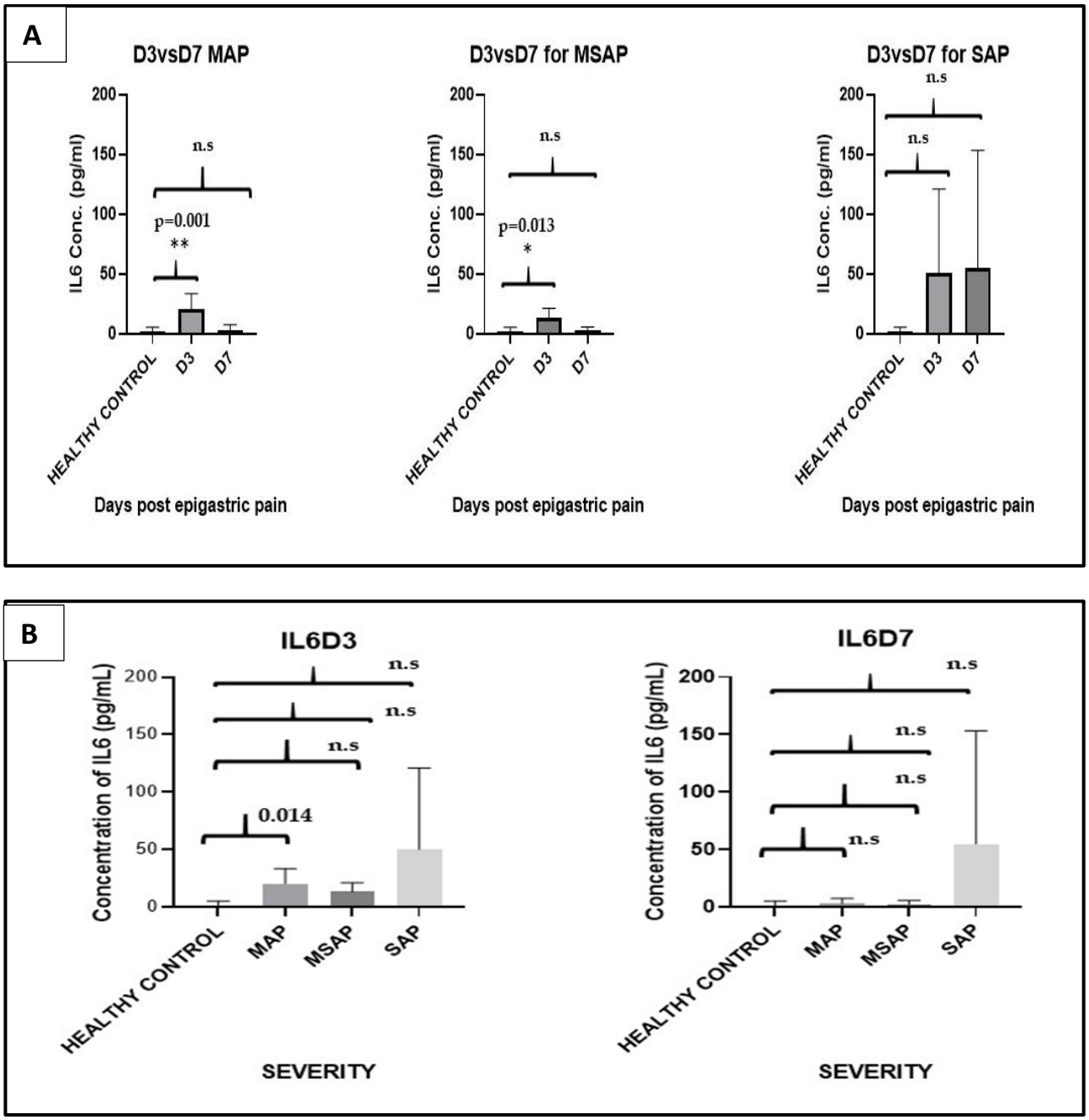


Figure 3.4 IL-6 secretion levels acquired using the MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit**. A:** Analysis was done on 23 patients sampled on Day 3 and 7, and 6 healthy controls were included. **B:** The concentration of IL-6 was highest in the SAP group 50 ± 50pg/mL and 65 ± 61pg/mL on Day 3 (*n*=2) and Day 7 (*n*=5) respectively. The MAP group IL-6 levels were 13± 8pg/mL (n=7) and MSAP 20 ± 13pg/mL groups (n=4) on Day 3. Significant differences were observed between the healthy controls (n=6) and MSAP at Day 3 *(n*=4) with *p*=0.014 and *p*=0.013, respectively. **Notes**: D: Day of the specific severity group; n: number; IL: interleukin; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

Grouping of MSAP and SAP group: The results show a significant difference between healthy control and MAP group at Day 3 with *p*=012. MSAP/SAP combined group showed no significance at both Day and 7, the mean concentration on Day 3 was 25 ± 37.6 pg/mL and Day 7 was 29 ± 71 pg/mL, refer to Figure 3.5. Refer to Appendix H for descriptive statistics of Day 3 data.

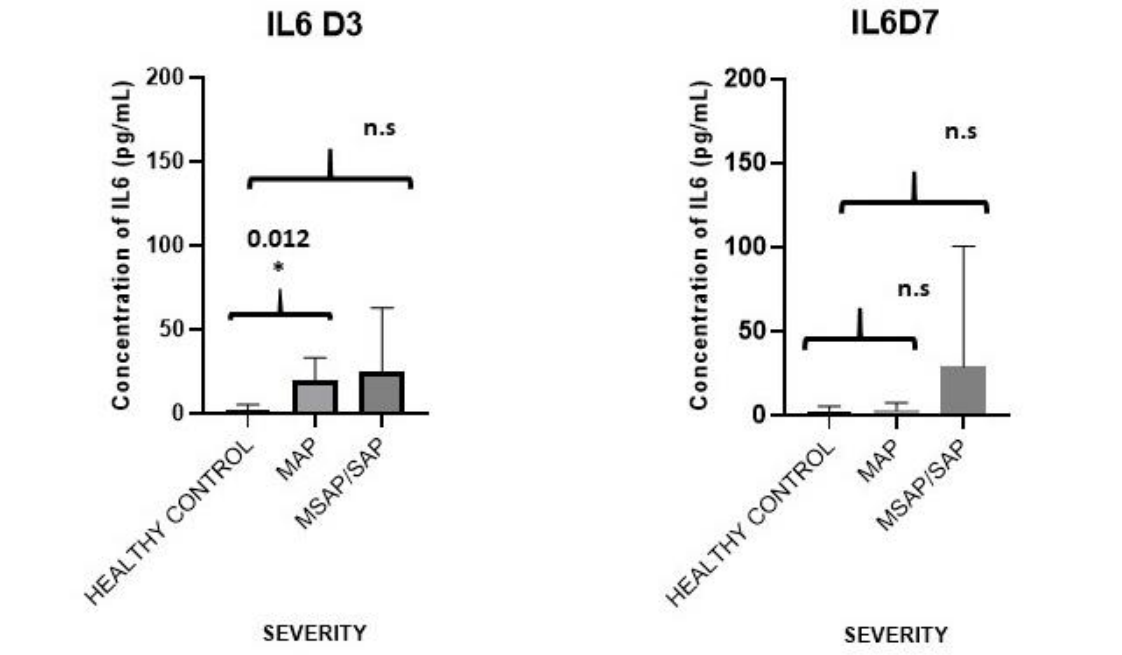


Figure 3.5 Interleukin (IL)-6 concentrations on Day 3 and Day 7. Analysis was done on 23 patients sampled on days 3 and 7, and 6 healthy controls were included. The MSAP and SAP groups concentrations were combined. MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit Significant differences were observed between the healthy controls (n=6) and MAP at Day 3 (n=7) with p=0.012. A Dunn's multiple comparison test was used as a post hoc to adjust p values. **Notes:** D: Day of the specific severity group; n: number; MAP: mild; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.5 Discussion

Linkages between the expression of plasma cytokines and the severity of AP patients have the potential to determine prognostic markers for effective and timely stratification of AP. Patients recruited for this study had comorbidities that included HIV and diabetes, as described in Chapter 2. The risk factors for AP, such as obesity was found in 39% of the patients who had biliary AP (Chapter 2 results). Cytokine analysis shows that IL-6 levels maintained an increasing trend in concentration in the SAP group from Day 1 to Day 7, compared to the healthy control group, the

MAP, and the MSAP group. This analysis is consistent with findings by Kay, Smith, and Brand (2017) that IL-6 remains elevated throughout the first week of onset of AP (Kay, Smith and Brand, 2017). However, the consistency in the concentration of IL-6 protein levels in the SAP group in the peripheral blood is likely due to the observed activated cell subsets such as monocytes and NK cells producing these cytokines, which are noted in the literature (Choy and Rose-John, 2017; Kim and Bajaj, 2014). These will be further discussed in Chapter 4.

CRP is a well-defined severity marker in AP and is initiated by the elevation of IL-6 (Mayerle et al., 2012; Stirling et al., 2017). The results showed that CRP at admission had a strong correlation with WBC, confirming the linkages between leucocyte subsets and the elevation of this cytokine in this cohort of patients, a finding observed by Machado. Elevated plasma levels of IL-6 and CRP are also associated with obesity and insulin resistance (Kim and Bajaj, 2014). The results showed that patients with a standard BMI index (18.5 to < 25kg/m^2) were also found to have high levels of IL-6. This cytokine is a common marker of inflammation in multiple diseases (Choy and Rose-John, 2017).

3.5.1 Do secreted cytokines exhibit a Th1, Th2, or Th17 polarisation?

IL-6 is elevated in multiple disease conditions that cause inflammation due to innate immune cell responses and may intensify the disease state through continuous recruitment to sites of inflammation (Choy and Rose-John, 2017; Kim and Bajaj, 2014; Peng, Li and Yu, 2021). At the site of injury within the pancreatic tissue, macrophages are known to produce TNF-α, IL-1, and IL-6 (Zhang and An, 2007; Fajgenbaum and June, 2020). Despite results showing no significant difference in TNF-α concentration at all time points, the analysis period needs to be considered, given that TNF-α has a short life span (Scheller et al., 2011). Sternby and colleagues did not observe any significant differences between AP severity groups in the concentration of this cytokine after 25 hours of the onset of pain due to AP (Sternby et al., 2020). Other studies reveal a strong association between IL-6 and TNF-α for predicting mortality in the first week in SAP patients (Sharma et al., 2017). In this cohort of patients, most presented at the hospitals 72 hours or Day 3 post-epigastric pain. Therefore, the concentration of TNF-α and its significance may be a consequence of the sampling period. Although our sampling times included Day 1 post epigastric pain, very few of our patients arrive at the hospital at this time. The CMJAH and CHBAH are referral hospitals, and hence the patients arrive long after the onset of epigastric pain.

IL-21 concentration was elevated in MAP and MSAP on both Day 3 and 7 and decreased in SAP on both days. IL-21 is produced by macrophages, NK cells, active CD4 T cells, Th17 cells, and Tfh cells (Solaymani-Mohammadi, Eckmann and Singer 2019; Thomson, 2019). Previous work in our laboratory implicated IL-21 in the immune imbalance in the late phase of SAP, also known as the second hit (Thomson et al. 2019). The current study shows that IL-21 decreased in concentration with the increase in severity. The higher IL-21 concentrations observed in healthy controls, MAP and MSAP, can be attributed to Tfh cells, which assist B cells to produce antibodies and differentiate in the presence of IL-6 and IL-21 (Zhu and Zhu, 2020). Whether the secreted cytokines exhibit a Th1, Th2, or Th17 polarisation will be further explored in Chapter 4.

3.5.2 Does admission data correlate to cytokine expression in patient samples?

Rao and Kunte (2017) found that at 48 hours post the onset of pain, IL-6 was a reliable predictive marker for severity in AP patients, performing even better than IL-8, IL-10, and CRP. The results showed that CRP had a very weak positive correlation to severity (r_s=0.234), though it had a very strong positive correlation to LOS (r_s=0.928). Singh and colleagues found that prolonged LOS resulted from ongoing AP-related symptoms even in MAP patients (Singh et al., 2017). Since LOS is linked to the disease course of AP, a case can be made for CRP as a marker for severity. In this case, the question would be how effective it is as an independent predictive marker. Mayerle et al. (2012) found that CRP had 85% sensitivity in predicting SAP; however, it was not specific to AP, and like IL-6, it is a marker for many inflammatory diseases.

On the other hand, WBC in the present study showed a strong positive correlation with severity (r_s=0.649), although not statistically significant. It aligns with the literature in that cytokines are predominantly produced by macrophages and CD4 T cells (Zhu and Zhu, 2020). Moreover, our study determined that IL-6 may be involved in the proliferation of the Th17 lineage of cells. The IL-21 elevated in MAP and MSAP patients may be due to the differentiation of Tfh cells (Zhu and Zhu, 2020). However, the limitation in sample size may have contributed to these findings, and further investigation in a larger cohort of patients may be required.

Furthermore, of the four (4) patients who died, one was both HIV positive and diabetic. This patient was on ARVs, including PIs and NRTIs, which are risk factors for severity in AP (Oliviera et al., 2014). HIV and, consequently, ARVs and AP in the South African population are not well expounded. Due to patients being on ARVs, it may be likely that the immune response observed

resulted from AP and may require further analysis. It is recommended that future studies in AP include CD4 cell counts and possibly viral load to ascertain whether the patient is immunocompromised. Additionally, it was not possible to predict whether patients in this study had AP as a result of ARVs or not; however, it is recommended that HIV as comorbidity be taken into consideration when designing other AP studies since IL-6 is a marker of inflammation in many disease states, including HIV (Breen et al., 1990; Zhu and Zhu, 2020).

3.5.3 Secreted IL-6 expression differentiates severity groups in early acute pancreatitis

In conclusion, of the cytokines analysed, IL-6 was found to increase with severity and over time, peaking at day 7. While this finding is not novel, it warrants the consideration of IL-6 as a potential predictive marker in a panel together with other markers. It has been established as a good early marker of AP (Manohar et al., 2017). These considerations are made in Chapter 4, especially concerning cell-associated IL-6 and not necessarily peripheral or circulating IL-6 as was the case here. The limitation of the techniques used to detect concentration was taken into account. Further analysis was done on a genetic level to establish linkages between cytokine data and genes expressed by innate and adaptive cells in different severities on AP.

CHAPTER 4

GENE EXPRESSION AND IMMUNE CELLS STUDIES

4.1 Introduction

4.1.1 Cell surface markers and disease state

Cell surface markers are proteins found on the cell surface membranes and are specific to cell types. The most common markers are the cluster of differentiation (CD), molecules; they can act as receptors in cell signalling or ligands. These cell surface markers may differ in expression depending on the stage of development of particular cells (Chan, Ng and Hui, 1988). For example, CCR7 distinguishes between memory (central) and effector memory T cells, where the former will express it and the latter will not (Unsoeld et al., 2002). The function of cell surface markers is for recognition and intercellular communication (Boumsell, 1984; Chan, Ng and Hui, 1988). Some important antigen recognition molecules include CD4 and CD8, expressed on CD3 positive T cells (Chan, Ng and Hui, 1988). These proteins can be distinguished in blood samples using immunophenotyping by flow cytometry (Roederer, 2002).

As mentioned in section 1.2.6.1, monocytes express CD14 and or CD16 depending on their function. The classical monocytes are $CD14^{+}CD16^{-}$, non-classical are $CD14^{-}CD16^{+}$, and intermediate are $CD14^{+}CD16^{+}$ (Yang et al., 2014; Peng, Li and Yu, 2021). Classical monocytes are reported to be highly expressed in AP patients within the first 48 hours of onset of disease compared to healthy controls due to acute inflammation (Zhang et al., 2019). The cell surface markers, CD56 and CD57, are expressed by lymphocytes, particularly NK cells (Ding et al., 2020). The $CD56^{-}CD16^{+}$ NK cells are the most abundant subset of this cell population found in blood (Poli et al., 2009).

4.1.2 Innate and adaptive immune response pathways in acute inflammation

The initial inflammation at the site of injury in AP is sterile, pro-inflammatory immune cells, including macrophages and neutrophils, are activated, and necrotising pancreatic cells release damage-associated molecular patterns (DAMPs), including high-mobility group box protein 1 (HMGB1) (Fonteh, Smith, and Brand, 2018; Peng, Li and Yu, 2021). The HMGB1 and other DAMPs will activate PRRs expressed by infiltrating immune cells, which are recognised by TLRs (Fonteh, Smith, and Brand, 2018). This action perpetuates sterile inflammation. The HMGB1

molecule initially activates TLR4, which mediate its adaptors through the NF-κB and mitogen-activated protein kinases (MAPK) pathways (Shen and Li, 2015).

Activation of NF-κB prompts the expression of IL-1β, IL-6, IFN-γ, and TNF-α and the eventual activation of macrophages. This activation, if exacerbated, is responsible for the ensuing systemic inflammation synonymous with AP pathology (Shen and Li, 2015; Peng, 2021). The MAPK pathway is critical in regulating pro-inflammatory cytokines, including IL-1, IL-6, IL-23 and TNF (Thalhamer, McGrath and Harnett, 2007). Since the systemic response in AP is concomitant with the production of pro-inflammatory cytokines, including TNF-α and IL-6, the different types of cells and genes expressed by the cytokines and produced by these activated pathways become of interest in the pathophysiology of AP.

The IL-6 receptor (IL-6R) is expressed by several immune cells, including lymphocytes, neutrophils, monocytes, and macrophages. This pro-inflammatory molecule is also responsible for the differentiation of Th17 cells and the balance between IL-17 cytokine-producing cells and T_{reg} cells (Luo and Zheng, 2016). The IL-6R plays a critical role in the transition from innate to adaptive immunity. Acute inflammation initiates the recruitment of neutrophils, replaced by monocytes and T cells after 48 hours, to prevent local tissue damage caused by neutrophil proteases and ROS (Scheller et al., 2011). Cytokines such IL-1β, IL-6 and TNF-α are then produced by cells at the injury site, increasing the recruitment of neutrophils. The neutrophils, which express the IL-6R, initiate a signalling pathway that causes a switch from neutrophil to monocyte recruitment by producing more C-C chemokines than CXC chemokines or neutrophil attractants (Scheller et al., 2011). This pathway, known as IL-6 trans signalling, promotes cell death in neutrophils and maintains Th17 differentiation (Scheller et al., 2011). At this stage, pancreatic necrosis can occur if the immune system continues to be dysregulated, and the late phase of AP kicks in, usually after day seven (7) post epigastric pain, commonly referred to as the "second hit" of SAP (Thomson et al., 2019).

At this point, an infection can occur at the site of injury, which results in decreased expression of HLA-DR, specifically on monocytes (Dabrowska et al., 2008; Sharma et al., 2017). HLA-DR is responsible for presenting antigens and pathogen-associated molecular patterns (PAMPs), including TLRs. In early SAP, downregulation of HLA-DR is linked to immunosuppression, resulting in translocation of the gut microbiome into the blood circulation (Sharma et al., 2017).

Once immunocompromised, CARs set in perpetuating the infection. The frequencies of HLA-DR PBMCs is a rudimentary predictive marker of severe outcomes in AP unless it is complemented with gene expression studies (Sharma et al., 2017). This chapter explored cell populations and linkages between gene expression studies and cytokine analysis from Chapter 3.

4.1 Objectives

The objectives of this chapter were

4.1.1 To determine genes expressed by innate and adaptive immune cells in the MAP, MSAP and SAP patients

4.1.2 Characterise innate and adaptive immune cells in the MAP, MSAP, and SAP patients to establish linkages to the disease severity in AP.

4.2 Materials and methods

4.2.1 Patient recruitment and sample collection

After informed consent, patients were recruited from the CHBAH and CMJAH, as elaborated in Chapter 2, section 2.2. Blood samples were collected as described in section 3.2.2. Figure 4.1 shows a flow diagram of the sampling methods used in this chapter.

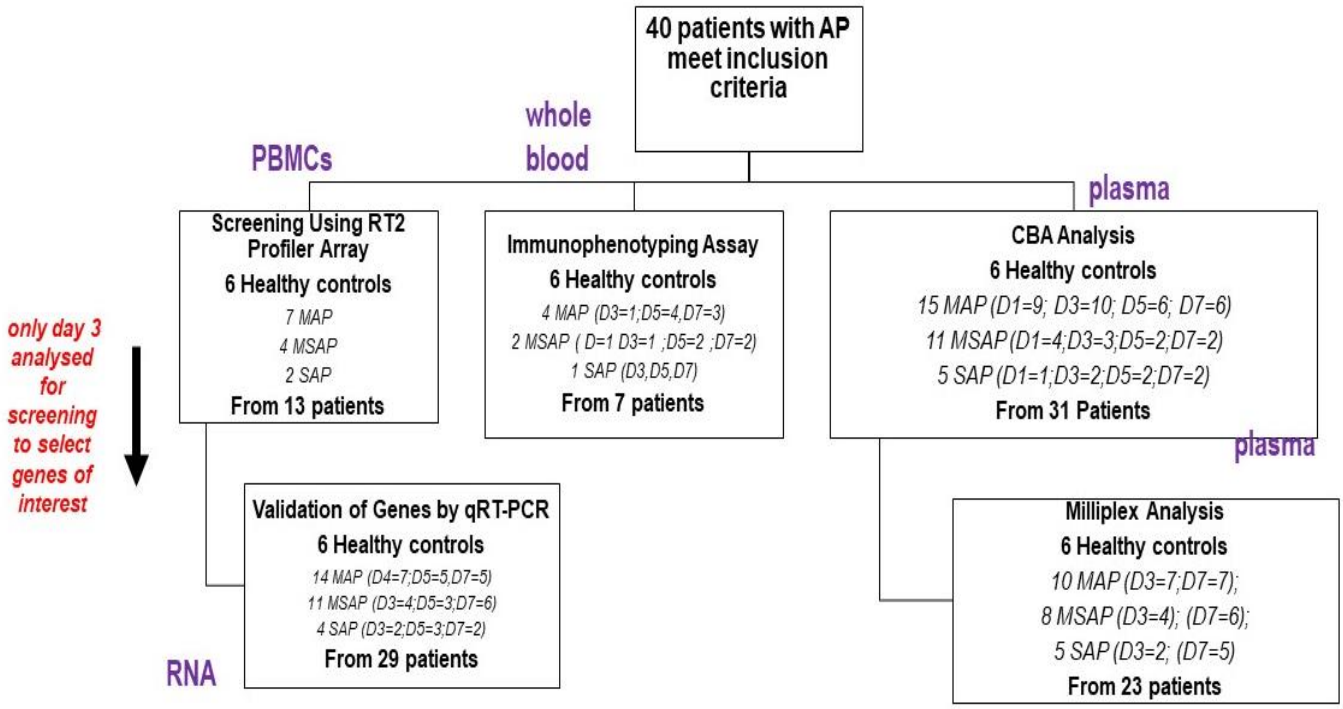


Figure 4.1 Flow diagram of patient recruitment. From the 40 patients and six(6) healthy controls recruited over the study period, peripheral blood mononuclear cells (PBMCs), whole blood, and plasma were used for the various study assays as shown. An exploratory study of seven Th1/Th2/Th17 cytokines was done on 31 patient samples, and 23 of these were selected for further analysis (available samples) using the MILLIPLEX® assay. PBMCs from 13 patients with Day 3 data were used to do a screening study of innate and adaptive immune cell genes using RT2 Profiler Array (Qiagen, Hilden, Germany). For immunophenotyping, 12 antibodies were selected to discriminate monocytes, lymphocytes, and granulocytes and their subpopulations from blood samples of seven (7) patients. **Notes:** D: Day;e.g. D3 is Day 3, after presentation; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

4.2.2 Sample processing

During sample processing, as described in sections 3.2.2 and 3.2.3, a 200 µL whole blood sample was reserved and used immediately for the immunophenotyping assay and the rest for isolating PBMCs for the gene expression studies. PBMCs were isolated by Ficoll-Paque gradient centrifugation while the reserved whole blood was prepared using the BD FACS lyse whole blood lyse method.

4.2.2.1 Whole blood lyse-wash method

Whole blood was transferred into a 50 mL tube (100µL per reaction) after separating the plasma (section 3.3.2). The blood was then washed with 2mL of PBS for every 100µl of blood by centrifugation at 277 x *g* for 5 minutes. The supernatant was drawn out with a pipette, and 100µL of blood was placed in 5mL Corning Falcon tubes (New York, USA). The titrated antibody volumes for the 12 colour panel were then added to the full stain tube. Then the blood was mixed by pipetting and incubated in the dark for 20 minutes. After that, 2 mL of BD FACS Lyse (BD Biosciences, New Jersey, USA) was diluted 10% (v:v) with deionized water. The mixture was then incubated for 12 minutes and then centrifuged at 277 x *g* for 5 minutes. The supernatant was discarded, and the cells were washed twice with 3 mL of 1XPBS (Sigma-Merck, Missouri, USA) and then suspended in 500µL of 1xPBS. The sample was then acquired by flow cytometry on a BD LSR II Fortessa (BD Biosciences, New Jersey, USA).

4.2.2.2 Peripheral blood mononuclear cells

With the aid of the Ficoll-Paque™ (GE Healthcare, Illinois, USA) separation method, as per the manufacturer's instructions: a concentration of $1x10^5$ to 10^6 cells/mL PBMCs were separated and stored in liquid nitrogen in a freezing medium (10% dimethyl sulphoxide, Sigma Aldrich, Missouri, USA, and 90% Gibco Foetal Bovine Serum (FBS), Thermo Fischer, Massachusetts, USA) until required. Briefly, after separating the plasma, the blood was diluted in a 1:1 ratio using Roswell Park Memorial Institute (RPMI) medium without FBS (Sigma Aldrich, St Louis, MI, USA). The diluted blood was layered onto Ficoll-Paque at a 2:1 ratio and followed by centrifugation at 1734 x *g*, for 30 min, at room temperature with the brakes off. The PBMCs were transferred to a new 50 mL falcon tube and washed (932 x *g*, 10 minutes room temperature) with 30 mL of RPMI (Sigma Aldrich). Red blood cells were lysed using ammonium chloride potassium, or ACK (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) for 5 minutes before washing with 15 mL of RPMI (277 x *g*, at room temperature for 10 minutes). The cells, at a concentration

of $1x10^5$ to 10^6 cells/mL were separated and stored in liquid nitrogen in a freezing medium (10% dimethyl sulphoxide, Sigma Aldrich, Missouri, USA, and 90% Gibco Fetal Bovine Serum FBS, Thermo Fischer, Massachusetts, USA) until required. Before analysis, samples were thawed once to preserve integrity. As mentioned in Chapters 2 and 3, clinical data from patient hospital files such as admission CRP, creatinine, platelets, and WBC were also recorded.

4.2.2.3 Ribonucleic acid extraction and complementary Deoxyribonucleic acid synthesis

Total RNA was extracted from the isolated PBMCs. This was followed by cDNA synthesis. The Day 3 samples were used to screen for innate and adaptive genes using a 96 well RT^2 Profiler PCR Array Human Innate & Adaptive Immune Responses (QIAGEN, Hilden, Germany). The RT^2 profiler genes are referred to in Appendix K (plate layout) and Appendix L (names of genes). As shown in Appendix M, a validation study was done using the same cDNA samples for selected genes. The selection of genes was based on their response patterns from the RT^2 profiler data.

RNA extraction using TRI Reagent® method: PBMCs were removed from the liquid nitrogen tank, thawed at room temperature, and then centrifuged at 500 × *g* for 5 minutes. The supernatant was then discarded, and the pellet retained. One mL of TRI Reagent® (Sigma-Aldrich, Missouri, USA) was used to lyse the PBMCs and left at room temperature for 5 minutes. 200µL of chloroform was added to the mixture and shaken vigorously for 15 seconds, then left at room temperature for 10 minutes. The sample was then centrifuged at 1610 × *g* for 15 minutes at 4 °C. The colourless upper aqueous phase (containing total RNA) was pipetted into a fresh tube, and 500µL of isopropanol was added. The sample was centrifuged at 1610 × *g* for 10 minutes at 4 °C. The RNA precipitate was washed three times with 1mL of 75% ethanol, then centrifuged at 629 × *g* for 5 minutes at 4°C. The RNA was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA). Samples with an $A_{260/280}$ ratio ≥ 1.8, representative of sample purity, were observed across all samples (Bustin et al., 2009).

cDNA synthesis: The total RNA samples in each severity group were pooled together after adjusting the concentration to 250ng/µL. cDNA was synthesised from 250 ng/µL of total RNA using the RT^2 First Strand Kit (QIAGEN, Hilden, Germany). A genomic DNA elimination mix was first prepared and incubated for 5 minutes at 42°C in a SimpliAmp™ thermocycler (Thermo Fischer Scientific, Massachusetts, USA), which was then placed on ice for 1 minute. Following

this, a 20 µL cDNA synthesis reaction was prepared and run at 42°C for 15 minutes, followed by incubation at 95°C for 5 minutes.

cDNA synthesis for the validation studies: the same kit was used above (RT2 First Strand Kit (QIAGEN, Hilden, Germany) and followed the same process. As indicated in Appendix I, the RNA concentration was worked out to 50ng/µL for the individual samples.

4.2.3 Screening with 96 well RT^2 Profiler using qRT-PCR for Day 3 pooled samples

Initial screening was performed on 13 (MAP *n*=7; MSAP *n*=4; SAP *n*=2) Day 3 samples. The RT^2 Profiler PCR Array had 96 built-in genes, 84 target genes, three positive PCR control (PPCs), three reverse transcription control (RTCs), and five reference genes (*ACTB, B2M, GAPDH, HPRT1 RPLP0*, and *HGDC)*[1], refer to Appendix K for plate layout and Appendix L for full names of genes (Gholamnezhadjafari et al. 2017). The study analysed gene expression of samples on the earliest day possible, Day 3, as most patients presented on Day 3 of pain.

Following the manufacturer's instruction, 102 µL of cDNA (prepared in section 4.4.2.3) was added to the PCR mixture and loaded onto the human innate and adaptive RT^2 Profiler 96-well PCR array plates Catalogue No. 330231 PAHS-052ZA (QIAGEN, Hilden, Germany). The samples were amplified on QuantStudio 1 (Applied Biosystems, California, USA). The PCR reaction was run for 40 cycles, including a 10-minute hot start at 95ºC for one cycle, 95ºC for 15 seconds, and 60ºC for 1 minute.

Using the QIAGEN GeneGlobe online tool (https://geneglobe.qiagen.com/za/analyze/), a fold-change of 2 was applied for differential analysis comparing the upregulated and downregulated genes in the three severity groups and normalized using the healthy control. This is done relative to the expression levels of the gene of interest in the healthy control samples.

4.2.4 Validation of selected gene targets in AP samples for Day 3, 5, and 7

After screening Day 3 samples for early immune markers with the RT^2 Profiler PCR Array Human Innate & Adaptive Immune Responses (QIAGEN, Hilden, Germany), selected markers listed in Appendix M were analysed based on their levels of expression in the three risk categories of AP. These markers included *CCR8, CCL1, CCL5, MPO, TLR1, IL-6, CXCL8* and *NOD1*. Twenty-nine

[1] https://geneglobe.qiagen.com/us/product-groups/rt2-profiler-pcr-arrays

(29) patients (MAP=14, MSAP=11, SAP=4) were included in this assay, as stated in Figure 4.1. The *RPL13A* (Hs04194366_g1, Thermo Fischer, Massachusetts, USA) was used as a reference gene (Yan et al. 2016).

qRT-PCR analysis for validation of genes: The TaqMan® Fast Advanced Master Mix (Thermo Fischer, Massachusetts, USA) was thawed on ice and then mixed as in Appendix J Table A. The cDNA concentration prepared in section 4.4.2.3) was diluted with nuclease-free water to 1ng.

The following controls were prepared:

a. No template control (NTC) ensures zero contamination in the master mix.
b. Positive PCR control (PPC) for checking for expression of the target gene.

Interplate calibrator (IPC): was used to compensate for the variation between qPCR runs from different plates (use sample in excess and with the highest concentration and purest quality).

The PCR reaction mix was added to each well of an optical reaction plate. A 2 µL of a 1ng cDNA template was then added to corresponding wells except for the NTC. The plate was centrifuged for 1 minute to eliminate bubbles. The samples were run on a QuantStudio 1 Real-Time PCR system (Thermo Fischer, Massachusetts, USA) using the set up in Appendix J.

4.2.5 Multicolour flow cytometry analysis for immunophenotyping innate and adaptive cells

Flow cytometry is a powerful technique that enables rapid multiparametric analysis of physical and chemical characteristics of single cells in a solution (Mckinnon, 2018). To effectively assess and characterise cells, a 12-colour panel was established. The panel was used to characterize heterogeneous cell populations in the three risk categories of AP using flow cytometry. The whole blood-lyse-wash method (described in section 4.4.2.1) was used to lyse red blood cells and isolate white blood cells from 200 µL (100 µL for the unstained and 100 µL for the stained sample) of blood collected from a BD vacutainer EDTA blood tube (BD Biosciences, New Jersey, USA) within 6 hours of phlebotomy. Antibodies used included those that stained for T and B cells, granulocytes, and NK cells. The antibodies, the respective fluorochrome-conjugated markers, the surface markers, and associated cells used for their analysis on the BD LSRII Fortessa flow cytometer (BD Biosciences, New Jersey, USA) are shown in Table 4.1. On a flow cytometer, the fluorochrome detects emission spectra on a specific filter on the instrument. The filters associated with specific detectors are shown in Appendix P.

Table 4.1 Optimised multicolour flow cytometry panel used to analyse and characterise white blood cells in acute pancreatitis patients

Fluorochrome	Surface Marker	Cell type[2]
APC-Cy7	CD11b	Neutrophils
Alexa Fluor 700	CD4	CD4 T cells
APC	CD45RO	Naïve cells
PE-Cy7	CD56	NK cells, $CD4^+$ T cells and $CD8^+$ T cells
PerCP-Cy5.5	CD14	Monocytes
PE-Cy5	CD16	Monocytes and NK cells
PE-CF594	CD19	B cells
FITC	CD57	Mature NK cells
BV650	HLA-DR	CD8 cells, monocytes
BV605	CD8	CD8 T cell
BV421	CCR7/CD197	Memory T cells. B and T lymphocytes and mature dendritic cells
BUV496	CD3	CD4 and CD8 cells

Notes: APC: Allophycocyanin; BUV: BD Horizon Brilliant™ Ultraviolet; BP: bandpass; Cy: Cyanine; CCR7: C-C chemokine receptor type 7; FITC: Fluorescein isothiocyanate; BV: Brilliant Violet™; HLA DR: human leukocyte D related; PE: Phycoerythrin; PE-CF594: Phycoerythrin Cyanide dye™; PerCP: Peridinin-Chlorophyll-protein. All antibodies were purchased from BD Biosciences (New Jersey, USA) clone numbers are listed in Appendix P).

Multicolour flow cytometry requires optimisation for instrument parameters as well as experimental parameters. The following subsections show the initial optimisation processes that were performed, such as antibody titrations (for optimal staining and resolution of subpopulations), photomultiplier tubes (PMT), voltage optimisation (for negative and positive controls), and compensation controls (to subtract spillover from overlapping fluorochromes). In performing the optimisation, the sample preparation processes used were similar to those used in the actual immunophenotyping assays, i.e. the same whole blood FAClyse methods described in 4.4.2.1 and staining conditions eventually used for the fully stained samples

[2] Sourced from BD Biosciences website, technical data sheets of antibodies. https://www.bdbiosciences.com/us/solrSearch?text=antibodies and ThermoFischer Scientific website technical information on antibodies. https://www.thermofisher.com/za/en/home/life-science/antibodies.html

4.2.5.1 Antibody titration

The required concentration of antibody volume for the 12 colour panel was determined using whole blood from healthy control participants collected in BD vacutainer EDTA tubes (BD Biosciences, New Jersey, USA). After removing the plasma through gravity separation, the blood was mixed by inversion, and the required volume (100 µL~ 1 million cells per reaction) for titration was transferred into a 50 mL (this volume was used to wash blood that was analysed, including extra volume for error) tube for washing . The blood was washed with 2 mL of PBS for every 100µL of blood by centrifugation at 277 x *g* for 5 minutes. The supernatant was then discarded.

Preparation of antibodies: antibodies were prepared by doubling dilutions of antibodies in seven (7) 5 mL BD polystyrene tubes (BD Biosciences, New Jersey, USA) as per the manufacturer's instruction. The results of the respective antibody titration findings are shown in Appendix Q.

Optimising PMT voltages: The PMT voltages are optimized to ensure the instrument performs optimally throughout a project, i.e. consistency in experiments performed over a long period; this setup also eliminates electronic noise from cell debris and others. In order to obtain consistent results, the voltages of the respective fluorochromes for the 12 colour panel had to be optimized. In so doing, the positive and negative populations of the target parameters were established. The stain index (SI) was calculated, which is the difference between the MFI of positive and negative populations divided by two times the standard deviation of the negative population. From this, a plot of antibody volume against the SI was used to decide on the optimal volume for the use, which was the volume corresponding to the highest stain index (see Appendix Q, Part A).

Also, the dynamic range, which is the total range of fluorescent values for the particular assay, are established in the process. An expanded protocol of how this optimisation was done is reported in the appendix (Appendix Q, Part B)

4.2.5.2 Acquiring the compensation controls and calculating compensation

After titration, the optimal titred antibody concentrations/volumes were used to set up compensation. Compensation is a process in multicolour flow cytometry where a correction in fluorescence spillover is applied (Roederer, 2002). Through compensation, a correction in this spillover is made to avoid false positives or false negatives. The compensation setup was

performed as directed by immunofluorescence protocols using Anti-Mouse Ig, κ/Negative Control Compensation Particles Set[3] (BD Biosciences, New Jersey, USA).

4.2.5.3 Gating Strategy

Cells were gated as singlets, then further as granulocytes, lymphocytes, and monocytes using forward scatter and side scatter properties as well as fluorescent antibody stains for specific subsets. Doublets were excluded using forward scatter height (FSC-H) and FSC area (FSC-A). FSC-A and Side Scatter Area (SSC-A) were used to discriminate white blood cells, namely, lymphocytes, granulocytes, and monocytes, based on their size and granularity or complexity, respectively (refer to Figure 4.2 A). The monocyte populations were discriminated against using CD16 PECy5, CD14 PerCPCy5.5, CD56 PECy7, and HLA-DR BV650. Subpopulations of monocytes included non-classical monocytes ($CD16^+$ $CD14^-$), intermediate monocytes ($CD16^+CD14^+$), classical monocytes ($CD16^-CD14^+$) and other monocytes subpopulations such as $CD14^+HLADR^{+/-}$ (Figure 4. 2 A to F). Neutrophils were gated with CD11bAPC, and CD56PECy7 was used to gate out granulocytes that express this marker (Figure 4.3 A to D). The CD3- subpopulations included B cells and NK cells. Natural killer cells were discriminated using CD3BUV496, CD16PECy5, and CD56PECy7 (Figure 4.4 A to E). The subpopulations of $CD3^+$ lymphocytes included CD4 Alexa Fluor 700 and CD8 BV605 cell populations. These were further discriminated into CCR7 BV421 and CD45RO APC (Figure 4.5 A to E). Refer to Table 4.1 for fluorochrome information and Appendix P for panel details. All populations were represented as percentages of their parent populations. Table 4.2 is a summary of these representative populations, while Figures 4.2, 4.3, 4.4, and 4.5 show how gating for the various cell populations was achieved. It should be noted that Figures A and B in Figures 4.2 to 4.5 show the same parent populations from which the different subpopulations were derived.

[3] https://www.bdbiosciences.com/en-eu/products/reagents/flow-cytometry-reagents/research-reagents/panels-multicolor-cocktails-ruo/anti-mouse-ig-negative-control-compensation-particles-set.552843

Table 4.2 Cell populations gated in the 12 colour panel

Cell population	Cell type	Flurochrome
$CD14^{+}CD16^{+}$	Non-classical monocytes	CD14 PerCPCy5.5 CD16 PECy5
$CD14^{+}CD16^{-}$	Intermediate monocytes	
$CD14^{-}CD16^{+}$	Classical monocytes	
$CD14^{+}HLA\text{-}DR^{+/-}$	HLA-DR positive or negative monocytes	HLA-DR BV650
$CD14^{+}CD56^{+/-}$	Monocytes	CD56 PECy7
$CD11b^{+}CD56^{-}$	Neutrophils	CD11b APCCy7
$CD3^{-}$CD16+CD56-	NK cells	CD3 BUV496
$CD3^{-}CD19^{+}$	B cells	CD19 PECF496
$CD4^{+}$	CD4 T helper cells	CD4 Alexa Fluor 700
$CD4^{+}CD45RO^{+}CCR7^{+}$	Naïve T cells	CD45ROAPC CCR7 BV421
$CD4^{+}CD45RO^{+}CCR7^{-}$	Effector memory cells	
$CD8^{+}$	CD 8 T cells	CD8 BV605

Notes: APC: Allophycocyanin; BUV: BD Horizon Brilliant™ Ultraviolet; BP: bandpass; Cy: Cyanine; CCR7: C-C chemokine receptor type 7; FITC: Fluorescein isothiocyanate; BV: Brilliant Violet™; HLA DR: human leukocyte D related; PE: Phycoerythrin; PerCP: Peridinin-Chlorophyll-protein; PE-CF594: Phycoerythrin Cyanine-based Fluorescent dye.

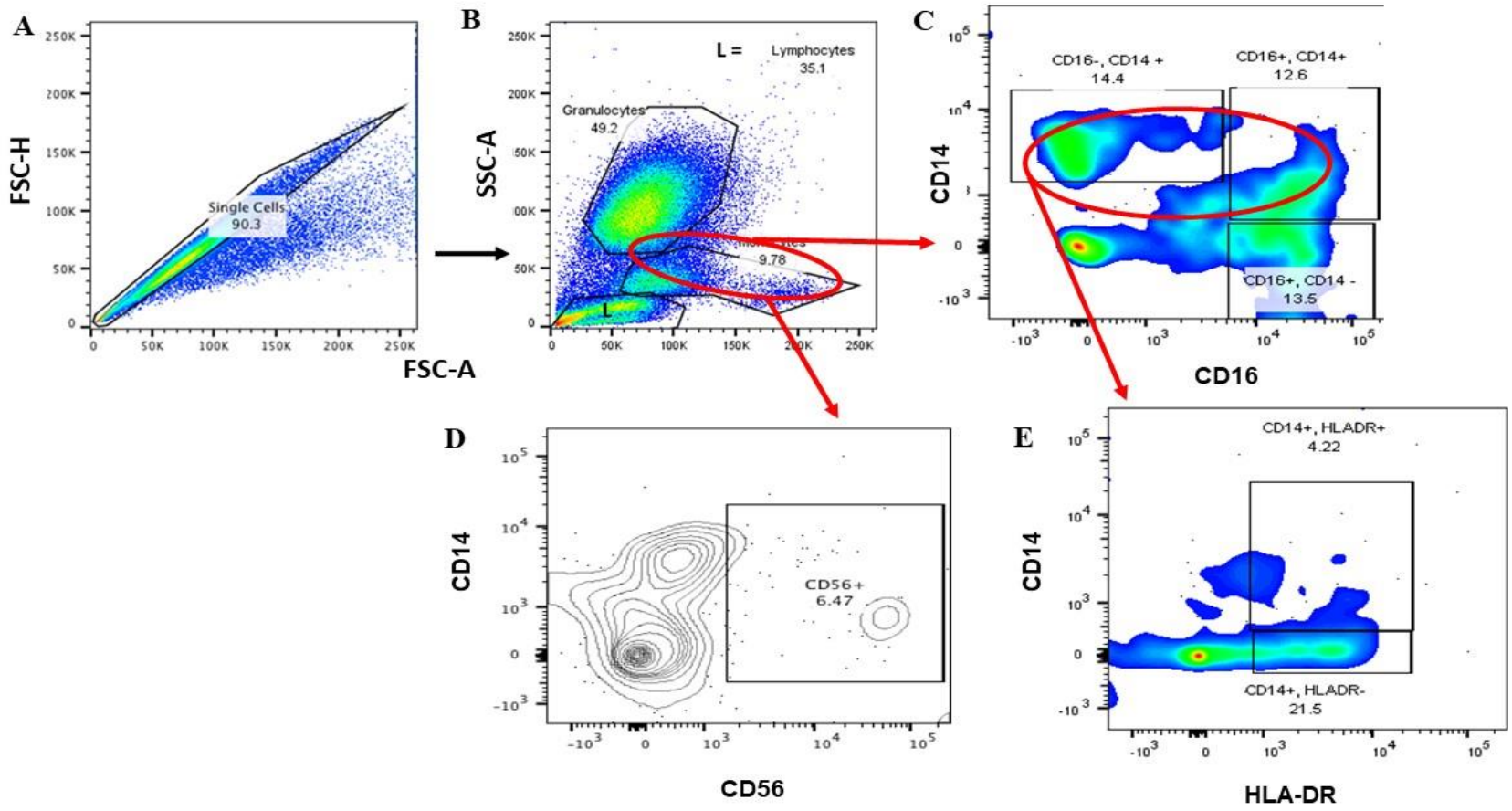


Figure 4.2 Gating strategy used to gate monocytes (A-E). **A** Doublets are clumped cells and may interfere in the reading of characteristics of single cells. They are excluded using FSC-H and FSC-A. **B:** In B, the cells are further discriminated based on granularity (SSC-A) and size (FSC-A). Lymphocytes (smallest and least granular), monocytes (bigger and more granular), and granulocytes (biggest and most granular) leucocyte subpopulations are shown. **C:** CD16CD14 cells were gated into Classical ($CD16^{-}CD14^{+}$), intermediate ($CD16^{+}CD14^{+}$) and non classical monocytes ($CD16^{+}CD14^{-}$). **D**: Gating to check HLA-DR expression of monocytes. **E:** Monocytes were gated with CD56 (a contour plot is used for better visualization as the cells were few). See Table 4.1 for full names of fluorochromes. CD: cluster of differentiation; FSH: forward scatter height; FSA: forward scatter area; HLA-DR:human leukocyte antigen D-related; SSC-A: side scatter area.

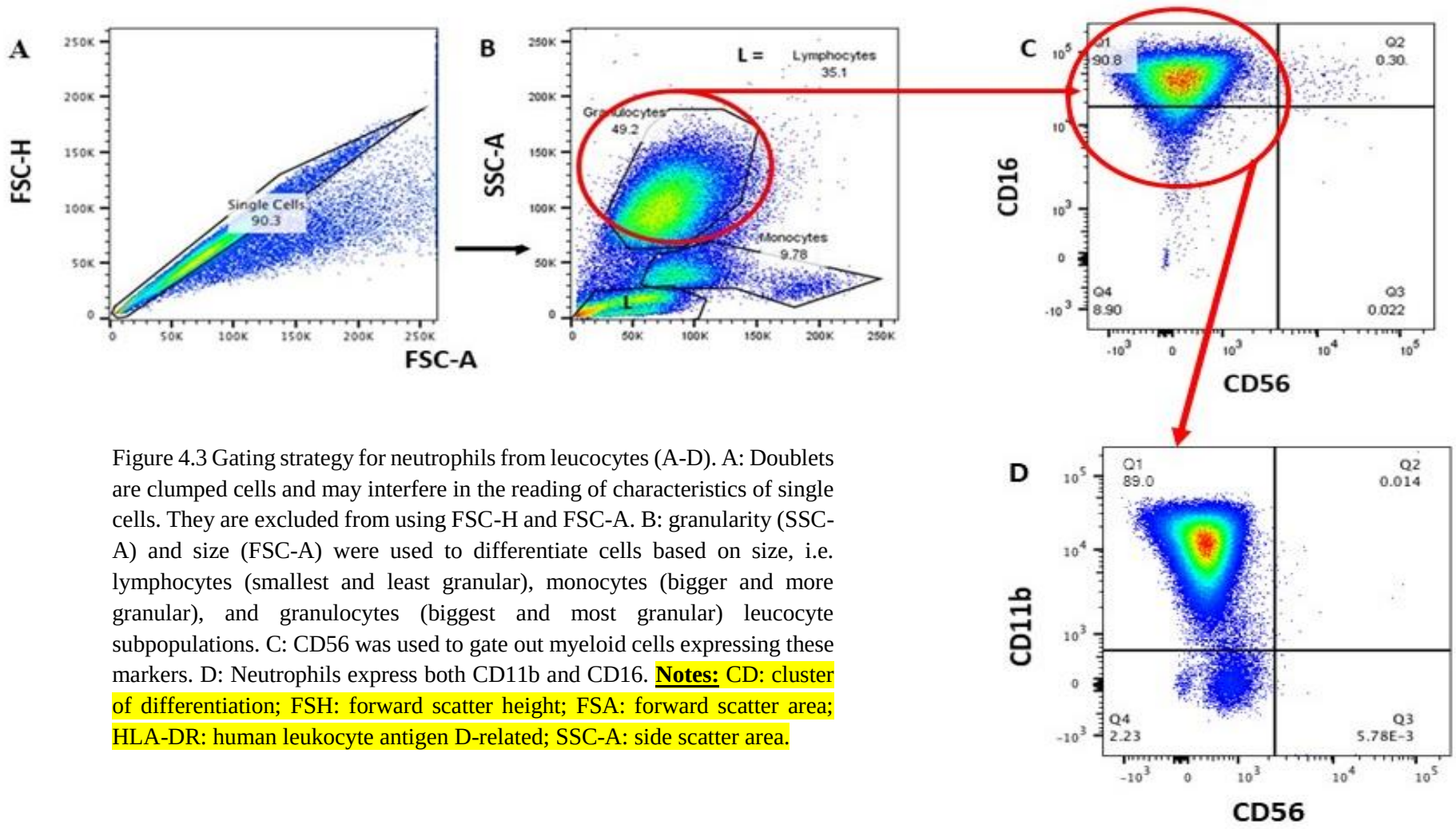


Figure 4.3 Gating strategy for neutrophils from leucocytes (A-D). A: Doublets are clumped cells and may interfere in the reading of characteristics of single cells. They are excluded from using FSC-H and FSC-A. B: granularity (SSC-A) and size (FSC-A) were used to differentiate cells based on size, i.e. lymphocytes (smallest and least granular), monocytes (bigger and more granular), and granulocytes (biggest and most granular) leucocyte subpopulations. C: CD56 was used to gate out myeloid cells expressing these markers. D: Neutrophils express both CD11b and CD16. **Notes:** CD: cluster of differentiation; FSH: forward scatter height; FSA: forward scatter area; HLA-DR: human leukocyte antigen D-related; SSC-A: side scatter area.

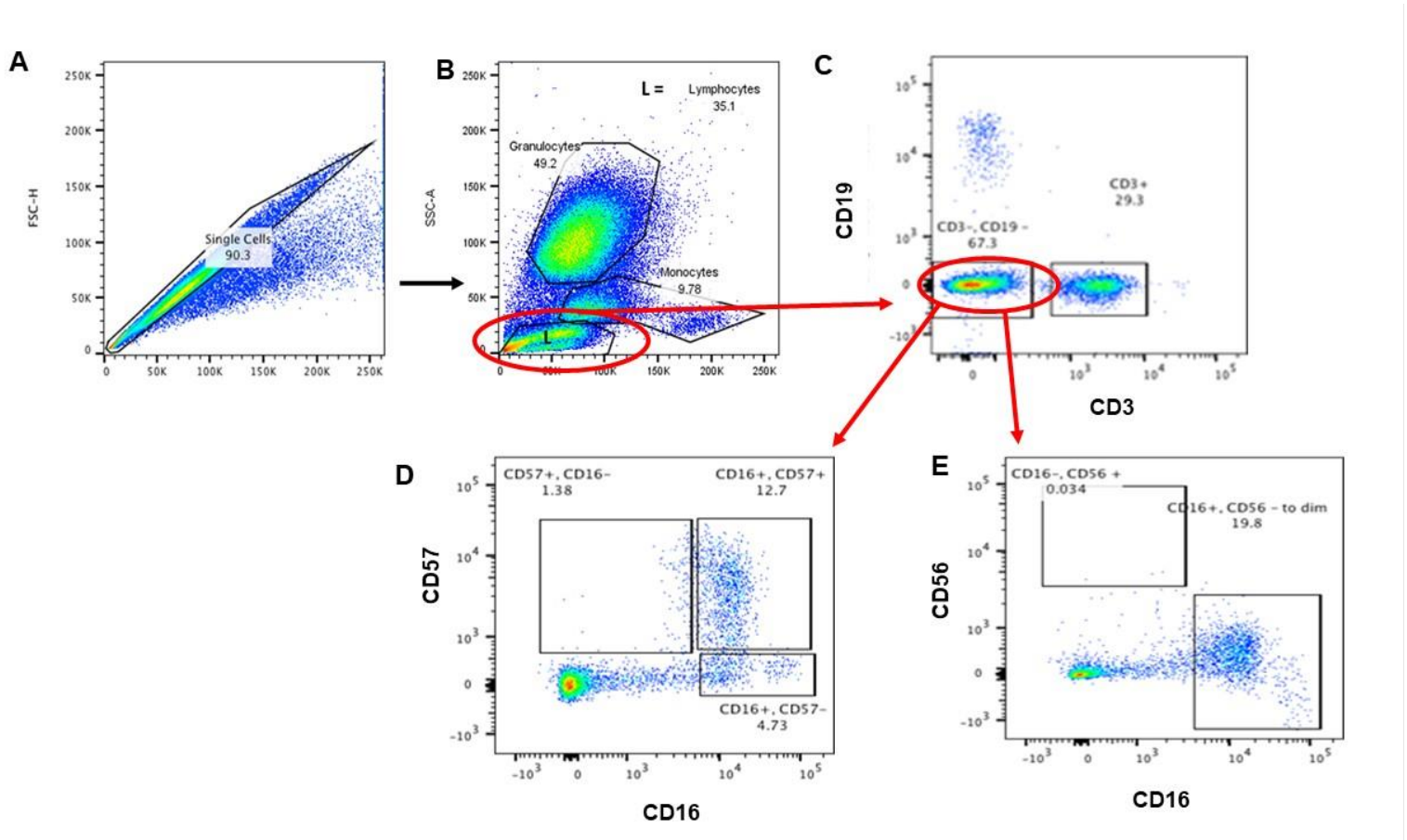


Figure 4.4 Gating strategy for natural killer cells. **A:** Doublets are clumped cells and may interfere in the reading of characteristics of single cells. They are excluded from using FSC-H and FSC-A. **B:** granularity (SSC-A) and size (FSC-A) were used to differentiate cells based on size, i.e. lymphocytes (smallest and least granular), monocytes (larger and more granular), and granulocytes (largest and most granular) leucocyte subpopulations. **C and D:** NK cell subsets expressing $CD16^{+}CD57^{+/-}$ or $CD16^{+}CD56^{-}$ were gated out from $CD3^{-}$ cells. CD:cluster of differentiation; FSH: forward scatter height; FSA: forward scatter area; HLA-DR:human leukocyte antigen D-related; NK: natural killer cells; SSC-A: side scatter area.

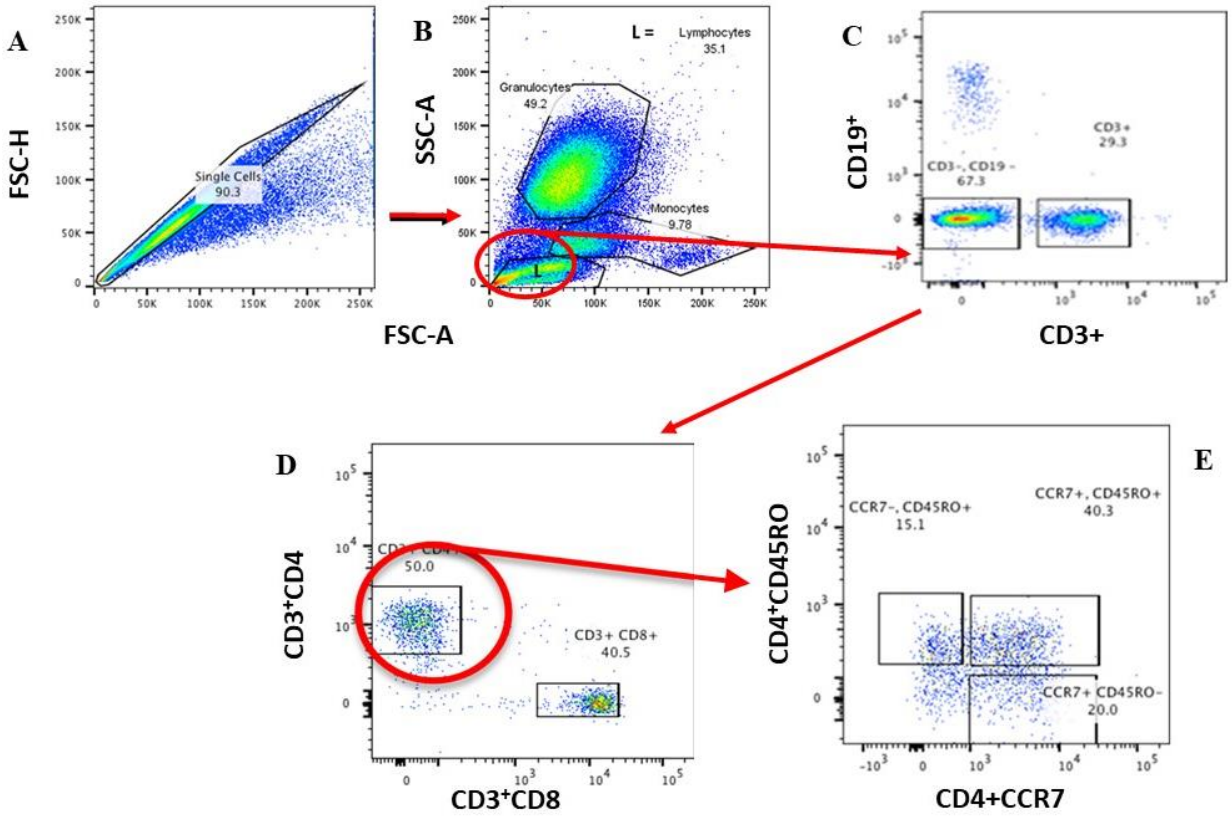


Figure 4.5 Gating strategy for lymphocytes (L). **A:** Doublets are clumped cells and may interfere in the reading of characteristics of single cells. They are excluded by using FSC-H and FSC-A. **B:** granularity (SSC-A) and size (FSC-A) were used to differentiate cells based on size, i.e. lymphocytes (smallest and least granular), monocytes (larger and more granular), and granulocytes (largest and most granular) leucocyte subpopulations. All graphs were gated using the corresponding unstained to distinguish positive and negative populations. **D**: CD4 and CD8 cells are both CD3+ and can be further gated into naïve and central memory and effector cells using CCR7 and CD45RO. **Notes:** CCR7:C-C chemokine receptor type 7; CD: cluster of differentiation; FSH: forward scatter height; FSA: forward scatter area; HLA-DR: human leukocyte antigen D-related; SSC-A: side scatter area.

4.3 Data analysis

For analyzing correlation, SPSS version 21 (IBM, Illinois, USA) was used. The Shapiro-Wilk test was used to test normality. Spearman's correlation was used to assess the correlation between clinical test results at admission and selected outcomes. The degree of association between selected clinical tests at admission (CRP, WBC, creatinine, and platelets) and patient outcomes (LOS and ICU admission) was analysed in Chapter 3 and detailed in sections 3.3.2 and 3.5.2.

For the RT^2 Profiler array: pooled cDNA samples (as described in 4.4.2.3) for the different AP severities were used for this study. Fold change was generated by inputting data into GeneGlobe software (https://geneglobe.qiagen.com/za/analyze/), (QIAGEN, Hilden, Germany). The Fold-Change (2^ (-Delta Delta CT) or $2^{-\Delta\Delta CT}$ was normalized gene expression $2^{\wedge-\Delta CT}$ in the AP pooled samples divided by the normalized gene expression $2^{-\Delta\Delta CT}$ in the healthy control sample (Livak and Schmittgen, 2001) (Bustin et al. 2009; Livak and Schmittgen, 2001). Fold-change values are greater than two represented changes in fold regulation. A negative fold regulation represented a down-regulation in gene expression, and a positive fold regulation represented upregulation. Fold-regulation is calculated as the negative inverse of the fold-change (RT^2 Profiler array protocol).

For the validation of gene expression in individual samples: normalisation was done using *RPL13A* on VIC dye (assay ID *Hs04194366_g1*, Thermo Fischer Scientific, Massachusetts, USA) (Yan et al. 2016). The target genes are listed in Appendix M. The Quant Studio™ 1 Real-Time System (Thermo Fischer Scientific, Massachusetts, USA) was used to run the RT-qPCR reactions. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression. The non-parametric Kruskal-Wallis test was performed to test for significant differences amongst groups and between days, where statistical significance was defined as $p \leq 0.05$.

Multicolour flow cytometry data were analysed using descriptive statistics, i.e. frequencies, due to the limitation in sample numbers in this aspect of the study

4.4 Results

4.4.1 Patient demographics

A breakdown of patients into the different analyses performed in this chapter is shown in Figure 4.1. For the demographics of the patients, refer to section 2.5.1.

4.4.2 Gene expression with RT^2 Profiler array (QIAGEN, Hilden, Germany)

A heatmap showing gene dysregulation in MAP, MSAP SAP patients compared to healthy controls is shown in Figure 4.6. Fold regulation was calculated for the 96 genes from the MAP, MSAP, and SAP for day 3 samples only and compared to healthy controls, refer as shown in Table 4.3. In the MAP group, a total of 31 genes were downregulated, and nine were upregulated. The *CXCL8* (fold change, median FC= -45.26) and *CD14* (FC= -21.58) genes were the most downregulated genes (refer to Table 4.3 and Appendix O).

In MSAP patients 68 genes were upregulated, and four were downregulated. The *CCL5* (FC= -3.76) and *APCS* (FC= 262.91) genes were the most downregulated and overexpressed, respectively. The MSAP patients had the highest number of upregulated genes, specifically those involved in inflammation such as *CRP* (FC=177.42), *FOX-p3* (FC=137.02*), IL-4* (FC=108.64), *IL-5* (FC=192.59*), IL-13* (83.66), *IL-10* (FC= 58.62) and *IL-23A* (FC=18.07), genes. Innate lymphoid cells group 2 (ILC2) transcription factor *GATA3* (FC =11.58) was also upregulated.

In the SAP group of patients, 34 genes were upregulated and 25 downregulated. The *CCR8* gene (FC) =1172.45) was upregulated, and *CD8A* (FC= -74.26) was the most downregulated (refer to Figure 4.5 A to B). *CCR8* increased steadily with disease severity and had the biggest fold change across all groups. The chemokine receptor *CCR6* was also downregulated in the MAP group (FC=-21.05). Other genes that increased with severity were *NOD1* and *TLR 1*. All these genes were closely associated with *CCR8*, as depicted in the heatmap branches in Figure 4.6.

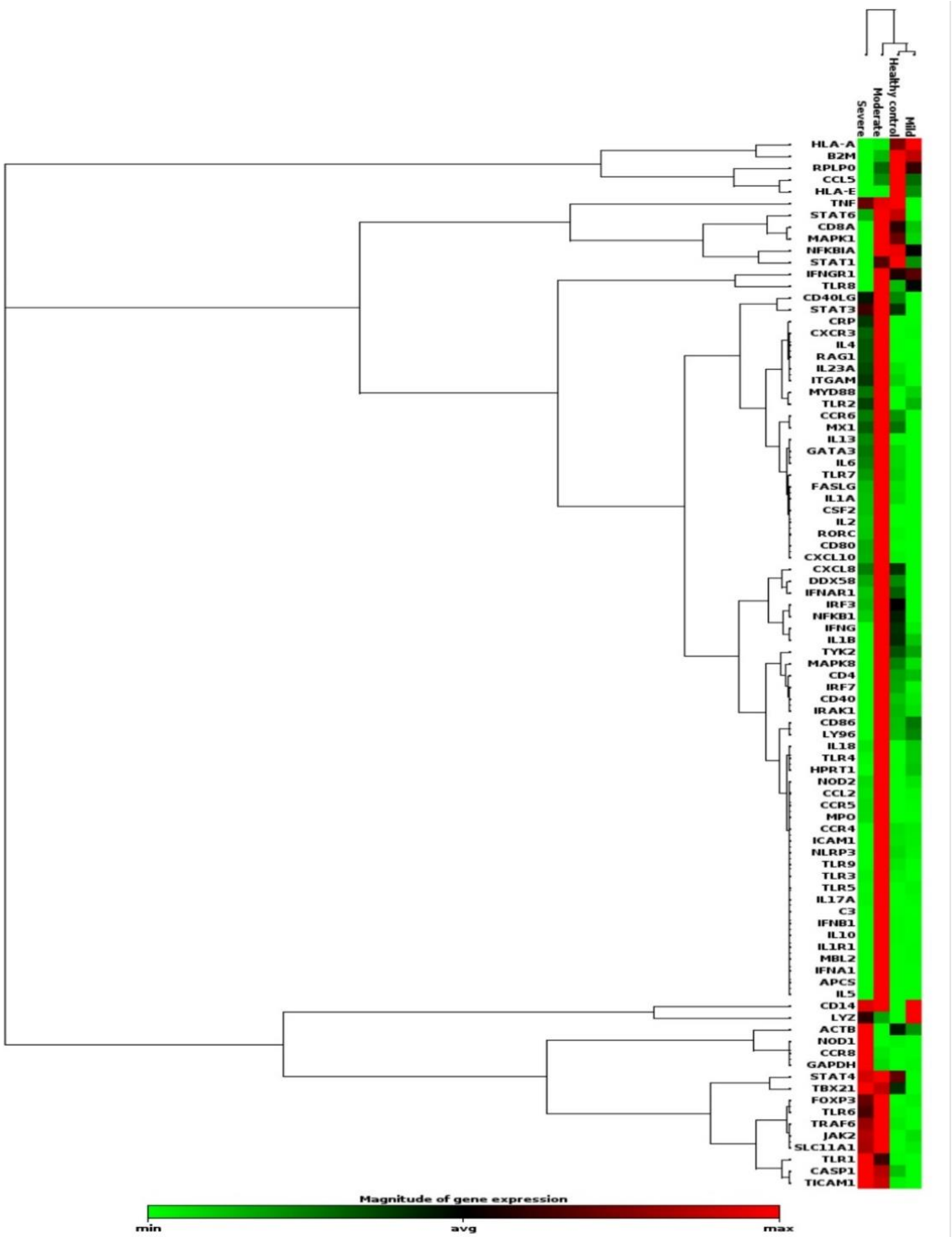


Figure 4.6. A heat map showing gene dysregulation in (a) mild (MAP), (b) moderate (MSAP) and (c) severe acute pancreatitis (SAP) patients compared to healthy controls (d). Hierarchical cluster of all the genes across patient severities. Red colour represents upregulated genes, green is downregulated, and black is unchanged. *CCR8* was the most upregulated in SAP, increasing with severity across the group. A list of the full names of genes is shown in Appendix L.

Based on the results from the analysis with the RT^2 Profiler array (QIAGEN, Hilden, Germany), *CCR8* was selected as the most upregulated gene in the SAP group. Six (6) *CCR8* related dysregulated genes and the *IL-6* gene, which was upregulated at the protein level in the cytokines studies (Chapter 3), were tested in validation studies reported in the next section (4.4.3). A detailed list of upregulated and downregulated genes can be found in Table 4.3.

Table 4.3 List of genes that were upregulated and downregulated in acute pancreatitis patients.

MAP				**MSAP**				**SAP**			
Upregulated genes		**Downregulated genes**		**Upregulated genes**		**Downregulated genes**		**Upregulated genes**		**Downregulated genes**	
CD14	10.99	*CCR6*	-21.05	*APCS*	262.91	*NOD1*	-14.62	***CCR8***	**1172.45**	*CD8A*	-74.26
LYZ	5.06	*IL1A*	-14.91	*IL1R1*	211.63	*B2M*	-5.02	*FOXP3*	96.27	*IFNG*	-21.58
FOXP3	3.9	*TBX21*	-13.82	***IL5***	**192.59**	*CCL5*	-3.76	***CRP***	**70.8**	***CCL5***	**-15.22**
TLR8	3.64	*STAT4*	-10.44	*CRP*	177.42	*HLA-E*	-2.6	***NOD1***	**64.21**	*TYK2*	-14.53
IL18	3.55	*IFNG*	-9.54	*RAG1*	169.88	*RPLP0*	-2.33	*RAG1*	56.67	*MAPK8*	-14.27
TLR4	3.27	***IL6***	**-9.15**	***FOXP3***	**137.02**	*HLA-A*	-1.88	*GAPDH*	44.64	*CD86*	-13.61
TLR2	2.95	***NOD1***	**-8.93**	*IL2*	134.43	*ACTB*	-1.54	*IL4*	36.83	*CD40*	-13.01
IL1R1	2.33	*CD40LG*	-7.62	*IL17A*	116.93	*STAT1*	-1.42	*TICAM1*	19.74	*B2M*	-12.61
CD86	2.14	*TLR9*	-6.18	***IL4***	**108.64**	*TNF*	-1.03	*IL13*	19.53	*IRF7*	-11.63
NOD2	1.84	*MX1*	-6.07	***MPO***	**91.77**	*NFKBIA*	1.02	*SLC11A1*	15.25	*IRAK1*	-9.81

Notes: For full names of genes, see Appendix L; CCR8 was the most upregulated gene in severe AP with a fold regulation of 1172.45, shown in bold. MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

4.4.3 Validation of selected genes in individual samples

Of the 40 patients recruited for this study, samples from 29 of them were included in the gene expression validation studies (Figure 4.1). A total of eight genes were analysed in the validation studies, namely *CCR8, CCL1, NOD1, IL-6, TLR1, CXCL-8, MPO,* and *CCL5.* Results from the gene validation studies were calculated using the $2^{-\Delta\Delta CT}$ method (Huggett et al., 2013; Livak and Schmittgen, 2001). The real-time PCR verification findings were analysed as the median fold change ($2^{-\Delta\Delta CT}$) in a severity group on specific days (i.e. day 3, 5 or 7 onset of pain) compared to the healthy controls. *RPL13A* gene was used to normalize the genes, and the average fold change of the control was used to calculate delta-delta C_T. The results of *CCL1* (*Hs00171072_m1*, Thermo Fischer, Massachusetts, USA) and *TLR1* (*Hs00413978_m1*, Thermo Fischer, Massachusetts, USA) gene expression in AP patients was undetected in most samples. Therefore, the average fold change could not be calculated in the different severity groups for these two genes. In the following

subsections, the results of the validation genes found to be dysregulated are presented. These are *CCR8, NOD1, I+L-6, CXCL-8, MPO,* and *CCL5.*

The results show that at Day 3 post-epigastric pain, the median FC of *CCR8* was most upregulated in the MSAP group with a median (IQR) of 28.1 (0-4130). This fold change dropped to 1.9 (0-202) on Day 5 and increased slightly by 2.1 fold (0-5078). In the SAP group, the Day 3 fold change was 2.4 (0-4362526). The upper quartile was affected by a patient who was a 69-year-old male who had no known comorbidities and was admitted to ICU for over two weeks. The patient's BMI was unknown. The median FC for *NOD1* was highest for the MAP group on Day 3, 5(1-15). For other groups at all time points, no change was observed. The *IL-6* gene maintained a median FC of 1 in all groups at all times. *CXCL8* gene had the most upregulated fold change on MAP Day 3 with a fold change of 6 (1-7). The most upregulated samples were in the MAP Day 5 with 3 (0-272). The *MPO* gene had a median FC of 72 (6 to 158) on Day 7 in the MAP group. This *MPO* gene was upregulated in the MSAP group on Day 7 (median FC=33 (18 to 524)) and the SAP group on Day 5 (median FC=27 (6 to 48)). The median FC for the *CCL5* gene was below 5 for MAP Day 5 and SAP Day 5.

Table 4.4 Median fold change $2^{\wedge-\Delta\Delta CT}$ and interquartile range for target genes in acute pancreatitis patients

Severity	*CCR8*	*NOD1*	*IL-6*	*CXCL-8*	*MPO*	*CCL5*
MAP D3	0.6 (0-1)	5(1-15)	1(1-1)	6 (1-7)	n/a	1 (0-2)
MAP D5	0.8 (0-2)	1(1-1)	1(0-2)	3 (0-272)	10 (1-19)	5 (1-5)
MAP D7	1.0 (0-2)	1(1-1)	1(0-2)	3 (1-10)	72 (6 to 158)	1 (0-1)
MSAP D3	28.1 (0-4130)	1(0-2)	1(0-2)	n/a	n/a	n/a
MSAP D5	1.9 (0-202)	n/a	n/a	0 (0-73)	10 (2 to 18)	0 (0-1)
MSAP D7	2.1 (0-5078)	0(0-1)	1(0-2)	1 (1-2)	33 (18 to 524)	1 (0-5)
SAP D3	2.4 (0-4362526)	n/a	1(1-2)	1 (1-2)	n/a	0 (0-1)
SAP D5	n/a	n/a	n/a	n/a	27 (6 to 48)	3 (0-5)
SAP D7	1.3 (0.9-1.8)	n/a	1(1-2)	4 (3-4)	n/a	n/a

Notes: MAP: mild AP, MSAP: moderately severe AP, SAP: severe AP; D: Day, e. g. D3 is Day 3 post-epigastric pain. *CCR8:* Chemokine receptor 8; *CCL1:* Chemokine ligand 1*; NOD1:* Nucleotide-binding oligomerization domain-containing protein 1*; IL-6:* interleukin-6*; TLR1:* Toll-like receptor 1*; MPO:* Myeloperoxidase; *CCL5:* Chemokine ligand 5 *CXCL8:* C-X-C Motif Chemokine Ligand 8. $2^{\wedge-\Delta\Delta CT}$ is the fold change used to measure the difference in the expression level of a gene (Livak and Schmittgen, 2001). n/a means not available due to single data points. *CCL1* and *TLR1* showed no expression in all samples.

4.4.4 Characterisation of innate and adaptive cells in acute pancreatitis patients

For the immunophenotyping analysis by flow cytometry, seven patients were recruited, 4 MAP, 2 MSAP, and 1 SAP (Figure 4.1). Only one patient in each group had Day 3 and 5 data, and the other patients came in on Day 5 and 7. Sampling limitations included patient dropout and late presentation at the hospital, and delays in the availability of the required antibodies for this aspect of the work. Therefore, the data presented here, for chapters 2 and 3, are preliminary. However, it should be noted that effort is made in the discussion to relate these findings with literature on similar work. For the healthy controls, six age-and sex-matched volunteers were recruited. The different cell populations and subpopulations analysed are listed in Table 4.1 and include B ($CD19^+$) and T lymphocytes and subsets (CD4, CD8, CD16, CD45RO, and CCR7), granulocytes (CD11b), and NK cells (CD16, CD56, and CD57).

Figure 4.7 shows how positively, and negatively stained cells were established for the different target populations. A representative stained sample of T lymphocytes ($CD3^+$cells) and B cells ($CD19^+$) is shown in A, and a representative unstained sample in B. These results show the separation of unstained cells in A from the stained cells, resulting from the establishment of negative controls (unstained B). A similar picture applies to all 12 fluorochromes used in the panel, shown in Table 4.1, albeit with slight differences due to the optimal antibody titre used.

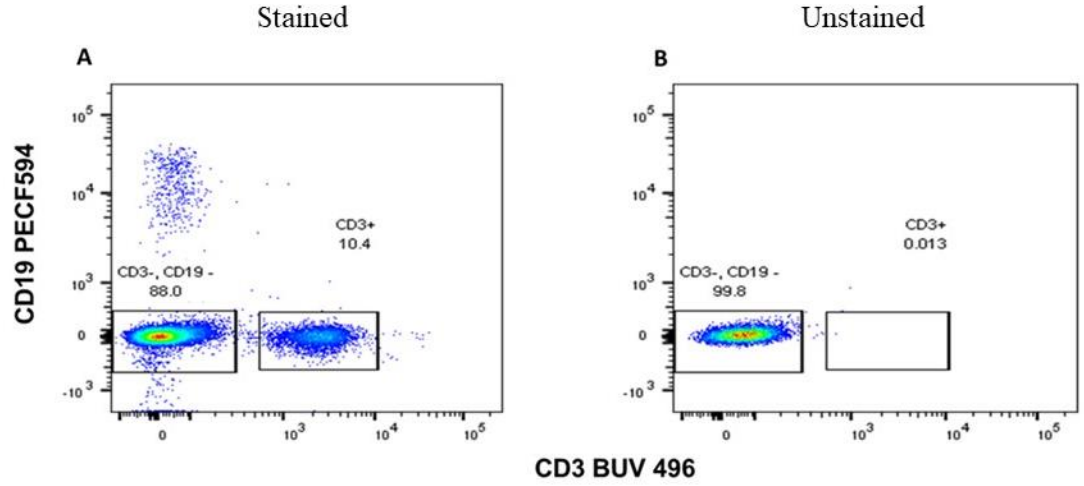


Figure 4.7 A shows the stained lymphocyte population for an MSAP patient, and B shows the same sample as an unstained population. These graphs were used to distinguish and gate the stained and unstained population to determine cell frequencies. The stained sample CD3+ cells (10.4% positivity) and the corresponding unstained sample (0.013%) are representative positive and negative gates. CD19 stained cells represent positively stained B cells in 4.7 A, absent in the unstained sample (4.7B). **Notes**: CD: cluster of differentiation; BUV: BD horizon Brilliant Ultraviolet™; PE-CF594: Phycoerythrin Cyanine-based Fluorescent dye.

4.4.4.1 Characterising innate immune cells

Monocyte cell populations and severity: Monocyte subsets were gated into three classes (Refer to Figure 4.2 for gating strategy and fluorochromes), classical monocyte ($CD14^{+}CD16^{-}$), intermediate monocytes ($CD14^{+}CD16^{+}$), and non-classical monocytes ($CD14^{-}CD16^{+}$). PerCPCy5. Monocytes expressing HA-DR were also gated using CD14 PerCPCY5.5 and HLA-DR BV650.

Classical monocyte subpopulations ($CD14^{+}$CD16-) were higher in more severe patients. The MSAP patients had 71% of the parent population on Day 3, which dropped to undetectable levels on Day 5 (Table 4.5). Classical monocytes increased from Day 3 (14%) to Day 7 (26%) in the SAP patient.

The percentage of HLA-DR^{-} monocytes decreased from 40% to 1% in the MSAP patient on Day 3 to Day 5 and decreased to 1% by Day 7, respectively (Table 4.5). In the SAP patient, the HLA-DR^{-} monocytes showed a slight decrease from 22% to 17% from Day 3 to Day 7. Representative data for MSAP and SAP patients are shown in Figures 4.8 and 4.9, respectively.

Table 4.5 Total percentage of parent population of natural killer cells and monocytes

		% NK cell subsets ($CD3^-$)					% Monocyte subsets					
Severity group	**No of Patients**	**$CD16^+$ $CD56^-$**	**$CD16^+$ $CD57^+$**	**$CD16^+$ $CD57^-$**	**$CD57^+$ $CD16^-$**	**Percentage of Monocytes in parent population**	**$CD14^+$ $HLA\text{-}DR^+$**	**$CD14^+$ $HLA\text{-}DR^-$**	**$CD16^+$ $CD14^-$**	**$CD16^+$ $CD14^+$**	**$CD16^-$ $CD14^+$**	**$CD14^+$ $CD56^+$**
Healthy Control	*n*=6	2	1	1	1	7	10	1	2	1	9	0
						DAY 3						
MAP	*n*=1	9	3	3	12	2	41	12	5	11	43	1
MSAP	*n*=1	20	13	5	1	10	4	4	7	8	71	1
SAP	*n*=1	8	6	2	4	2	4	22	14	13	14	6
						DAY 5						
MAP	*n*=4	0	0	0	0	10	0	0	0	0	0	0
MSAP	*n*=2	50	28	19	2	2	47	40	16	10	0	3
SAP	*n*=1	16	14	4	2	2	14	19	8	14	21	6
						DAY 7						
MAP	*n*=3	24	17	7	1	26	3	1	4	4	1	0
MSAP	*n*=1	1	0	0	0	16	1	1	1	1	1	0
SAP	*n*=1	1	0	0	0	3	15	17	10	16	26	7

Notes: CD: cluster of differentiation; HLA-DR: human leukocyte antigen D- related; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis; *n*: number of patients; NK: natural killer cells. These cells were gated, as represented in Figure 4.2 monocytes and 4.4, respectively.

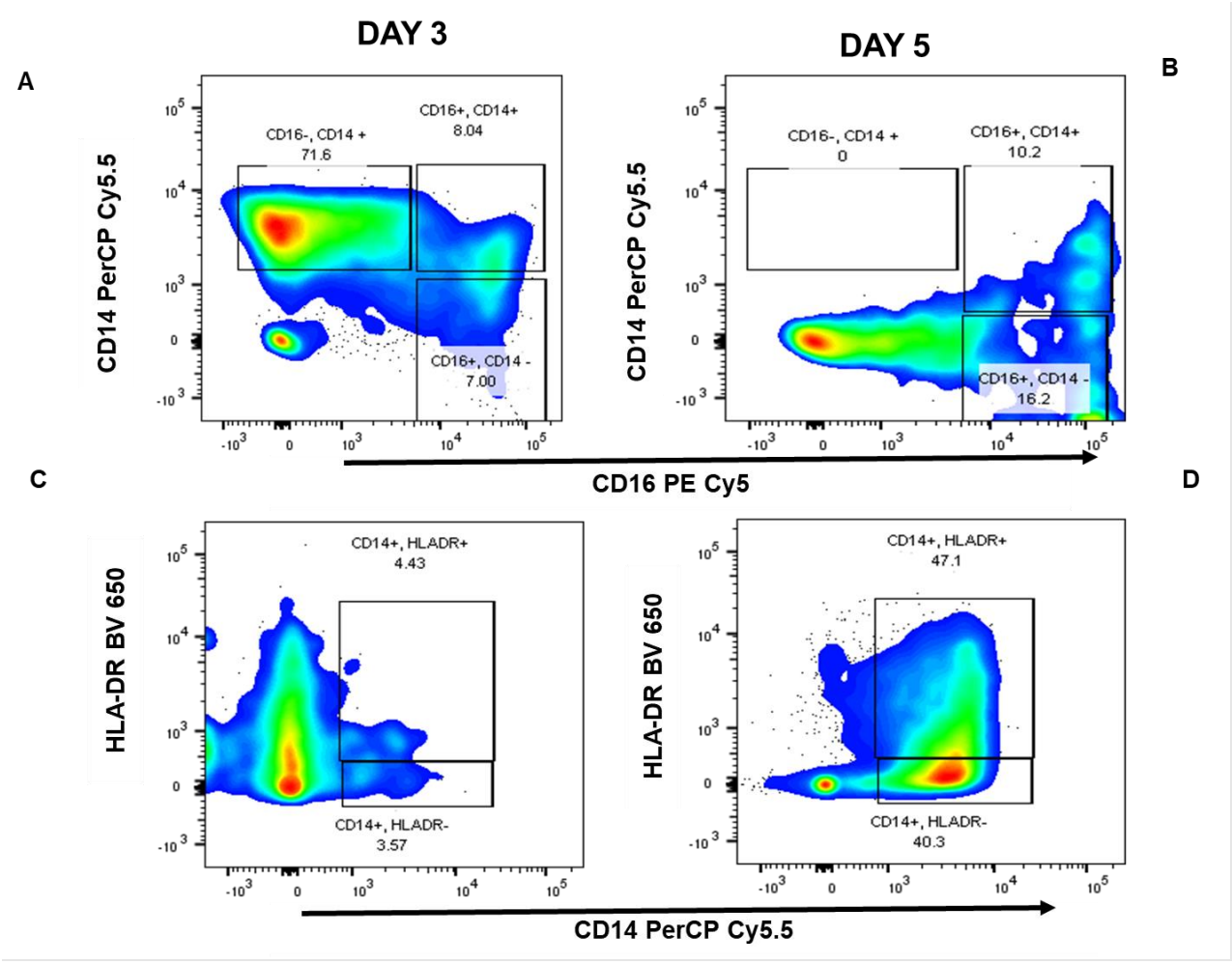


Figure 4.8 Moderately severe acute pancreatitis profile of the monocyte subpopulation. Cells were gated into intermediate ($CD14^{+}CD16^{+}$), classical ($CD14^{+}CD16$-), non-classical monocytes ($CD16^{+}CD14$-) and $HLA\text{-}DR^{+}$/- monocytes. A and B show CD14 PerCP Cy5.5 and CD16 PECy5 plots for days 3 and 5, respectively. **C** and **D**: show a plot of CD14 PerCP Cy5.5 and HLA-DR BV650 for days 3 and 5, respectively. **Notes:** BUV: BD Horizon Brilliant™ Ultraviolet; Cy: Cyanine; B: Brilliant Violet™; HLA DR: human leukocyte D related; PE: Phycoerythrin; PerCP: Peridinin-Chlorophyll-protein. All antibodies are from BD Biosciences (New Jersey, USA).

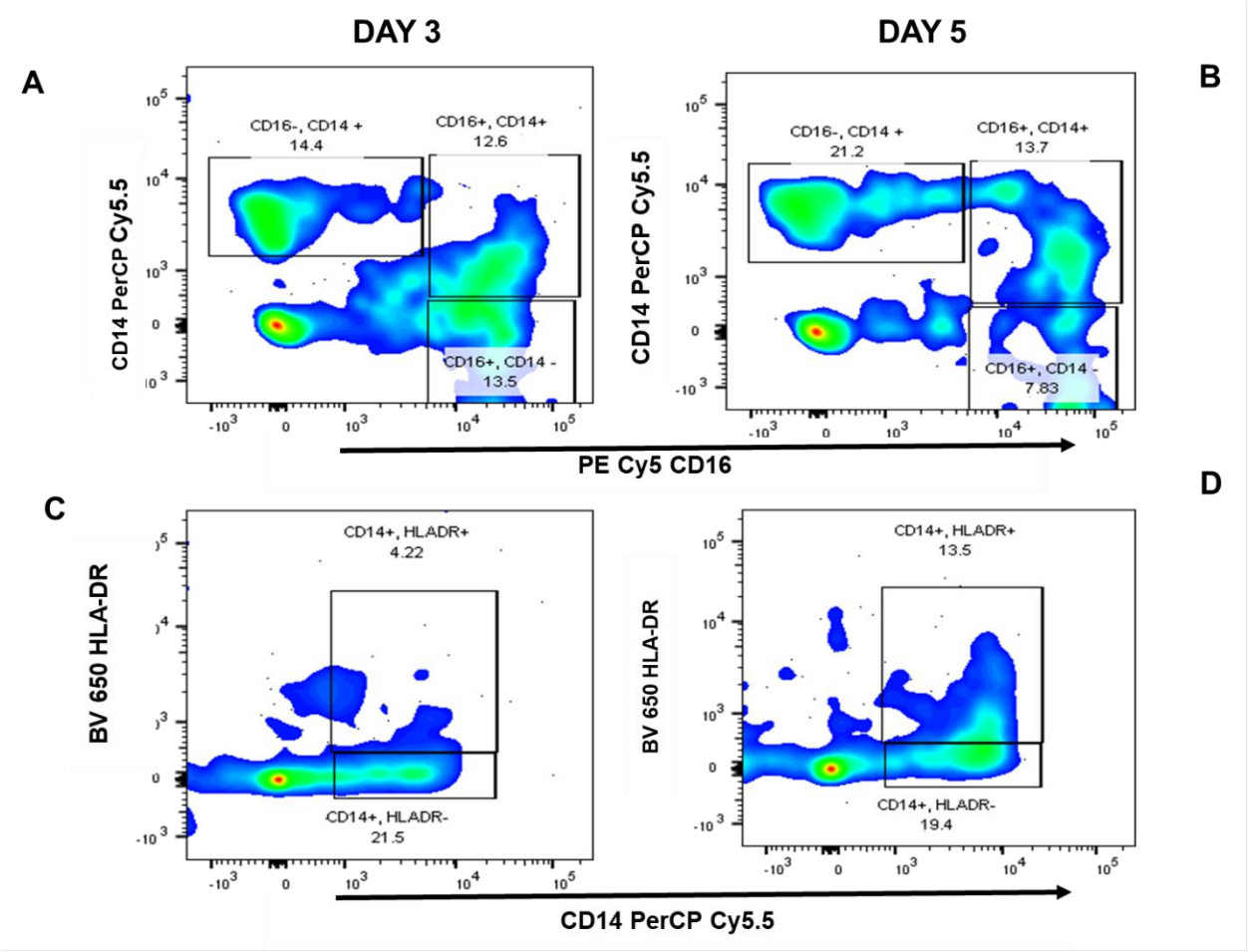


Figure 4.9 Monocyte subpopulation and associated markers in a severe acute pancreatitis patient sample. The plot was generated using FlowJo™ version 10 (Oregon, USA). Cells were gated into intermediate ($CD16^+CD14^+$), classical ($CD16^-CD14^+$) and non-classical monocytes ($CD16^+CD14^-$). Figures 4.9 A and B show the CD14CD16 plot for Day 3 and Day 5, respectively. Figures 4.9 C and D show a plot of CD14HLA-DR for Ddays3 and 5, respectively. The percentage of $HLA\text{-}DR^+$ monocytes increased from 4 % on Day 3 to 14% on Day 5. **Notes:** BUV: BD Horizon Brilliant™ Ultraviolet; Cy: Cyanine; B: Brilliant Violet™; HLA DR: human leukocyte D related; PE: Phycoerythrin; PerCP: Peridinin-Chlorophyll-protein. All antibodies are from BD Biosciences (New Jersey, USA).

Natural killer subsets in AP patients and immune suppression: Both $CD56^-$ and $CD57^-$ NK subsets did not show a clear trend in all three severity categories. Of note is the complete depletion of these cell subsets in MSAP and SAP patients by Day 7. The cells were also depleted in the MAP group at Day 5 post epigastric pain (refer to Table 4.5)

4.4.4.2 Characterising adaptive immune cells

Lymphocyte B and T cells were frequent in acute pancreatitis patients: CD4 and CD8 cells were gated using CD3BUV496, CD4Alexa Fluor, and CD8BV605 shown in the gating strategy in Figure 4.5. Naive CD4 and CD8 T cells are $CCR7^+CD45RO^–$, central memory T cells (T_{CM} cells) are $CD4^+CD45RO^+CCR7^+$, and effector memory T cells are $CD4^+CD45RO^+CCR7^–$ (T_{EM} cells). There was much variation in the frequencies of cells in the three severity groups. Table 4.6 shows that on days 3 and 5, all three subsets were depleted in the SAP patient with undetectable frequencies of these cells. On Day 7, the T_{EM} cells frequency was relatively high with 44% and more than twofold that of the healthy control sample.

The $CD8^+$ cells were relatively stable in all three risk categories, despite the *CD8A* gene being downregulated at Day 3 in the pooled sample (FC= -74.26) in the MSAP group in the gene analysis (Table 4.3). The B cells were gated using $CD3^-CD19^+$ gates. B cell frequency was also quite low for both MAP and SAP on days 5 and 7 with less than 2% frequencies. MSAP B cell frequency reached 14% on Day 5, a more than three-fold increase from Day 3.

Table 4.6 The percentage of parent populations of B and T lymphocytes in acute pancreatitis patients at different days

					$CD4^+$ T cells					Total B Cells
	No of patients/ volunteers	**Total $CD3^+$ lymphocytes**	**Total $CD4^+$**	**Total $CD8^+$**	**T_{CM} $CD4^+CCR7^+$ $CD45RO^-$**	**Naïve $CD4^+CCR7^+$ $CD45RO^+$**	**T_{EM} $CD4^+CCR7^-$ $CD45RO^+$**	**$CD4^+$ $CD16^+$**	**CD4+ $CD57^+$**	**CD3- CD19+**
Healthy control	*n*=6	27	65	28.0	35	30	15	2	27	4
					DAY 3					
MAP	*n*=1	58	61	25.5	8	36	25	1	28	6
MSAP	*n*=1	29	57	36.3	20	40	15	18	6	3
SAP	*n*=1	3	0	34.7	0	0	0	0	0	4
					DAY 5					
MAP	*n*=4	0	58	47.1	3	53	27	1	27	0
MSAP	*n*=2	62	46	43.0	0	1	72	8	11	14
SAP	*n*=1	20	0	57.7	0	0	0	0	0	2
					DAY 7					
MAP	*n*=3	58	46	48	15	26	39	27	6	2
MSAP	*n*=1	29	24	72	25	26	37	6	24	1
SAP	*n*=1	3	46	39	13	28	44	1	3	1

Notes: T_{CM} ($CD4^+CCR7^+$ $CD45RO^-$) are central memory cells; T_{EM} ($CD4^+CCR7^-$ $CD45RO^+$) are effector cells. Where there was more than one patient, the mean frequency was recorded.

4.3.5 Evaluating the neutrophil-lymphocyte ratio:

The total parent population of neutrophils was gated from granulocytes using FSC-A and SSC-A, and then $CD56^{-}CD11b^{+}$ gated as described in Figure 4.3B to D. The total lymphocyte population gate in Figure 4.5B was used as the total lymphocyte population to calculate the neutrophil-lymphocyte ratio (NLR). The NLR was assessed using one patient from each group on days 3, 5, and 7 compared to an age and sex-matched healthy control sample. The NLR ratio was calculated for the MAP, MSAP, and SAP patients (Table 4.7). Normal NLR is within 0.78 and 3.53 (Forget et al., 2017).

The mean NLR was 0.52 in six (6) healthy controls in the patients' samples at day 3. Each group included one patient. The NLR for day 3 was 3.00 (MAP), 4.00 (MSAP), and 0.02 (SAP). On day 5, the NLR was 0.75 (MAP), 2.6 (MSAP) and 0.3 (SAP). On day 7, the NLR was 0.94 (MAP), 1.2.1 (MSAP) and 0.98 (SAP). The MSAP patient had a higher percentage of neutrophils than lymphocytes throughout the seven (7) day period, whilst the SAP group had 50 times more lymphocytes on day 3, which decreased to 1:1 by day 7.

Table 4.7 The total neutrophil and lymphocyte populations

Severity /Day	No of patients/ volunteers	Total lymphocytes	Granulocytes	Neutrophils $CD56^{-}$ $CD16^{+}$	Neutrophils $CD56^{-}$ $CD11b^{+}$	Total population of $CD56^{-}CD11b^{+}$ in granulocyte population*	Neutrophils $CD56^{+}$ $CD16^{+}$	Neutrophils $CD56^{+}CD16^{-}$	Neutrophils $CD56^{-}$ $CD16^{-}$
					(%)				
Healthy Control	*n* = 6	***27***	14	96	97	***14***	0.7	0.0	3.5
					DAY 3				
MAP	*n* = 1	***20***	61	99	99	***60***	0.3	0.0	0.5
MSAP	*n* = 1	***11***	49	91	89	***44***	0.3	0.0	8.9
SAP	*n* = 1	***5***	88	65	1	***0.1***	2.1	1.3	32.0
					DAY 5				
MAP	*n* = 4	***28***	28	71	75	***21***	0.3	0.0	28.7
MSAP	*n* = 1	***19***	51	96	99	***50***	1.7	0.0	2.6
SAP	*n* = 1	***7***	74	96	0.3	***0.2***	1.3	0.0	2.7
					DAY 7				
MAP	*n* = 3	***18***	19	75	87	***17***	2.3	0.0	22.8
MSAP	*n* = 1	***23***	30	89	93	***28***	0.5	0.0	10.3
SAP	*n* = 1	***46***	46	96	98	***45***	1.3	0.1	2.6

Notes: All numbers are represented as a percentage (%); The table shows data to one decimal place were minor (i.e. ≤ 1%) differences between parent and subsets populations. (*)The total population of $CD56^{-}CD11b^{+}$ in the granulocyte population was calculated as a percentage of the total granulocytes (i.e. 97% of total granulocytes were $CD56^{-}CD11b^{+}$, therefore 97% of 14%= 14%).CD: cluster of differentiation; D: Day; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

4.4 Discussion

4.5.1 Monocytes and acute pancreatitis

Characterization of innate and adaptive immune cells and related genes show promising linkages to disease severity in AP. Macrophages and monocytes have been implicated in the excessive recruitment of neutrophils that cause immune dysregulation and ultimately OF (Manohar et al., 2017; Fonteh, Smith and Brand, 2018). The neutrophil chemoattractant gene, *CXCL-8,* and *CD14* gene, expressed on the surface of macrophages and monocytes, respectively, were downregulated in the pooled MAP group sample. At the site of inflammation, resident macrophages express the *CXCL-8* gene to recruit neutrophils. These monocytes were once circulating classical monocytes (Prame Kumar, Nicholls and Wong, 2018; Peng, Li and Yu, 2021). Characterization of the immune cell population in whole blood samples showed an increase in monocyte cell subsets over time, specifically, classical monocytes and depletion of NK cell subsets. Classical monocyte subpopulations ($CD14^{+}CD16$-) were higher in the MSAP and SAP patients than healthy controls at Day 3. Acinar cell death in the MSAP and SAP patients is necrotic and produces proinflammatory molecules which attract the M1 phenotype of macrophages. The fate of classical monocytes in tissue is more than likely into the M1 macrophage phenotype (Hu et al., 2020). Therefore the presence of classical monocytes in both the MSAP and SAP patient is expected.

Furthermore, $HLA\text{-}DR^{-}$ monocytes decreased in the MSAP patient on Day 5 and 7, respectively. The Day 7 $HLA\text{-}DR^{-}$ monocyte frequency of the MAP and MSAP group was at 1%, indicating resolving inflammation. This marker may distinguish between the permanent and transient OF used to diagnose SAP and MSAP, respectively. Dabrowski et al. found that $HLA\text{-}DR^{-}$ monocytes were associated with organ dysfunction, and Zhang et al. demonstrated that these cell subsets led to a compromised T cell response rendering more SAP patients immunocompromised (Dabrowski et al., 2008; Zhang et al., 2019)

4.5.2 T helper (Th) 2 Response in moderately severe acute pancreatitis

The cytokine IL-4 and transcription factor GATA-3 are involved in the differentiation of Th2 cells and the regulation of Th2 cell differentiation, respectively (Vivier et al., 2020; Zhou et al., 2020; Zhu and Zhu, 2020). The genes that express these molecules, namely, *IL-4* and *GATA-3*, were upregulated in the pooled MSAP sample group. The *IL-5* and *IL-13* are regulated by transcription factor GATA-3 and are involved in the maturation of lymphocytes and inhibiting pro-inflammatory cytokines (Vivier et al., 2020; Zhu and Zhu, 2020). The presence of these molecules

shows evidence of a strong type 2 response in MSAP patients. The CD4 T cell subsets, including T_{EM} cells ($CD45RO^{+}CCR7$), were depleted in the SAP in the first five (5) days post epigastric pain, then they increased to 44% on Day 7. When read together with frequencies of monocytes and NK cells, these findings confirm the current literature proposed by Zhang and colleagues that downregulation of HLA-DR on monocytes is linked to immune suppression in AP patients and a compromised T cell response (Zhang et al., 2019). These studies also support the resulting strong correlation observed between WBC and LOS in sampled patients.

4.5.3 Chemokine (C-C motif) receptor 8 gene expression

The most upregulated gene in the RT^2 profiler array analysis and in the pooled sample of the SAP group was *CCR8* (FC =1172.45). The *CCR8* gene is a chemokine with a C-C motif and is expressed on cells that induce IL-6 cytokine production through the MAPK signalling pathway (Oshio et al., 2014). This gene is mainly expressed by Th2 lymphocytes and peritoneal macrophages (PMφ) (Oshio et al., 2014). Since MAPKs are critical in regulating gene expression, differentiation, apoptosis and other cell functions, it is crucial to consider the linkages between CCR8 gene upregulation and this signalling pathway (Irrera et al., 2014). Studies have demonstrated that MAPK and NF-κB pathways are activated by TLR-4 pathways downstream, resulting in the production of proinflammatory molecules, including IL-6 cytokine (Scheller, 2011; Oshio et al., 2014). The acute inflammation occurring in AP may be due to the IL-6 trans signalling pathway, producing C-C chemokines and continuously attracting monocytes from the periphery (Scheller, 2011). The results demonstrate a consistent presence of monocytes in the SAP group.

Findings presented in Chapter 3 show that IL-6 was consistently elevated in the SAP patient group, establishing a linkage between CCR8 gene expression and IL-6 cytokine elevation. When expressed by T_{regs}, *CCR8* drives immunosuppression (Barsheshet et al., 2017). Additionally, the *CCR8* chemokine is known to stimulate cytokine production in tissue-resident macrophages through the MAPK pathway, which regulates the production of IL-10, IL-6, and NF-κB (Oshio et al. 2014). All these molecules play different roles in AP pathogenesis. The IL-10 cytokine is an anti-inflammatory marker, whilst IL-6 and NF-κB are key pro-inflammatory molecules in the initial innate response due to inflammation (Mayerle et al., 2012; Peng, Li and Yu, 2021). The *Fox-p3* gene is a transcription factor that is expressed on $CD4^{+}$ T_{regs}, which are responsible for homeostasis. T_{regs} produce IL-10 and TGF-β, which resolve the immune imbalance caused by the acute inflammatory response by suppressing the immune system and preventing autoimmunity

(Zhu and Zhu, 2020). The *IL-10* gene was upregulated in MSAP (FC= 58.62). Barsheshet and coauthors found that $CCR8^{+}$ T_{regs} made up 30% of this cell population in the bloodstream. These cells will activate STAT 3 mediated FOXp3 and IL-10, resulting in homeostasis (Barsheshet et al., 2017). Therefore, *CCR8* gene expression has the potential to be a promising marker to distinguish MSAP and SAP patients.

4.5.4 Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) gene expression

The *NOD1* gene, a PRR, detects peptidoglycan derivatives from bacteria and triggers innate immune responses by activating macrophages and peritoneal neutrophils amongst other inflammatory cells (Jeon et al., 2012; Shay and Kang, 2013; Uhlen et al. 2019). The initial screening of pooled samples showed upregulation of *NOD1* in the SAP group (median FC=64.21) and downregulation in MSAP (median FC=-14.62) and MAP (median FC= -8.93). In AP, the *NOD1* receptor is known to activate the NF-κB signalling pathway that activates monocytes and macrophages to the injury site through secretion of MCP-1 (Hu et al., 2020). However, in individual analysis, the *NOD1* gene was most upregulated in MAP Day 3 patients with a median FC=5. In the higher severities, there was no change. The SAP group had only one data set for each day, and the FC ranged from 1 to 3. Out of the three (3) SAP patients initially included in the pooled sample, only one sample showed expression of the *NOD1* gene. In SAP, upregulation of the NOD1 receptor regulates NF-κB expression and recruitment of inflammatory molecules, including IL-6 (Yan et al., 2017). Therefore in the MAP, group the NF-κB signalling pathway may have influenced the observed decrease in IL-6 cytokine levels over time and is unlikely due to bacterial infection because inflammation in MAP patients remains sterile throughout the course of the disease.

4.5.5 Toll-like receptor 1 (TLR1) gene expression

The *TLR1* gene was also linked to *CCR8* gene expression, and it is a receptor expressed by neutrophils, classical, non-classical, and intermediate monocytes (Uhlen et al., 2019). This receptor acts as a PRR for PAMPs and activates innate immunity (Shay and Kang, 2013; O'reilly and Duffy, 2016). The median FC from the RT^2 profiler array for MAP, MSAP, and SAP groups was -1.09, 8.4, and 12.95, respectively. The TLR1 gene was therefore upregulated in the MSAP and SAP group, which is expected as monocyte subpopulation is higher in more severe AP risk categories (Zhang et al. 2019). However, none of the individual samples showed any expression

of this gene. The downstream effect of the TLR1 receptor expression in AP is the activation of the NF-κB signalling pathway, which may result in the elevation of IL-6 (Vaz, Akbarshahi and Andersson, 2013). The result may be a reflection of the time of measurement of the blood sample.

4.5.6 Interleukin-6 gene expression

The *IL-6* gene was selected for the validation studies because of the IL-6 cytokine elevated concentration observed in MSAP and SAP group in Chapter 3. The *IL-6* receptor is found on blood monocytes, neutrophils, and naïve CD4 T cells (Shay and Kang, 2013). IL-6 cytokine plays a role in differentiating Tfh cells and Th17 cells (Zhu and Zhu, 2020). The *IL-6* gene encodes the IL-6 cytokine that induces inflammation and activates the mature B cells in the presence of the Bcl6 transcription factor and IL-21 cytokine (Kang et al. 2019). In chapter 3 results, IL-21 was elevated in the plasma of healthy controls and on Day 3 and 7 in MAP and MSAP. They showed antibody production in these two groups compared to the low concentration observed on days 3 and 7 in the SAP group. In the event of IL-6 being elevated in the presence of TGF-β, naïve $CD4^+$ T cells will differentiate into Th17 cells (Kang et al. 2019; Zhu and Zhu, 2020). An imbalance in the ratio of Th17 and T_{regs} will result in dysregulation (Kang et al., 2019). The low levels of IL-21 cytokine in SAP and elevated levels of IL-6 point towards a Th17 response.

4.5.7 Chemokine ligand 5 (CCL-5) gene expression

The *CCL5* gene was downregulated in the SAP group with FC=-15.22 and was of interest because of its role as an HIV-suppressive factor and its ability to attract memory T cells (Shay and Kang, 2013; Santopaolo, 2021). In the SAP group, two (2) out of the five (5) patients were HIV positive; one had type II diabetes and was one of four (4) mortalities (refer to Chapter 2 for more details). A link between downregulated *CCL5* and HIV status could not be made as most patients in South Africa are placed on HIV treatment when they test positive (Nel et al., 2020). The potential role of AP in a population with a high HIV rate requires further investigation.

4.5.8 Myeloperoxidase (MPO) gene expression

The MPO molecule, which forms part of components of NETs, is released by activated neutrophils and has been implicated in lung injury in SAP patients (Merza et al., 2015).The *MPO* gene, which had an FC= 91.77 in the MSAP group from the pooled sample, is expressed mainly by classical monocytes. However, it is also found on intermediate and non-classical monocytes and neutrophils

(Uhlen et al., 2019). Although this was not the case in individual sample expression, this gene is discussed further in the cell frequencies in different risk categories.

In the individual samples, the *MPO* gene was upregulated on Day 5 in all severity groups in the individual validation samples, especially the MSAP group. The MPO protein is an antimicrobial substance found on activated neutrophils and is responsible for forming NETs that cause sterile inflammation and may lead to OF in AP (Carestia, Kaufman and Schattner, 2016; Peng, Li and Yu, 2021). The upregulation of the *MPO* gene at Day 5 and 7 in the MSAP group aligns with the observed increase in classical monocytes in the MSAP patients on Day 5, which are major CXCL-8 chemoattractant producers. Further research on distinguishing the MSAP and SAP, using *MPO* gene expression levels as a good predictor of transient and permanent OF at admission with a larger cohort of patients and earlier time points is required.

4.5.9 Neutrophil lymphocyte ratio

Azab and colleagues were the first to describe NLR as a potential marker in determining severity, explicitly determining which patients required ICU admission and prolonged LOS. Their 2011 study found that an NLR greater than 4.7 predicted adverse outcomes (Azab et al., 2011). A review of the prognostic potential of NLR in AP argues that without organ failure data and very low in-hospital mortality, the Azab paper is flawed. The Azab study included a cohort of 146 AP patients, with many having a mild form of the disease (Cho et al., 2018). In addition, it is not clear how many of the patients had moderate disease, and before the revisions on the RAC (before 2012), there were only two classifications, MAP and SAP (Banks et al., 2013). Only seven (7) patients were included in the current study, and the NLR was mainly for individuals. The limitations of the NLR analysis include a small sample size and missing data in terms of organ failure assessment from hospital files. Although Cho and colleagues have highlighted that NLR is not a significant independent prognostic marker, the data in the current study is not sufficient to refute or support this claim.

4.5.10 Potential markers in acute pancreatitis

In conclusion, there is potential for *CCR8* and *MPO* genes as severity markers. The link between these markers and IL-6 cytokine elevation, the increase in monocyte cell population frequency, and the depletion of NK cells in MSAP and SAP groups requires further investigation.

CHAPTER 5
DISCUSSIONS, RECOMMENDATIONS, AND CONCLUSIONS

This study investigated the expression patterns of several inflammatory and immune response-related molecules at the early stages of AP to identify markers that can assist with stratification and complement existing grading and scoring systems. The challenge with existing markers for predicting severity is that they are not disease-specific and are mainly inflammatory markers that can be applied to multiple diseases.

5.1. Discussion

The international prevalence of AP is currently 80 per 100,000 (Anderson and Thomson, 2017). There is a paucity of data on AP in South Africa. Reported risk factors of SAP include Age, obesity, ARVs, comorbidities, and BMI (Weitz et al., 2016; Shah, Mourad and Bramhall, 2018). The South African population consists of approximately 80% of people younger than 60 years old (Stats SA, 2019). Age as a risk factor for AP translates to prolonged LOS (Yadav and Lowenfels, 2006; Roberts et al., 2017). This study showed that 87.5% of the patients (35/40) presented with either MAP or MSAP, and the remaining 12.5% (5/40) were SAP patients. The most common comorbidity reported in the patient cohort was HIV. Other comorbidities included hypertension, non-diabetic hyperglycaemia, and type II diabetes. HIV has been previously reported as a comorbidity of AP in the South African population (Anderson and Thomson, 2017). Data on disease prevalence, however, remains a challenge. The LOS, on average, was longer in more severe patients, and ICU admission was equally more common in severe patients. Important observations regarding admission data were made.

The research findings highlight the role of monocytes, lymphocytes, and neutrophils in predicting the severity of AP patents. Linkages between clinical outcomes and immune markers were considered to determine the translational potential of the results in clinical practice. The cytokine, IL-6, was the only protein that showed a trend with a significant difference in the three risk categories of AP. The cytokine IL-21 showed a downward trend with increasing severity with the lowest concentration in the SAP group. The consistently high levels in the concentration of IL-6

protein (>50pg/mL) in the SAP group in peripheral blood are likely due to activated monocyte subsets. The results suggest that the possible link between monocytes and NK cells may determine whether a patient will present with MSAP or SAP. The cells and genes expressed are downstream products of the MAPK and NF-κB pathways mainly related to IL-6 trans signalling (Scheller et al., 2011).

The upregulated expression of the *CCR8* gene in MSAP and SAP may provide evidence of macrophage activity initiated in the peritoneal cavity and elevated monocytes in MSAP and SAP in the periphery. The gene expression results show evidence of linkages with specific CD4 T cell subsets. The data supported the possibility of a Th2 response driving MSAP disease pathology and a Th17 response driving the SAP group pathophysiology and maintained inflammation that is observed in many autoimmune diseases. The *MPO* gene also emerged as a potential prognostic marker, and together with *CCR8*, require further investigation in a larger cohort.

5.2 Revisiting the Research Objectives

Taken together, we have shown the role of Th1, Th2 and Th17 related cytokines and how they are polarized in AP with IL-6 being a prominent mediator. Novel markers (*CCR8* and *MPO*) with potential for prognosticating AP were identified. Characteristic phenotypic changes were shown, where lymphocytes tend to decrease with increasing severity and classical monocytes decreased and increased with time in MSAP and SAP patients respectively. We were also able to correlate the immune status of AP with the clinical outcomes such as LOS, which was longer in more severe patients.

5.3 Study Limitations, Conclusions, and Recommendations

5.3.1 Limitations of the study

Like many clinical studies, obtaining an ideal study cohort that is adequate (not too small or too big) for interpreting is essential for producing accurate and reliable reproducible data. The sample size at this point is more of a pilot.

The initial recruitment of neutrophils to the site of injury in the case of acute inflammation is within the first 48 hours, after which monocytes and CD T cells are recruited (Scheller et al., 2011). This action alone needs to be well understood to predict which proinflammatory molecules may act as disease predictors and potential immune therapy markers. Upon reflection, analysis of prognostic markers in disease may benefit from complementary studies in animal models. A controlled

environment measuring the same parameters as those analysed in patient samples can give a clearer picture of what molecules are being produced at what time.

Other study limitations included several logistical challenges. After consenting, the patient drop-out rate; some patients were too weak or sick to participate, especially from the SAP group. Arrival at the hospital was also usually delayed, and this could be attributed to the socio-economic status of the patients who tended to delay seeking treatment. Although payment for treatment at these hospitals is free, it does not guarantee that patients will access government facilities as there is a preconceived notion that affordable health care is of a compromised quality (Burger and Christian, 2018). Access to health care is further compounded by the fact that South Africa has one of the highest income inequalities in the world, which disproportionally affect black Africans (Burger and Christian, 2018). The distance of patients' residences from the hospitals may also play a role in the delay of seeking treatment. It is recommended that future studies should include recruitment at regional hospitals and not be limited to referral hospitals as in this study.

Another challenge that influenced the immunophenotyping studies was the procurement of antibodies. Significant delays and antibodies for the desired panel were only available long after sampling of the bigger cohort had begun. For this reason, concurrent immunophenotyping was only done for the last few patient samples.

In addition, given that the diagnosis of patients and patient care was at the clinicians' discretion, it was observed that clinical tests for routine monitoring differed from patient to patient. These differences resulted in differences and hence missing data points on patient outcomes, especially information on CRP, creatinine and platelets. We also found that sampling times and the study methodology did not align most of the time with published literature. Given the time-sensitive nature of the immune response in AP, studies must be aligned to make informed inferences. The sampling period coincided with the COVID-19 pandemic. Between March 2020 and November 2020, access to hospitals and patients was restricted due to COVID-19 regulations, and sampling and recruitment were discontinued. Nevertheless, significant trends were observed to benefit future studies on AP.

5.3.2 Future work

For future studies, a closer look at white blood cells, especially CD3 negative cells, neutrophils, and monocytes known to produce IL-6, may help understand the pathophysiology of AP and

identify possible targets for immune therapy. Gene studies should be complemented by controlled animal studies looking specifically at different stages of the MAPK and NF-κB signalling pathways. It will be essential to know at which stage of the pathway the different types of cells are recruited. The focus of these studies should be on MSAP and SAP and using MAP as a control. The study should be expanded to include a bigger sample size to enable inferences to the larger population.

Some theories and or questions that require further exploration are:

- Determining the ratio of cell subsets producing IL-6 in each risk category of AP. An MSc study is currently ongoing in our laboratory to determine the expression of intracellular IL-6 expression in the different disease severities
- What is the implication of monocyte subsets association on severity, i.e. what is the relationship with severity?
- Given that IL-6 is a Th2 marker and an ILC2 cytokine, it would be helpful to know if the Th2 pathway or its innate counterpart determines severity?

It would be helpful in correlate cytokine expression with the cell phenotype in a one-pot experiment to provide helpful information. One way of doing this is through intracellular cytokine staining. There is an ongoing MSc study where IL-6 expression is being analysed simultaneously with cell surface marker expression, specifically of monocytes and NK cells.

5.2.3 Overall Conclusions

This study established that biliary and alcohol-related AP were the most common causes of AP in this South African cohort. This conclusion requires further investigation over a longitudinal study. A link between the upregulation of the CCR8 gene and IL6 cytokine elevation with disease severity was shown. The *MPO* gene also emerged as a potential prognostic marker to distinguish between MSAP and SAP patients. The study showed that monocytes, ILCs, Th2, and Th17 lymphocyte frequencies might differentiate patients' into MSAP and SAP groups. These findings may be beneficial as prognostic parameters in early AP stratification. This data highlights IL-6 associated cell populations as a promising predictive marker for patient risk stratification in AP. These findings contribute to a better understanding of the disease mechanism and potential early stratification options for AP.

REFERENCES

Afghani, E. (2014). Introduction to Pancreatic Disease : Acute Pancreatitis, *Pancreapedia*, 1(4), pp. 1–9.

Amarante-Mendes, G.P., Adjemian, S., Branco, L.M. et al. (2018). Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. *Frontiers in Immunology*. 9, p. 2379.

Anderson, F., Thomson, S. R., Clarke, D. L. et al. (2008). Acute pancreatitis: Demographics, aetiological factors and outcomes in a regional hospital in South Africa. *South African Journal of Surgery*, 46(3), pp. 83–86.

Anderson, F. and Thomson, S. R. (2017). Pancreatitis in a high HIV prevalence environment. *South African Medical Journal*, 107(8), pp. 706–709.

Augustine, T.N., Duarte, R., Candy, G.P. (2020). Breast Cancer Cells Induce a Pro-inflammatory Response to Mitigate Immune Mediation in a 3D Culture Model. Anticancer Research. 40, pp.6179–6193.

Azab, B., Jaglall, N., Atallah, J. P. et al. (2011). Neutrophil-lymphocyte ratio as a predictor of adverse outcomes of acute pancreatitis, *Pancreatology*. 11(4), pp. 445–452.

Banks, P.A., Bollen, T.L., Dervenis, C. et al. (2013). Classification of acute pancreatitis—2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 62, pp.102–111.

Barsheshet, Y., Wildbaum, G., Levy, E. et al. (2017). CCR8 + FOXp3 + T reg cells as master drivers of immune regulation. *Proceedings of the National Academy of Sciences of the United States of America. 114*, pp. 6086–6091.

Bateman C. (2018). National Doctor's Day – conserving one of South Africa's greatest assets. South African Journal of Medicine. 108(11) pp. 891-892.

Bhanou, N.M.S., Balachandran, G. and Jain, N.B. (2018). Neutrophil to lymphocyte ratio (NLR) in acute pancreatitis as an early predictor of severity and outcome. *International Surgery Journal*. 5(11), p. 3545.

Bhurwal, A., Sapru, S., Ramasamy, D. (2018). Diffuse Pancreatic Inflammation in an HIV Infected Individual with Elevated IgG4 Levels. Journal of Medical Cases. 9, pp. 109–111.

Biassoni, R., Cantoni, C., Pende, D., et al. (2001). Human natural killer cell receptors and co-receptors: Human NK-cell receptors. *Nature Reviews Immunology*. 181, pp 203–214.

Bollen, T. L. (2016). Acute pancreatitis: International classification and nomenclature. Clinical Radiology.

Boumsell, A.B. (1984). Human leukocyte differentiation antigen. *Press medicale*. 13(38), pp. 2311-16.

Breen EC, Rezai AR, Nakajima K, et al. (1990). Infection with HIV is associated with elevated IL-6 levels and production. *The Journal of Immunology*. 144(2). pp.480-484.

Burger, R. and Christian, C. (2018). Access to health care in post-apartheid South Africa: availability, affordability, acceptability. *Health Economics, Policy and Law*. X, pp.1–13.

Bush, Z.M., and Kosmiski, L.A. (2003). Acute Pancreatitis in HIV-Infected Patients: Are Etiologies Changing Since the Introduction of Protease Inhibitor Therapy?. *Pancreas* 27, pp. e1–e5.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, et al. (2009). The MIQE Guidelines: Minimum Information for **Publication** of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* 55, pp. 611–622.

Campos, T. de, Parraira, J., Cesar Assef, J. et al. (2013). Classification of acute pancreatitis, *Journal of the Brazilian College of Surgeons*. 40(2), pp. 164–168.

Cantor, J.,and Haskins, K. (2007). Recruitment and Activation of Macrophages by Pathogenic CD4 T Cells in Type 1 Diabetes: Evidence for Involvement of CCR8 and CCL1. *Journal of Immunology*. 179, pp.5760–5767.

Carestia, A., Kaufman, T., and Schattner, M. (2016). Platelets: New Bricks in the Building of Neutrophil Extracellular Traps. *Frontiers in Immunology*. 7(271).

Chan, J.K.C., Ng, C.S., and Hui, P.K. (1988). A simple guide to the terminology and application of leucocyte monoclonal antibodies. Histopathology. 12 pp. 461-480.

Chandra, S., Murali, A., Bansal, R. et al. (2017). The Bedside Index for Severity in Acute Pancreatitis: a systematic review of prospective studies to determine predictive performance. *Journal of Community Hospital Internal Medicine Perspectives*. 7(4), pp. 208–213.

Coillard, A., and Segura, E. (2019). In vivo Differentiation of Human Monocytes. *Frontiers in Immunology*. 10 (1907).

Cho, J. H., Kim, T. N., Chung, H. H., et al. (2015). Comparison of scoring systems in predicting the severity of acute pancreatitis. *World Journal of Gastroenterology*, 21(8), pp. 2387–2394.

Cho, S.K., Jung, S., Lee, K.J. *et al.* (2018). Neutrophil to lymphocyte ratio and platelet to lymphocyte ratio can predict the severity of gallstone pancreatitis. *BMC Gastroenterology*, 18(1), p. 18.

Choy, E., Rose-John, S. (2017). Interleukin-6 as a Multifunctional Regulator: Inflammation, Immune Response, and Fibrosis. *Journal of Scleroderma and Related Disorders*. 2, pp.S1–S5.

Dabrowski, A., Osada, J., Dabrowska, M.I., Wereszczynska-Siemiatkowska, U. (2008). Monocyte Subsets and Natural Killer Cells in Acute Pancreatitis. *Pancreatology*. 8, pp.126–134.

Dellinger, E. P., Forsmark, C. E., Layer, P. et al. (2012). Determinant-Based Classification of Acute Pancreatitis Severity. *Annals of Surgery*. 256(6), pp. 875–880.

Deng, L. H., Hu, C., Cai, W. H. et al. (2017). Plasma cytokines can help to identify the development of severe acute pancreatitis on admission. *Medicine*. 96(28).

Department of Health (DOH) South Africa. (2016). Strategy for the prevention and control of obesity in South Africa, Government of South Africa.

Ding, L., Yang, Y., Li, H. et al.. (2020). Circulating Lymphocyte Subsets Induce Secondary Infection in Acute Pancreatitis. *Frontiers in Cellular and Infection Microbiology*. 10, pp. 128.

Do, J. H. (2015). Mechanism of Severe Acute Pancreatitis: Focusing on Development and Progression. *Korean Journal of Pancreas and Biliary Tract*, 20(3), pp. 115–123.

Dragovic, G. (2013). Acute pancreatitis in HIV/AIDS patients: an issue of concern. *Asian Pacific Journal of Tropical Biomedicine*. 3, pp. 422–425.

Duque, A. G.,and Descoteaux, A. (2014). Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. *Frontiers in Immunology*. 5(491).

Endo, Y., Hirahara, K., Yagi, R., Tumes, D.J., Nakayama, T. (2014). Pathogenic memory type Th2 cells in allergic inflammation. Trends in Immunology. 35, pp. 69–78.

Fajgenbaum, D.C.,and June, C.H. (2020). Cytokine Storm. *New England Journal of Medicine*. 383, pp.2255–2273.

Ferreira, A. de F., Bartelega, J. A., Urbano, H. C. et al. (2015). Acute pancreatitis gravity predictive factors: which and when to use them? ABCD, *Arquivos Brasileiros de Cirurgia Digestiva*. 28(3), pp. 207–211.

Folias, A.E., Penaranda, C., Su, A.L. et al. (2014). Aberrant Innate Immune Activation following Tissue Injury Impairs Pancreatic Regeneration. *PLoS ONE*. 9, pp. e102125.

Fonteh, P., Smith, M., Brand, M. (2018). Adaptive Immune Cell Dysregulation and Role in Acute Pancreatitis Disease Progression and Treatment. *Archivum Immunologiae et Therapiae Experimentalis*. 66, pp. 199–209.

Forget, P., Khalifa, C., Defour, J. P. et al. (2017). What is the normal value of the neutrophil-to-lymphocyte ratio? BMC Research Notes 10(1), pp. 1–4.

Frandy, D. and Wirawan, A. D. (2019) Acute Pancreatitis-Etiology Pathogenesis, Pathophysiology and the current trend in its management and prevention. *Indonesian Journal of Gastroenterology*, Hepatology & Digestive disorders. 20(1), pp. 27–38.

Gholamnezhadjafari, R., Tajik, N., Falak, R. et al. (2017. Innate inflammatory gene expression profiling in potential brain-dead donors: detailed investigation of the effect of common corticosteroid therapy. *Innate Immunology*. 23 pp. 440–448.

Gulati K, Guhathakurta S, Joshi J, Rai N, Ray A (2016) Cytokines and their Role in Health and Disease: A Brief Overview. *MedCrave Online Journal of Immunology*. 4(2) pp. 00121.

Habtezion, A. (2015). Inflammation in acute and chronic pancreatitis. *Current Opinion in Gastroenterology*, 31(5), pp. 395–399.

He, F., Zhu, H., Li, B et al. (2020). Factors predicting the severity of acute pancreatitis in elderly patients. *Aging Clinical Experimental Research*. 33, 183–192 (2021).

Hlafa, B., Sibanda, K., and Hompashe, D.M. (2019). The Impact of Public Health Expenditure on Health Outcomes in South Africa. *International Journal of Environmental Research and Public Health*. 16 (2993).

Hofman, K. (2014). Non-communicable diseases in South Africa: A challenge to economic development. *South African Medical Journal*. 104(10).

Holdsworth, S.R., and Gan, P.-Y. (2015). Cytokines: Names and Numbers You Should Care About. *Clinical Journal of the American Society of Nephrology*. 10, pp. 2243–2254.

Hu, F. Lou, N., Jiao J. (2020). Macrophages in pancreatitis: Mechanisms and therapeutic potential. *Biomedicine & Pharmacotherapy*. 131, p. 110693.

Irrera, N. Bitto,A., Interdonato,M., et al. (2014). Evidence for a role of mitogen-activated protein kinases in the treatment of experimental acute pancreatitis. *World Journal of Gastroenterology*. 20(44), p. 16535.

Index Mundi Database. (2020). IndexMundi - Country Factshttps://www.indexmundi.com

Italiani, P., and Boraschi, D. (2014). From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Frontiers in Immunology*. 5.

Jakkampudi, A., Jangala, R., Reddy, B.R. et al. (2016). NF-κB in acute pancreatitis: Mechanisms and therapeutic potential. *Pancreatology*. 16, pp. 477–488.

Jee, S. L., Jarmin, R., Lim, K. F. et al. (2018). Outcomes of early versus delayed cholecystectomy in patients with mild to moderate acute biliary pancreatitis: A randomized prospective study. *Asian Journal of Surgery*. Elsevier Taiwan LLC, 41(1), pp. 47–54.

John, K., Segal, I., Hassan, H. et al. (1997). Acute Pancreatitis in Sowetan Africans. *International Journal of Pancreatology*. 21(2), pp. 149–155.

Jeon, D.I., Park, S.R., Ahn, M.Y., et al. (2012). NOD1 and NOD2 stimulation triggers innate immune responses of human periodontal ligament cells. *International Journal of Molecular Medicine*. 29, pp.699–703.

Kay, P. S., Smith, M. D. and Brand, M. (2017). The Initiating Immune Response of Acute Pancreatitis May be Mediated by the T-Helper 17 Pathway. *Journal of the Pancreas*. 18(1), pp. 241–245.

Kang, S., Tanaka, T., Narazaki, M., Kishimoto, T. (2019). Targeting Interleukin-6 Signaling in Clinic. *Immunity*. 50, pp.1007–1023.

Kang, L., Schmalzl, A., Leupold, T., et al. (2021). CCR8 Signaling via CCL1 Regulates Responses of Intestinal IFN-γ Producing Innate Lymphoid CelIs and Protects From Experimental Colitis. *Frontiers in Immunology*. 11, pp.609400.

Khatua, B., El-Kurdi, B., and Singh, V.P. (2017). Obesity and pancreatitis. *Current Opinion in Gastroenterology* .33, pp. 374–382.

Kim, J., and Bajaj, M., (2014). Normal Adipose Tissue Biology: Adipocytokines and Inflammation. *Pathobiology of Human Disease*. pp. 488–497.

Korn T, Bettelli E, Oukka M. et al. (2009). IL-17 and Th17 Cells. *Annual Review of Immunology*. 27 pp.485–517.

Koziel, D., Gluszek-Osuch, M., Suliga, E. et al. (2018). Elderly persons with acute pancreatitis-specifics of the clinical course of the disease. *Clinical Interventions Aging*. 14, 33–41.

Prame Kumar, K., Nicholls, A.J. and Wong, C.H.Y. (2018). Partners in crime: neutrophils and monocytes/macrophages in inflammation and diseases. *Cell and Tissue Research*. 371(3), pp. 551–565.

Kylänpää, L., Rakonczay, Z. and O'Reilly, D. A. (2012). The clinical course of acute pancreatitis and the inflammatory mediators that drive it. *International Journal of Inflammation*, 2012.

Leal, C., Almeida, N. (2019). Predicting Severity in Acute Pancreatitis: A Never-Ending Quest. *Journal of Gastroenterology*. 26, pp. 232–234

Leppäniemi, A., Tolonen, M., Tarasconi, A., et al. (2019) 2019 WSES guidelines for the management of severe acute pancreatitis. *World Journal of Emergency Surgery*. 14(1), pp. 1–20.

Lerch, M. M. and Gorelick, F. S. (2013). Models of Acute and Chronic Pancreatitis, *Gastroenterology*. Elsevier Inc., 144(6), pp. 1180–1193.

Leung, P. S. and Ip, S. P. (2006). Pancreatic acinar cell : Its role in acute pancreatitis. The *International Journal of Biochemistry & Cell Biology*. 38, pp. 1024–1030.

Li, Y., Zhao, Y., Feng, L. et al. (2017). Comparison of the prognostic values of inflammation markers in patients with acute pancreatitis: A retrospective cohort study. *British Medical Journal Open*. 7(3), pp. 1–8.

Li, X., Guo, X., Ji, H. et al. (2019). Relationships between Metabolic Comorbidities and Occurrence, Severity, and Outcomes in Patients with Acute Pancreatitis: A Narrative Review. BioMed Research International. 2019.

Liu, Y.-J., Soumelis, V., Watanabe, N. et al. (2007). TSLP: An Epithelial Cell Cytokine that Regulates T Cell Differentiation by Conditioning Dendritic Cell Maturation. *Annual Review of Immunology*. 25, pp.193–219.

Littner, M. R., Cotten, D., Talchman, D., et al. (2011). In the Clinic: acute pancreatitis, *Annals of Internal Medicine*. pp. 2–16

Livak, K. J. and Schmittgen, T. D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*. 25(4), pp. 402–

Luckheeram, R.V., Zhou, R., Verma, A.D., Xia, B. (2012). CD4 + T Cells: Differentiation and Functions. *Clinical and Developmental Immunology*. 2012, pp. 1–12.

Lundberg, A.H., Eubanks, J.W., Henry, J., et al. (2000). Trypsin Stimulates Production of Cytokines from Peritoneal Macrophages In Vitro and In Vivo: Pancreas. 21, pp.41–51.

Luo, Y. and Zheng, S.G. (2016). Hall of Fame among Pro-inflammatory Cytokines: Interleukin-6 Gene and Its Transcriptional Regulation Mechanisms. *Frontiers in Immunology*. 7 (604)

Ma, W.T., Gao, F., Gu, K., Chen, D. K. (2019). The Role of Monocytes and Macrophages in Autoimmune Diseases: A Comprehensive Review. *Frontiers in Immunology*. 10, pp 1140.

Machado, M.M.C., and Coelho, A.M.M (2012). Role of Peritoneal Macrophages on Local and Systemic Inflammatory Response in Acute Pancreatitis. *InTechopen*.

Manohar, M., Verma, A. K., Venkateshaiah, S. U. et al. (2017). Pathogenic mechanisms of pancreatitis. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, 8(1), p. 10.

Marshall, J.S., Warrington, R., Watson, W. et al. (2018). An introduction to immunology and immunopathology. *Allergy, Asthma and Clinical Immunology*. 14, 49.

Mayerle, J., Dummer, A., Sendler, M. et al. (2012). Differential roles of inflammatory cells in pancreatitis, *Journal of Gastroenterology and Hepatology*. 27(2), pp. 47–51

McKinnon, K.M. (2018). Flow cytometry: an overview. *Current Protocols in Immunology*, 120(1), pp.5-1.

Meher, S., Mishra, T. S., Sasmal, P. K., et al. (2015). Role of Biomarkers in Diagnosis and Prognostic Evaluation of Acute Pancreatitis. *Journal of Biomarkers*. pp. 1–13.

Meintjes, G., Moorhouse, M. A., Carmona, S. et al. (2012). Adult antiretroviral therapy guidelines 2017. *Southern African Journal of HIV Medicine*. 13(3) p. 106.

Merza, M., Hartman, H., Rahman, M. et al. (2015). Neutrophil Extracellular Traps Induce Trypsin Activation, Inflammation, and Tissue Damage in Mice With Severe Acute Pancreatitis. *Gastroenterology*. 149, pp. 1920-1931

Mukaka, M.M. (2012). Statistics Corner: A guide to appropriate use of Correlation coefficient in medical research 3. *Malawi Medical Journal*. 24(3), pp. 69-71.

Nel, J., Dlamini, S., Meintjes, G et al. (2020). Southern African HIV Clinicians Society guidelines for antiretroviral therapy in adults: 2020 update. Southern African Journal of HIV medicine. pp 1-39

Nesvaderani, M., Eslick, G. D., Vagg, D. et al. (2015). Epidemiology, aetiology and outcomes of acute pancreatitis: A retrospective cohort study. *International Journal of Surgery*. 23, pp. 68–74

Nesvaderani, M., Eslick, G.D., and Cox, M.R. (2015). Acute pancreatitis: update on management. *Medical Journal of Australia*. 202, pp. 420–423.

NHLS. (2021). TrackCare Lab Reports. The Republic of South Africa.

Nicholson, L.B., 2016. The immune system. *Essays in Biochemistry*. 60, pp. 275–301.

Nieminen, A., Maksimow, M., Mentula, P. et al. (2014). Circulating cytokines in predicting development of severe acute pancreatitis. *Critical Care*. 18, pp. R104.

Oiva, J., Mustonen, H., Kylänpää, M.L. et al. (2010). Acute pancreatitis with organ dysfunction associates with abnormal blood lymphocyte signaling: controlled laboratory study. *Critical Care*. 14, pp. 1-11.

Oliveira, N. M., Ferreira, F. A. Y., Yonamine, R. Y. et al. (2014). Antiretroviral drugs and acute pancreatitis in HIV/AIDS patients: is there any association? A literature review. *Einstein*. 12(1), pp. 112–119.

Orekhov, A.N., Orekhova, V.A., Nikiforov, N.G. et al. (2019).Monocyte differentiation and macrophage polarization. *Vessel Plus*. 3 pp.10.

O'reilly, S., and Duffy, L., (2016). Toll-like receptors in the pathogenesis of autoimmune diseases: recent and emerging translational developments. *Immunotargets and therapy*. 5, pp.69–80.

Oshio, T., Kawashima, R., Kawamura, Y. I. et al. (2014). Chemokine receptor CCR8 is required for lipopolysaccharide-triggered cytokine production in mouse peritoneal macrophages. *PLoS ONE*, 9(4).

Pang, Y., Kartsonaki, C., Turnbull, I. et al. (2018). Metabolic and lifestyle risk factors for acute pancreatitis in Chinese adults: A prospective cohort study of 0.5 million people, *PLoS Medicine*. 15(8), pp. 1–19.

Peng, C., Li, Z., Yu, X. et al. (2021). The Role of Pancreatic Infiltrating Innate Immune Cells in Acute Pancreatitis. *International Journal Medical Science*. 18, pp. 534–545.

Poli, A., Michel, T., Thérésine, M. et al. (2009). CD56 bright natural killer (NK) cells: an important NK cell subset. *Immunology* 126, 458–465.

Prame Kumar, K., Nicholls, A.J., Wong, C.H.Y. (2018). Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell Tissue Research*. 371, pp. 551–565

Rao, S.A., and Kunte, AR. (2017). Interleukin-6: An Early Predictive Marker for Severity of Acute Pancreatitis. *Indian Journal of Critical Care Medicine*. 21(7), pp. 424-428.

Richardson, A., and Park, W.G. (2021). Acute pancreatitis and diabetes mellitus: a review. *Korean Journal of Internal Medicine*. 36, pp.15–24.

Roberts, S. E., Akbari, A., Thorne, K. et al. (2013). The incidence of acute pancreatitis: Impact of social deprivation, alcohol consumption, seasonal and demographic factors. *Alimentary Pharmacology and Therapeutics*. 38(5), pp. 539–548.

Roberts, S.E., Morrison-Rees, S., John, A. et al. (2017). The incidence and aetiology of acute pancreatitis across Europe. *Pancreatology* 17, 155–165.

Roederer, M. (2002). Compensation in flow cytometry. *Current protocols in cytometry*. 22(1), pp.1-14.

Routy, J.P.,and Isnard, S. (2021). Beyond the Absence of CD4 T-Cell Count: A Novel Genetic CD4 T-Cell Deficiency Disorder with a Contingency Plan. *Journal of Infectious Diseases*. 223, 547–549.

SAGE. (2019). Learn to Use the Eta Coefficient Test in R With Data From the NIOSH Quality of Worklife Survey. *SAGE Research Methods Datasets.*Newcastle, UK

Santopaolo, M., Sullivan N., Thomas A.C. et al. (2021). Activation of Bone Marrow Adaptive Immunity in Type 2 Diabetes: Rescue by Co-stimulation Modulator Abatacept. *Frontiers in Immunology*. 12:609406.

Scheller, J., Chalaris A.,Schmidt-Arras, D., Rose-John, S. (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1813(5), pp. 878–888.

Schepers, N. J., Besselink, M. G. H., van Santvoort, H. C. et al. (2013). Early management of acute pancreatitis. *Best Practice and Research Clinical Gastroenterology*. 27(5), pp. 727–43.

Shah, A. P., Mourad, M. M. and Bramhall, S. R. (2018). Acute pancreatitis: Current perspectives on diagnosis and management, *Journal of Inflammation Research*, 11, pp. 77–85.

Sharma, D., Jakkampudi, A., Reddy, R. et al. (2017). Association of Systemic Inflammatory and Anti-inflammatory Responses with Adverse Outcomes in Acute Pancreatitis: Preliminary Results of an Ongoing Study. *Digestive Diseases and Sciences*. 62(12), pp. 3468–3478.

Shay T, and Kang J. (2013). Immunological Genome Project and systems immunology. *Trends in Immunolology*. 34(12), pp. 602-609.

Shen, X., Sun, J., Ke, L. et al. (2015). Reduced lymphocyte count as an early marker for predicting infected pancreatic necrosis, *BMC Gastroenterology*. 15(1), p. 147.

Shen, X. and Li, W.Q. (2015). High-mobility group box 1 protein and its role in severe acute pancreatitis. *World Journal of Gastroenterology*, 21(5). p. 1424.

Shrivastava, P. and Bhatia, M. (2010). Essential role of monocytes and macrophages in the progression of acute pancreatitis. *World Journal of Gastroenterology*. 16, pp.3995.

Singh, P. and Garg, P. K. (2016). Pathophysiological mechanisms in acute pancreatitis: Current understanding. *Indian Journal of Gastroenterology*. 35(3), pp. 153–166

Singh, H., Gougol, A., Mounzer, R. et al. (2017). Which Patients with Mild Acute Pancreatitis Require Prolonged Hospitalization? *Clinical and Translational Gastroenterology*. Nature Publishing Group, 8(12).

Solanki, N.S., Barreto, S.G., Saccone, G.T.P. (2012). Acute pancreatitis due to diabetes: The role of hyperglycaemia and insulin resistance. *Pancreatology*. 12 pp. 234–239.

Solaymani-Mohammadi, S., Eckmann, L., and Singer S.M. (2019). Interleukin (IL)-21 in Inflammation and Immunity during Parasitic Diseases. *Frontiers Cellular and Infection Microbiology.* 9, pp.401.

Stagg, A.J., and Knight, S.C. (2001). Antigen-presenting Cells, in: John Wiley & Sons, Ltd (Ed.), *Encyclopedia of Life Sciences*., p. a0000903.

Stats SA. (2016). South Africa Demographic and Health Survey 2016: Key Indicator Report. The Republic of South Africa.

Stats SA. (2019). Mid-year population estimates 2019. Department of Statistics. The Republic of South Africa.

Staubli, S.M., Oertli, D. and Nebiker, C.A. (2015) 'Laboratory markers predicting severity of acute pancreatitis', Critical Reviews in Clinical Laboratory Sciences, 52(6), pp. 273–283.

Sternby, H., Hartman, H., Thorlacius, H., Regnér, S. (2021). The Initial Course of IL1β, IL-6, IL-8, IL-10, IL-12, IFN-γ and TNF-α with Regard to Severity Grade in Acute Pancreatitis. *Biomolecules.* 11 (591).

Stirling, A. D., Moran, N. R., Kelly, M. E., et al. (2017). The predictive value of C-reactive protein (CRP) in acute pancreatitis – is interval change in CRP an additional indicator of severity? *Journal of the International Hepato Pancreato Biliary Association*. 19(10), pp. 874–880.

Swain, S.L., McKinstry, K.K. and Strutt, T.M. (2012). Expanding roles for CD4+ T cells in immunity to viruses. *Nature Reviews Immunology*. 12, 136–148.

Szatmary, P., Gukovsky, I. and Angeles, L. (2016). The Role of Cytokines and Inflammation in the Genesis of Experimental pancreatitis. *Pancreapedia*, 1(36)

Talukdar, R. and Vege, S. S. (2015). Classification Systems for the Severity of Acute Pancreatitis. *Pancreapedia*, (23), pp. 1–11.

Tenner, S., Baillie, J., DeWitt, J. et al. (2013). American College of Gastroenterology Guideline: Management of Acute Pancreatitis. *The American Journal of Gastroenterology*. 108(9), pp. 1400–1415.

Thaker, A. M., Mosko, J. D. and Berzin, T. M. (2015).Post-endoscopic retrograde cholangiopancreatography pancreatitis. *Gastroenterology Report*, 3(1), pp. 32–40.

Thalhamer, T., McGrath, M.A. and Harnett, M.M. (2007). MAPKs and their relevance to arthritis and inflammation. *Rheumatology*. 47(4). pp. 409–414.

Thomson, J. E., Brand, M. and Fonteh, P. (2018). The immune imbalance in the second hit of pancreatitis is independent of IL-17A. *Pancreatology*. 18(3), pp. 246–252.

Thomson, J.E., Nweke, E.E., Brand, M., et al. (2019). Transient Expression of Interleukin-21 in the Second Hit of Acute Pancreatitis May Potentiate Immune Paresis in Severe Acute Pancreatitis. *Pancreas*. 48, pp. 107–112.

Ting, J., Wilson, L., Schwarzenberg, S. J. et al. (2017). Direct costs of acute recurrent and chronic pancreatitis in children in the INSPPIRE registry, *Journal of Paediatric Gastroenterology Nutrition*. 176(1), pp. 139–148.

Trikudanathan, G., Wolbrink, D. R. J., van Santvoort, H. C. et al. (2019). Current Concepts in Severe Acute and Necrotising Pancreatitis: An Evidence-Based Approach. *Gastroenterology*. 156(7), pp. 1994-2007.

Ugwu, B., Obekpa, P. and Kidmas, A. (1997). Acute Pancreatitis at Jos University Teaching Hospital.

Uhlen, M., Karlsson, M.J., Zhong, W. et al. (2019). A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. *Science*. 366(6472)

van den Berg, F.F., de Bruijn, A.C., van Santvoort, H.C. et al.(2020) 'Early laboratory biomarkers for severity in acute pancreatitis; A systematic review and meta-analysis. *Pancreatology*. 20(7), pp. 1302–1311.

van Geenen, E.J.M., van der Peet, D.L., Mulder, C.J.J. et al. (2009). Recurrent acute biliary pancreatitis: the protective role of cholecystectomy and endoscopic sphincterotomy. *Surgical Endoscopy*. 23 pp. 950–956.

van Walbeek, C. and Blecher, E. (2014). The Economics of Alcohol Policy in South Africa. WHO. Geneva, Switzerland

Vasseur, P., Devaure, I., Sellier, J. et al. (2014). High plasma levels of the pro-inflammatory cytokine IL-22 and the anti-inflammatory cytokines IL-10 and IL-1ra in acute pancreatitis. *Pancreatology*. 14(6), pp. 465–469.

Vaz, J. Akbarshahi, H. and Andersson, R. (2013). Controversial role of toll-like receptors in acute pancreatitis. *World Journal of Gastroenterology*, 19(5), p. 616-630.

Vivier, E., Artis, D., Colonna, M. et al. (2018). Innate Lymphoid Cells: 10 Years On. Cell 174, pp. 1054–1066.

Wang, W., Xiang, H., Wang. E., et al. (2017). CD4 + CD25 + CD127 high cells as a negative predictor of multiple organ failure in acute pancreatitis. *World Journal of Emergency Surgery*, 12(1), p. 7.

Weitz, G., Woitalla, J., Schmidt, K. J. et al. (2016). Comorbidity in acute pancreatitis relates to organ failure but not to local complications. *Gastroenterology*. 54(3), pp. 226–230.

WHO. 2016. Global report on diabetes. World Health Organisation. Geneva, Switzerland

WHO. (2020). Noncommunicable diseases: progress monitor 2020. World Health Organization.

Willinger, T. (2019). Metabolic control of innate lymphoid cell migration. *Frontiers in Immunology*, 10(8).

Wu, B. and Banks, P. (2013) Clinical Management of Patients with Acute Pancreatitis. *Gastroenterology*. 144, pp. 1272–1281.

Yadav, D. (2014). Acute Pancreatitis: Too many classifications - what is a clinician or researcher to do?. *Clinical Gastroenterology and Hepatology*. 12(2), pp. 317–319.

Yadav, D. and Lowenfels, A. B. (2006). Trends in the epidemiology of the first attack of acute pancreatitis: A systematic review. *Pancreas*. 33(4), pp. 323–330.

Yadav, D. and Lowenfels, A. B. (2013). The epidemiology of pancreatitis and pancreatic cancer. *Gastroenterology*. 144(6), pp. 1252–1261.

Yan, Z., Jinhang G., Lv, X. et al. (2016). Quantitative Evaluation and Selection of Reference Genes for Quantitative RT-PCR in Mouse Acute Pancreatitis. Biomedical Research International. pp. 1–11.

Yan, Y., Lu, B., Li, P. et al. (2017). NOD receptor and TLR9 modulation in severe acute pancreatitis-induced intestinal injury.*Molecular Medicine Reports*. 16(6), pp. 8471–8476

Yang, J. Zhang, L., Yu, C., et al. (2014) 'Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases', *Biomarker Research*, 2(1), p. 1.

Yang, Z., Meng, X. and Xu, P. (2015). Central role of neutrophil in the pathogenesis of severe acute pancreatitis. *Journal of Cellular and Molecular Medicine*. 19(11), pp. 2513–2520.

Zambrano-Zaragoza, J.F., Romo-Martínez, E.J., Durán-Avelar, Ma. de J. et al .(2014). Th17 Cells in Autoimmune and Infectious Diseases. *International Journal of Inflammation* 2014, pp. 1–12.

Zhang, J.M. and An, J. (2007). Cytokines, Inflammation, and Pain. *International Anesthesiology Clinics*. 45. pp. 27–37.

Zhang, R., Shi, J., Zhang, R. *et al.* (2019). Expanded $CD14^{hi}$ $CD16^{-}$ Immunosuppressive Monocytes Predict Disease Severity in Patients with Acute Pancreatitis. *The Journal of Immunology*. 202(9), pp. 2578–2584.

Zheng, L., Xue, J., Jaffee, E. et al. (2013). Role of immune cells and immune-based therapies in pancreatitis and pancreatic ductal adenocarcinoma. *Gastroenterology*. 144(6), pp. 1230–1240.

Zhou, Q., Tao, X., Xia, S. et al. (2020). T Lymphocytes: A Promising Immunotherapeutic Target for Pancreatitis and Pancreatic Cancer? *Frontiers in Oncology*. 10, pp. 382.

Zhu, J., Yamane, H., and Paul, W.E. (2010). Differentiation of Effector CD4 T Cell Populations. *Annual Reviews of Immunology*. 28, pp. 445–489.

Zhu, X. and Zhu, J. (2020). CD4 T Helper Cell Subsets and Related Human Immunological Disorders. *International Journal of Molecular Science*. 21, pp. 8011.

APPENDICES

APPENDIX A: Information sheet for patients

INFORMATION SHEET: PATIENT SAMPLE

EARLY IMMUNE RESPONSES IN ACUTE PANCREATITIS AND THEIR ROLE IN PREDICTING DISEASE SEVERITY

Good day,

We are researchers from the University of the Witwatersrand doing research on acute pancreatitis. Research is a process of learning and solving problems. In this research we want to find out what happens in the body when someone has this disease, why do other people stay in hospital for a shorter time and others go for surgery or they get released and come back with the same problem. Why is it a very serious disease for some people but not for others?

You are invited to take part in this study. This research study is looking for **at least 15 participants diagnosed with acute pancreatitis of different severity** to donate **2½ teaspoons (about 12 mL)** of blood. The blood will be drawn from a vein in your arm using a syringe at the Hepatopancreaticobiliary Unit of the academic hospital in which you are admitted and transported safely protected in a secondary container to the University of the Witwatersrand where the research will be done. Transport will be by private means or through the NHLS transport services. From the blood, the liquid portion called plasma and cells will be separated and used for the study. Some of these samples such as the plasma cannot be used immediately and will be frozen at -20 or -80C until needed (within 2 months to 5 years). The results from these samples will be compared to those of participants who lack a history of pancreatic diseases.

Sampling will be part of routine management procedures and you may be subjected to some pain or discomfort caused by the needle, but a qualified and experienced medical practitioner (nurse or doctor) has been recruited to perform the procedure and to minimize the risks. The procedure will only take about 5 minutes and blood will be obtained from you once you consent and every 48 hours afterwards up to 7 days post admission.
Participating in this study might not benefit you now but we hope that the results from these studies will benefit patients in future. Significant new findings developed during the course of the research which may relate to your willingness to continue participating in the study will be provided to you.
Participating in this study will not result in any "out of pocket' expenses. Refusal to participate will involve no penalty or loss of benefits normally entitled to you. You may discontinue participation at any time without penalty or prejudice. Blood samples will be collected anonymously to ensure confidentiality. Personal information may only be disclosed if required by law or organizations that inspect and/or copy research records for quality assurance and data analysis such as the Research Ethics Committee (REC). At the end of the study, leftover blood will be discarded in 10% bleach, sterilized by heating to 120 degrees Celsius and disposed in bio-hazardous/ medical waste boxes.
For further information/ reporting study related adverse effects/complaints contact:

Researchers:
Miss Mwangala Nalisa (Research student)
Tel: 0117172478, Email: 1742389@students.wits.ac.za
Dr Pascaline Fonteh (Supervisor)
Tel: 0117172476, pascaline.fru-fonteh@wits.ac.za
Dr John Devar (Surgical Gastroenterologist, CHBAH), Lecturer, General Surgery
Tel: 082 770 5178, John.devar@wits.ac.za
REC Chairperson – Prof Cleaton-Jones: peter.cleaton-jones1@wits.ac.za
REC Administrators - Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng
Tel 011 717 2700/2656/1234/1252, Email: HREC-Medical.ResearchOffice@wits.ac.za

APPENDIX B: Consent Form for patients

CONSENT FORM FOR PATIENTS

Dear Sir/madam,

You are currently admitted to a University of Witwatersrand affiliated academic hospitals. This hospital not only renders treatment but is also actively involved in conducting research aimed at improving the quality of care that is provided. From time to time such research involves the use of patient samples and records from which information is extracted. The use of such information is subject to the following:

1. Approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand.
2. Identity of a patient from whose file information is extracted is never revealed to anyone but the researcher unless specific consent is obtained to do so. The information gathered does not contain the name of the patient but only a coded number so as to maintain anonymity.

Whilst we are not currently involved in research that requires us to use any information now but rather are interested in collecting a blood sample from you, this may change in the future when you may have already been discharged. We would like to obtain your consent to use information from your file for the purpose of research, subject to the aforementioned conditions. We anticipate that such information will be accessed up to 5 years after sample collection. 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, phone: Ms Mwangala Nalisa at (011) 717-2574, Dr Pascaline Fonteh at (011)717-2476. If you agree to participate please place your signature below under the participant section.

PARTICIPANT

Print Name	**Signature**	**Date**

RESEARCHER

Print Name	**Signature**	**Date**

WITNESS

Print Name	**Signature**	**Date**

APPENDIX C: Information sheet for Healthy controls

INFORMATION SHEET: HEALTHY CONTROL SAMPLE
EARLY IMMUNE RESPONSES IN ACUTE PANCREATITIS AND THEIR ROLE IN PREDICTING DISEASE SEVERITY

Good day,

We are researchers from the University of the Witwatersrand doing research on acute pancreatitis. Research is a process of learning and solving problems. In this research we want to find out what happens in the body when someone has this disease, why do other people stay in hospital for a shorter time and others go for surgery or they get released and come back with the same problem. Why is it a very serious disease for some people but not for others?

You are invited to take part in this study as a healthy volunteer. This research study is looking for at least 15 participants with no history of pancreatic disease to donate 2½ teaspoons (about 12 mL) of blood. The blood will be drawn from a vein in your arm using a syringe at the Department of Surgery, 9 Floor, and Faculty of Health Sciences Building of the University of the Witwatersrand. Research on your sample will be done in the same department. From the blood, the liquid portion called plasma and cells will be separated and used for the study. Some of these samples such as the plasma cannot be used immediately and will be frozen at -20 or -80C until needed (within 2 months to 5 years). If you choose to participate your sample will help us to compare with other samples collected from patients with acute pancreatitis.

During sampling you may be subjected to some pain or discomfort caused by the needle, but a qualified and experienced medical practitioner (nurse or doctor) has been recruited to perform the procedure and to minimize the risks. The procedure will only take about 5 minutes and blood will be obtained from you only once if you consent. Participating in this study might not benefit you now but we hope that the results from these studies will benefit patients in future. Significant new findings developed during the course of the research which may relate to your willingness to continue participating in the study will be provided to you.

Participating in this study will not result in any "out of pocket' expenses. Refusal to participate will involve no penalty. Blood samples will be collected anonymously to ensure confidentiality. Personal information may only be disclosed if required by law or organizations that inspect and/or copy research records for quality assurance and data analysis such as the Research Ethics Committee (REC). At the end of the study, leftover blood will be discarded in 10% bleach, sterilized by heating to 120 degrees Celsius and disposed in bio-hazardous/ medical waste boxes.
For further information/ reporting study related adverse effects/complaints contact:
Researchers:
Miss Mwangala Nalisa (Research student)
Tel: 0117172478, Email: 1742389@students.wits.ac.za
Dr Pascaline Fonteh (Supervisor)
Tel: 0117172476, pascaline.fru-fonteh@wits.ac.za
Dr John Devar (Surgical Gastroenterologist, CHBAH), Lecturer, General Surgery
Tel: 082 770 5178, John.devar@wits.ac.za
REC Chairperson – Prof Cleaton-Jones: peter.cleaton-jones1@wits.ac.za
REC Administrators - Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng
Tel 011 717 2700/2656/1234/1252, Email: HREC-Medical.ResearchOffice@wits.ac.za

APPENDIX D: Consent Form for Healthy controls

CONSENT FORM: USE OF BLOOD SAMPLE AS HEALTHY CONTROL

Dear Sir/madam,

If you are reading this form it means you have responded to a request to be recruited as part of the study on acute pancreatitis. Before signing this document the researcher would have related to you the details of the study in which you are participating.
You are required to donate to this study approximately 2½ teaspoons of blood. This sample will only be collected once. The information generated from this study is subject to the following:

1. Approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand.
2. Identity of a volunteer form is never revealed to anyone but the researcher unless specific consent is obtained to do so. The information gathered does not contain the name of the volunteer but only a coded number so as to maintain anonymity.

Volunteering in this study means that you give permission to the researcher to analyse your blood to make sure that you do not currently suffer from any illness that may compromise your eligibility to serve as a healthy control in this study. We anticipate that this information will be required up to 5 years after sample collection.

If you choose not to give consent, this will not compromise you 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, phone. Ms Mwangala Nalisa at (011) 717-2574, Dr Pascaline Fonteh at (011)717-2476. If you agree to participate please place your signature below under the participant section.

PARTICIPANT

Print Name	**Signature**	**Date**

RESEARCHER

Print Name	**Signature**	**Date**

WITNESS

Print Name	**Signature**	**Date**

APPENDIX E: Datasheet

DATASHEET FOR ACUTE PANCREATITIS DIAGNOSIS

DEMOGRAPHICS

Sample No: _____

Hospital No: _____

Sex: M ☐ F ☐

Age: _____

Cause of pancreatitis:

_____

BMI _____kg/m^2

DETERMINANTS OF SEVERITY

ON ADMISSION

WBC _____ uL Glucose _____mmol /L LDH _____ IU/L

SGOT _____ IU/L

C - reactive protein _____mg/L

Procalcitonin (PCT) _____ng/mL

SGOT- Serum glutamic-oxaloacetic transaminase, LDH- Lactate dehydrogenase

AFTER 48HRS ADMISSION

Hematocrit_____%

Increase in Blood Urea Nitrogen _____ mg/dL or mmol/L

Calcium _____ mg/dL or mmol/L

PO_2 _____ mmHg

Base deficit _____ mmol/L

Fluid sequestration _____L

C - reactive protein _____ mg/L

Procalcitonin (PCT) _____ 2ng/mL

(The above will be compared to thresholds of Ranson's criteria scores for the respective indicators, depending on whether the AP is alcohol related or gallstone related)

SEQUENTIAL ORGAN FAILURE ASSESSMENT (SOFA) SCORE (if applicable)

Creatinine: _____ mmol/L

Hematocrit:_____ L/L

Platelets: _____ 10^9 /L

Bilirubin: _____ umol/L

Glasgow Coma Scale (GCS): _____

PaO_2/FiO_2: _____mmHg

COMORBIDITY AND PATIENT OUTCOMES

Has the patient been diagnosed with any other disease apart from AP (circle relevant option)?

Y N

If yes, state disease

_____ _____

Is the patient HIV positive **Y N**

If HIV positive state whether they are on ARVs

CD4 count_____ cell/mm^3

Did the patient receive any antibiotics (*circle relevant option*)? **Y N**

If yes, what antibiotics were administered?

How were the administered: _____ (*e.g. IV or Oral*)

On what day post onset of symptoms were they started: _____

Duration of antibiotics (in days): _____

Were CT scans performed on the patient? If yes, state major finding

Date of scan _____ ___(*DD/MM/YYYY*)

Were further radiological/surgical interventions performed?

Additional comments/observations

APPENDIX F: Ethics Clearance certificate and Plagiarism Report

Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms M Nalisa
School of Clinical Medicine
Department of Surgery
Medical School
University

E-mail: Mwangalan@gmail.com

CC: Supervisor: Drs P Fru-Fonteh and T Augustine <Pascaline.Fru-Fonteh@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 05/06/2018

REF: *R14/49*

PROTOCOL NO: **M180133** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Early immune responses in acute pancreatitis and their role in predicting disease severity*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms M Nalisa

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M180133

NAME: (Principal Investigator)	Ms M Nalisa
DEPARTMENT:	School of Clinical Medicine Department of Surgery Medical School University
PROJECT TITLE:	Early immune responses in acute pancreatitis and their role in predicting disease severity
DATE CONSIDERED:	26/01/2018
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Drs P Fru-Fonteh and T Augustine
APPROVED BY:	Professor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	05/06/2018

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on 3rd floor, Phillip V Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/We fully understand the conditions under which I am/we are authorised 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 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 in **January** and will therefore be due in the month of **January** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Plagiarism (Turnitin) Report

a0046051:11_11_2021_MNalisa_Thesis_corrections_TIN.docx

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APPENDIX G: Interpretation of Spearman's correlation (r_s) and Eta Coefficient

Table A: Spearman's correlation interpretation

Positive Spearman's correlation (r_s)	Negative Spearman's correlation (r_s)
0.00 to 0.19 "very weak positive"	-0.80 to -1.0 "very weak negative"
0.20 to 0.39 "weak positive"	-0.60 to -0.79 "weak negative"
0.40 to 0.59 "moderate positive"	-0.40 to -0.59 "moderate negative"
0.60 to 0.79 "strong positive"	-0.20 to -0.39 "strong negative "
0.80 to 1.0 "very strong positive"	-0.19 to less than 0 "very strong negative"

Notes: Values are between 0 to 1 for positive correlation and a -1 to less than 0 for the negative correlation. Depending on the strength of the correlation, the significance is achieved at α= 0.01 level (2-tailed) and α= 0.05 level (2-tailed). Notes: Adapted from Mukaka (2012).

Table B: Eta coefficient interpretation

Notes: Adopted from Sage 2019

Range η coeeficient	Interpretation
0.00	No association between the two variables
0.01–0.19	No or negligible association between the variables
0.2–0.39	Weak association between the variables
0.4–0.69	Medium association between the variables
0.70-1.0	Strong association between the variables

APPENDIX H: MILLIPLEX® descriptive data results with MSAP and SAP grouped for Day (D) 3.

Table showing Day 3, IL-6 from the MAP group descriptive statistics

	Healthy controls	MAP	MSAP/SAP
Number of values	*n*=6	*n*=7	*n*=6
Minimum	0.0	4.0	0.0
25% Percentile	0.0	9.0	1.5
Median	0.0	14	14
75% Percentile	2.3	31	42
Maximum	9.0	40	100
Mean	1.5	20	25
Standard Deviation	3.7	13	37
Standard Error of Mean	1.5	5.0	15

APPENDIX I: RNA concentration of samples used in the validation of gene targets associated with intracellular innate and adaptive immune systems of AP patients

Sample No	Day	Severity	Concentration (ng/µL)	Final volume to add to get 50ng/µL	Volume of water to add for dilution(µL)
C1	N/A	healthy control	169.10	2.96	5.0
C2	N/A	healthy control	16.40	3.05	5.0
C3	N/A	healthy control	108.60	4.60	3.4
C4	N/A	healthy control	254.30	1.97	6.0
C5	N/A	healthy control	65.10	7.68	0.0
C6	N/A	healthy control	147.40	3.39	4.6
C6	N/A	healthy control	8.00	6.25	1.8
CHAP0015	D7	MSAP	4.60	10.87	0.0
CHAP0015	D5	MSAP	10.00	5.00	3.0
CHAP0020	D3	SAP	6.20	8.06	0.0
CHAP0023	D5	SAP	7.80	6.41	1.6
CHAP0024	D3	MAP	10.00	5.00	3.0
CHAP0028	D3	MAP	38.30	1.31	6.7
CHAP0029	D3	MAP	141.30	3.54	4.5
CHAP0032	D3	MAP	85.70	5.83	2.2
CHAP0037	D5	MAP	5.20	9.62	0.0
CHAP0037	D7	MAP	11.00	4.55	3.5
CHAP006	D7	MAP	5.80	8.62	0.0
CHAP006	D3	MAP	73.10	6.84	1.2
CHAP007	D7	MSAP	32.50	1.54	6.5
CHAP007	D5	MSAP	6.50	7.69	0.0
CHAP007	D3	MSAP	5.80	8.62	0.0
CHAP008	D7	MSAP	51.60	0.97	7.0
CHAP008	D3	MSAP	99.00	5.05	2.9
CHAP014	D7	MSAP	872.70	1.00	7.0
CHAP018	D7	MSAP	28.10	1.78	6.2
CHAP019	D3	MSAP	25.90	1.93	6.1
CHAP020	D5	SAP	5.80	8.62	0.0
CHAP021	D7	MSAP	47.30	1.06	6.9
CHAP022	D5	MAP	9.00	5.56	2.4
CHAP023	D7	SAP	86.00	5.81	2.2
CHAP023	D3	SAP	378.80	1.32	6.7
CHAP024	D3	MAP	120.80	4.14	3.9
CHAP024	D5	MAP	64.90	7.70	0.0
CHAP025	D3	MSAP	191.80	2.61	5.4
CHAP026	D4	MAP	19.2	2.60	5.4
CHAP026	D7	MAP	68.70	7.28	0.7
CHAP030	D7	MAP	156.50	3.19	4.8
CHAP031	D3	SAP	130.50	3.83	4.2
CHAP031	D7	SAP	130.60	3.83	4.2
CHAP031	D5	SAP	41.40	1.21	6.8
CHAP033	D5	MSAP	18.50	2.70	5.3
CHAP035	D7	MSAP	246.50	2.03	6.0
CHAP036	D7	MAP	593.50	1.00	7.0
CHAP038	D3	MAP	280.90	1.78	6.2

CHAP038	D7	MAP	130.90	3.82	4.2
CHAP038	D3	MAP	280.90	1.78	6.2
CHAP038	D5	MAP	25.00	2.00	6.0
CMAP003	D5	MAP	5.40	9.26	0.0

Notes: CHAP: patient was recruited at CHBAH; CMAP: the patient was recruited at CMAJH; C:Healthy controls, D: Day of pain.

APPENDIX J: Calculations and volumes of PCR Master Mix (MM) and Set up for PCR run to determine the cycle threshold (CT) of targets in AP samples

Table A Calculations and volumes of PCR Master Mix (MM)

Component	**Volume/ Reaction (µL) (X1)**	**Volume/ Reaction (µL) X*n** (+1)**
Taqman Fast Advanced Mm (2x)	5.0	*5n*+1
Taqman Assay (20x, Primers Listed In Materials)	0.5	*0.5n*+1
Endogenous control (RPL13A)	0.5	*0.5n*+1
Taqman Assay (20x)	1.0	*1n*+1
Nuclease Free H_20	4.0	*7n*+1
Total Per Reaction	10.0	*8n*+1

Notes: number of samples (*n*); ribosomal protein L13a (RPL13A);* *Xn* means volume multiplied by the number of samples; Polymerase chain reaction (PCR)

Table B Set up for PCR run to determine the cycle threshold (CT) of targets in AP samples

	Incubation	**PCR Activation**	**PCR (40 cycles)**	
	Hold	Hold	Denature	Anneal /Extend
Temp	50°C	95°C	95°C	60°C
Time	2 Minutes	2minutes	1 Seconds	20seconds

Notes: *C_T < 29 are strong positive reactions sufficient target gene in the sample*
C_T is 30-37 are positive reactions, reasonable amounts of target gene
C_T is 38-40 are weak reactions negligible amounts of target gene representing an infection state or environmental contamination.

APPENDIX K: The 96-well plate layout for the RT^2 Profiler PCR array including representative genes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	APCS	C3	CASP1	CCL2	CCL5	CCR4	CCR5	CCR6	CCR8	CD14	CD4	CD40
B	CD40LG	CD80	CD86	CD8A	CRP	CSF2	CXCL10	CXCR3	DDX58	FASLG	FOXP3	GATA3
C	HLA-A	HLA-E	ICAM1	IFNA1	IFNAR1	IFNB1	IFNG	IFNGR1	IL10	IL13	IL17A	IL18
D	IL1A	IL1B	IL1R1	IL2	IL23A	IL4	IL5	IL6	IL8	IRAK1	IRF3	IRF7
E	ITGAM	JAK2	LY96	LYZ	MAPK1	MAPK8	MBL2	MPO	MX1	MYD88	NFKB1	NFKBIA
F	NLRP3	NOD1	NOD2	RAG1	RORC	SLC11A1	STAT1	STAT3	STAT4	STAT6	TBX21	TICAM1
G	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TNF	TRAF6	TYK2
H	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

APPENDIX L: Gene table: RT² Profiler PCR Array Position UniGene, GenBank Symbol, Description

A01 Hs.507080 NM_001639 APCS Amyloid P component, serum

A02 Hs.529053 NM_000064 C3 Complement component 3

A03 Hs.2490 NM_033292 CASP1 Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)

A04 Hs.303649 NM_002982 CCL2 Chemokine (C-C motif) ligand 2

A05 Hs.514821 NM_002985 CCL5 Chemokine (C-C motif) ligand 5

A06 Hs.184926 NM_005508 CCR4 Chemokine (C-C motif) receptor 4

A07 Hs.450802 NM_000579 CCR5 Chemokine (C-C motif) receptor 5

A08 Hs.46468 NM_004367 CCR6 Chemokine (C-C motif) receptor 6

A09 Hs.113222 NM_005201 CCR8 Chemokine (C-C motif) receptor 8

A10 Hs.163867 NM_000591 CD14 CD14 molecule

A11 Hs.631659 NM_000616 CD4 CD4 molecule

A12 Hs.472860 NM_001250 CD40 CD40 molecule, TNF receptor superfamily member 5

B01 Hs.592244 NM_000074 CD40LG CD40 ligand

B02 Hs.838 NM_005191 CD80 CD80 molecule

B03 Hs.171182 NM_006889 CD86 CD86 molecule

B04 Hs.85258 NM_001768 CD8A CD8a molecule

B05 Hs.709456 NM_000567 CRP C-reactive protein, pentraxin-related

B06 Hs.1349 NM_000758 CSF2 Colony stimulating factor 2 (granulocyte-macrophage)

B07 Hs.632586 NM_001565 CXCL10 Chemokine (C-X-C motif) ligand 10

B08 Hs.198252 NM_001504 CXCR3 Chemokine (C-X-C motif) receptor 3

B09 Hs.190622 NM_014314 DDX58 DEAD (Asp-Glu-Ala-Asp) box polypeptide 58

B10 Hs.2007 NM_000639 FASLG Fas ligand (TNF superfamily, member 6)

B11 Hs.247700 NM_014009 FOXP3 Forkhead box P3

B12 Hs.524134 NM_002051 GATA3 GATA binding protein 3

C01 Hs.181244 NM_002116 HLA-A Major histocompatibility complex, class I, A

C02 Hs.650174 NM_005516 HLA-E Major histocompatibility complex, class I, E

C03 Hs.643447 NM_000201 ICAM1 Intercellular adhesion molecule 1

C04 Hs.37026 NM_024013 IFNA1 Interferon, alpha 1

C05 Hs.529400 NM_000629 IFNAR1 Interferon (alpha, beta and omega) receptor 1

C06 Hs.93177 NM_002176 IFNB1 Interferon, beta 1, fibroblast

C07 Hs.856 NM_000619 IFNG Interferon, gamma

C08 Hs.520414 NM_000416 IFNGR1 Interferon gamma receptor 1

C09 Hs.193717 NM_000572 IL10 Interleukin 10

C10 Hs.845 NM_002188 IL13 Interleukin 13

C11 Hs.41724 NM_002190 IL17A Interleukin 17A

C12 Hs.83077 NM_001562 IL18 Interleukin 18 (interferon-gamma-inducing factor)

D01 Hs.1722 NM_000575 IL1A Interleukin 1, alpha

D02 Hs.126256 NM_000576 IL1B Interleukin 1, beta

D03 Hs.701982 NM_000877 IL1R1 Interleukin 1 receptor, type I

D04 Hs.89679 NM_000586 IL2 Interleukin 2

D05 Hs.98309 NM_016584 IL23A Interleukin 23, alpha subunit p19

D06 Hs.73917 NM_000589 IL4 Interleukin 4

D07 Hs.2247 NM_000879 IL5 Interleukin 5 (colony-stimulating factor, eosinophil)

D08 Hs.654458 NM_000600 IL6 Interleukin 6 (interferon, beta 2)

D09 Hs.624 NM_000584 IL8 Interleukin 8

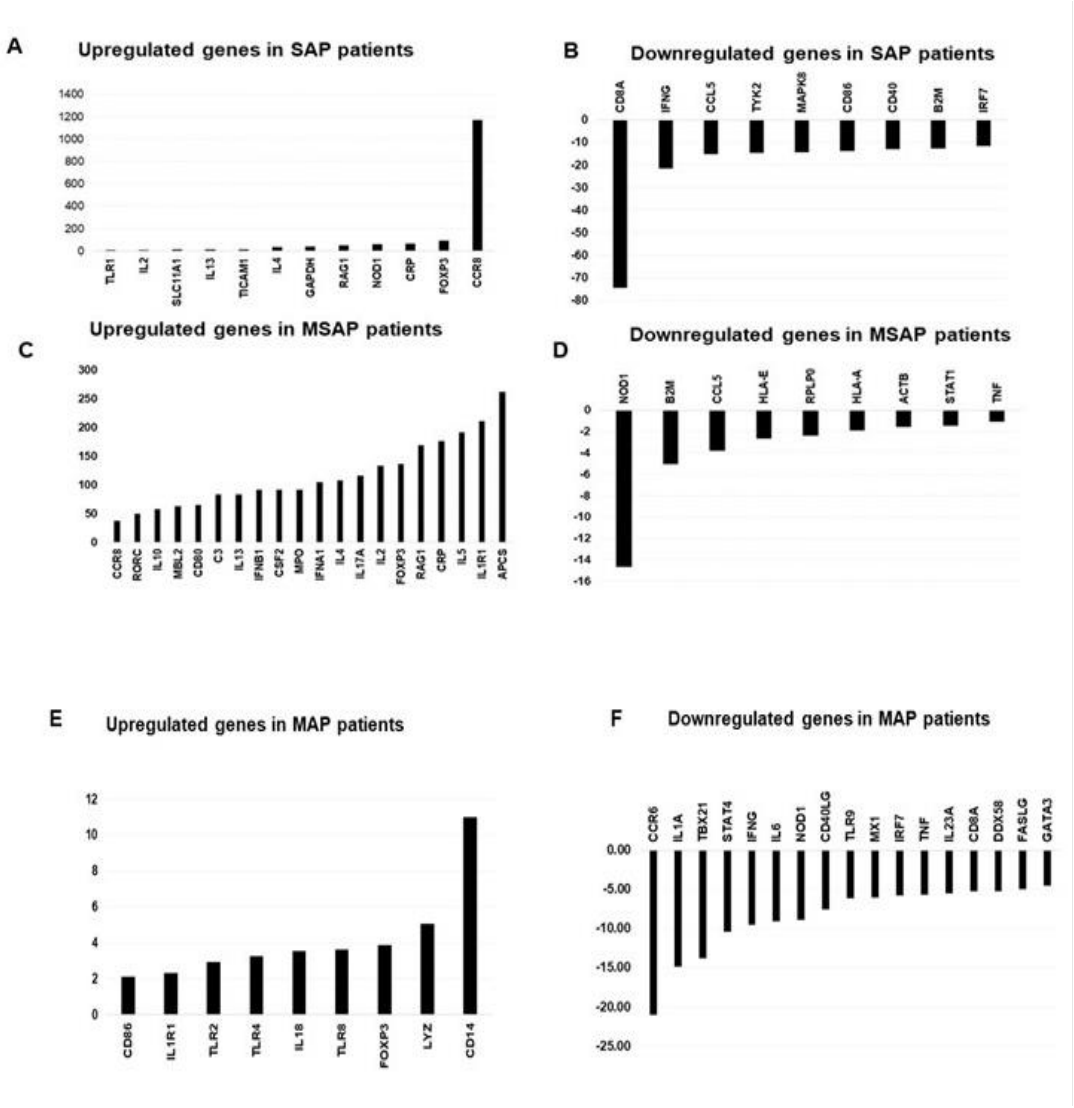
APPENDIX M: A list of selected genes used for the validation assay and their functions

Gene of interest	Function of genes	Cells expressing the genes
CCR8	• *CCR8* expressed on T_{regs} drives immunosuppression (Barsheshet et al. 2017). • Expressed on the ILC1 group of cells which produce IFN-γ (Shay and Kang et al. 2013; Kang et al. 2021)	• Th2 lymphocytes • peritoneal macrophages (PMφ) (Oshio et al. 2014).
CCL1	• ligand of CCR8, • activates the expression of CCR8 in the peritoneal cavity (Oshio et al. 2014)	• peritoneal macrophages (Oshio et al. 2014).
NOD1	• pattern recognition receptor (PRR) • recognises peptidoglycan derivatives from bacteria and triggers innate immune responses (Shay and Kang, 2013)	• macrophages • dendritic cells • peritoneal neutrophil (Jeon et al. 2012; Shay and Kang, 2013; Uhlen et al. 2019)
IL-6	• encodes the IL-6 cytokine • induces inflammation through an acute phase response and activates the mature B cells. • Required for differentiation of naïve CD 4 T cells into Th17 cells (Shay and Kang, 2013; Zhu and Zhu, 2020)	• naïve CD4 T cells • classical monocytes • non-classical monocytes • intermediate monocytes (Shay and Kang, 2013;Uhlen et al. 2019)
TLR1	• receptor that acts as PRR for pathogen-associated molecular patterns (PAMPs) • activates innate immunity (Shay and Kang, 2013; O'reilly and Duffy, 2016).	• neutrophils, classical • non-classical • Intermediate monocytes (Uhlen et al. 2019).
CXCL8	• a neutrophil chemoattractant • recruits basophils and T-cells to the site of inflammation (1.09, 8.4, and 12.95)	• Neutrophils (Uhlen et al. 2019)
MPO	• MPO component of neutrophil extracellular traps implicated in lung injury in SAP patients (Merza et al. 2015)	• classical monocytes • intermediate monocytes • non-classical monocytes • neutrophils (Uhlen et al. 2019).
CCL5 Gene	• a chemokine that attracts monocytes, memory T helper cells, and eosinophils to the site of inflammation • act as an HIV-suppressive factor (Shay and Kang, 2013; Santopaolo, 2021)	• naïve and memory CD4 and CD8 cells (Uhlen et al. 2019).

Notes: Chemokine receptor 8 (*CCR8*); Chemokine ligand 1(*CCL1*); Nucleotide-binding oligomerization domain-containing protein 1 (*NOD1*), interleukin (IL-6). Toll-like receptor 1 (*TLR1*); Myeloperoxidase (MPO) Chemokine ligand 5(CCL5); C-X-C Motif Chemokine Ligand 8 (CXCL8).

APPENDIX O: A to F shows bar graphs of fold regulation of genes in pooled samples.

SAP (n=2) graph A (upregulated genes) and B (downregulated genes). Figure 4.5C and D show MSAP (n=4) group where C has upregulated genes and D is downregulated. Figure 4.4E and F the MAP (n=7) group fold regulation for upregulated and downregulated genes, respectively. These were compared to healthy controls (n=6) at Day 3.



APPENDIX O: Gene expression results colour coded with cells expressing them

Green=lymphocytes; Purple = monocytes and neutrophils; Red: *CCR8* is expressed on macrophages, monocytes, neutrophils, and Th2 lymphocytes as well as ILC2 group cells.

GENE SYMBOL	MILD(MAP)	MODERATE (MSAP)	SEVERE (SAP)
APCS	**1.33**	**262.91**	**-1.01**
CASP1	**-1.43**	**3.02**	**3.38**
CCR8	**1.33**	**38.28**	**1172.45**
IL10	**-1.30**	**58.62**	**-1.47**
IL13	**-1.92**	**83.66**	**19.53**
IL17A	**1.72**	**116.93**	**2.56**
IL23A	**-5.60**	**18.07**	**6.57**
IL4	**-1.13**	**108.64**	**36.83**
IL5	**1.33**	**192.59**	**1.21**
NOD1	**-8.93**	**-14.62**	**64.21**
MPO	**1.33**	**91.77**	**6.82**
GAPDH	**1.38**	**3.47**	**44.64**

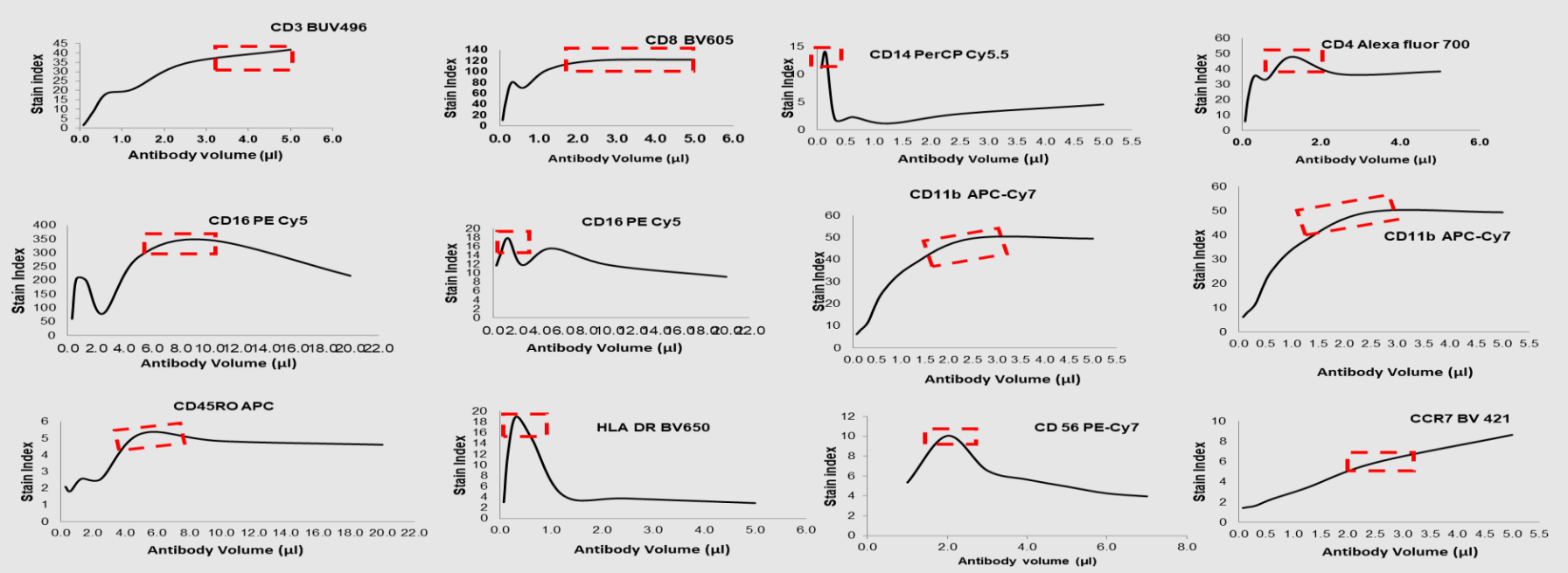
APPENDIX P: 12 colour panel designed for this study according to configuration of BD LSR Fortessa II

Laser Name	Filter	Parameter	Emission	Lymphocytes: B cells	Lymphocytes: T Cells	Lymphocytes :NK Cells	Myeloid cells: Monocytes & granulocytes	Vol per test (µL)	Optimised volume(µL)	Catalogue No; Clone
Red 640										
Power 40	780/60 BP	APC-Cy7	780				CD11b	5	1.0	557754, clone ICRF44
	730/45 BP	Alexa Fluor 700	720		CD4			5	1.0	557922, Clone RPA-T4
	660/20 BP	APC	660		CD45RO			20	4.0	559865, Clone UCHL1
Blue 488										
A	780/60 BP	PE-Cy7	780			CD56		5	1.0	560916; Clone B159
B	695/40	PerCP-Cy5-5	695				CD14	5	2.5	561116; Clone MφP9
C	660/20	PE-Cy5	670			CD16		20	1.0	555408; Clone 3GB
D	610/20 BP	PE-CF594	612	CD19				5	1.0	562321, Clone HIB19
F	530/30BP	FITC	515		CD57			5	1.0	555619, Clone G155-228
Violet 405										
	655/8	BV650	650		HLA-DR		HLA-DR	5	2.5	564231;Clone G46-6
Power 50	605/12	BV605	605		CD8			5	0.5	564115,Clone SK1
	450/50 BP	BV421	421		CCR7/CD197			5	1.0	562555, Clone 150503
UV 355										
	530/30	BUV496	496		CD3			5	1.0	564810, Clone UCHT1

APPENDIX Q: Optimisation of 12 colour panel: Antibody titration and verifying cell populations

PART A. To determine optimum concentrations for antibodies to as in the panel. Serial dilutions were made of each antibody and acquired using LSR II Fortessa. (BD Biosciences, New Jersey, USA). The volume corresponding to the highest stain index was selected for use in the experiment for volumes less than 0.5µL. It was assumed that 0.5µL was sufficient. The dotted red line represents the peak and the optimum volume for respective antibodies. The formula used to determine Stain index(SI) is:

$$SI = \frac{MFI_{positive} - MFI_{Negative}}{2 \times SD_{Negative}}$$



PART B: VERIFICATION OF CELL POPULATION

Verifying negative populations: unstained whole lysed blood (described in 4.4.2.1) was used for establishing negative controls for immunophenotyping. A 100µL of a whole blood sample from a healthy control was washed and fixed with FACs Lysing solution (BD Biosciences, New Jersey, USA), and events were acquired on an LSR Fortessa II flow cytometer (BD Biosciences, New Jersey, USA). Whilst the unstained sample was running, and the PMT voltages were adjusted so that the robust standard deviation (rSD) values were 2.5 times the standard deviation of the electronic noise (SD_{EN}). These values are obtained from the baseline report generated when the quality

control (QC) and set up for the instrument were performed. Precautions were taken to eliminate the electronic noise, which can interfere with measurements, especially where dim fluorescence and negative populations are involved, to rule out false positives

Verifying positive populations: After setting PMT voltages, the cell populations were gated using FACSDiva™ software (BD Biosciences, New Jersey, USA). The mean for all fluorochromes used in the panel was selected. Single stain samples, prepared using the BD Whole Blood and a Lyse wash Procedure (BD Biosciences, New Jersey, USA), were used to adjust the fluorescence to minimise spillover from other dye falling within the same spectrum by subtracting it from the primary detector. These settings, also known as application settings, were maintained throughout the experiments to enable consistency in instrument performance.

APPENDIX R: Optimisation of 12 colour panel: Mean Fluorescence intensity

The BD Biosciences 8 peak bead range per antibody peak six was selected for monitoring. The below shows the expected range for each analysis.

Parameter (clone)	Application (MFI)	Variation allowed (MFI)
BLUE LASER		
CD57 FITC	15005	13505 < MFI < 16505
CD19 PECF594	14196	12776 < MFI <15616
CD14 PerCPCy5.5	4001	3601 <MFI <4401
CD16 PECy5	2420	2174 <MFI <2662
CD56 PECy7	986	888 <MFI < 1084
RED LASER		
CD45RO APC	15918	14327 <MFI <17509
CD4 Alexa Flour 700	6077	5470 <MFI <6684
CD11b APC Cy7	4255	3830 <MFI <4680
VIOLET LASER		
CCR7 BV421	71444	64300 <MFI <78858
HLA DR BV605	1595	1436 <MFI <1754
CD8 BV650	547	493 <MFI <601
UV LASER		
CD3 BUV496	9460	8514 <MFI <10406

Notes: MFI is median fluorescence intensity