IL-6 AND ASSOCIATED IMMUNE MARKERS IN ACUTE PANCREATITIS: POTENTIAL FOR MONITORING DISEASE SEVERITY

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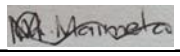
DECLARATION

I, Murunwa Grace Maimela, declare this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)  Date: 25 April 2023

ABSTRACT

Numerous scoring systems for diagnosing, stratifying, and prognosticating acute pancreatitis (AP) have been developed; however, these systems do not fully reflect the evolving understanding of AP immunopathogenesis. Assessment of AP severity and possible complications using inflammatory mediators such as cytokines and dysregulated immune cells may serve as a better option in choosing rational and effective therapeutic options. This study aimed to monitor inflammatory mediators such as interleukin-6 (IL-6), chemokine ligand 4 (CXCL-4), reactive oxygen species (ROS) and associated immune cells to assess their potential in predicting AP severity and determining outcomes of severe AP patients for better management. Twenty-four AP-patients, 17 mild AP (MAP), four moderately severe AP (MSAP), and three severe AP (SAP) patients were recruited from the Chris Hani Baragwanath Academic Hospital (CHBAH), Johannesburg, South Africa. Approximately 16mL of blood was collected on days (D) 3, 5, 7, 9, 13 and 15 post-epigastric pain. These samples were assayed with once-off samples from eight age- and sex-matched healthy controls (HCs). ROS were studied using a spectrophotometric assay, and IL-6 and CXCL4 were analysed by intracellular cytokine staining (ICS) and enzyme-linked immunosorbent assays (ELISA). Among the 24 patients, biliary AP (n=14, 58%) was the predominant aetiology. IL-6, CXCL4 and ROS increased with increasing AP severity (SAP>MSAP>MAP), especially from D9-D15. These findings agreed with routine clinical biochemical test results such as C-reactive protein (CRP), which were shown to increase with severity ($p=0.019$). Additionally, natural killer cells (NKs) and activated $CD62p^{+}$ platelets were expressed more in the severe patient group and further linked to IL-6 and CXCL4 secretion in AP patients, respectively. Statistically significant differences were only observed on D9 and D13 for the ROS data between SAP patients and HCs, $p=0.0050$ and $p=0.0071$, respectively. These preliminary findings further support that IL-6, CXCL4 and ROS combined with clinical parameters may be useful in monitoring and predicting the outcome of patients, especially in SAP, for improved care and better AP management. Importantly, for the SAP patient who enters a second phase of disease, the findings provide important implications of early intervention and can potentially inform management guidelines.

DEDICATION

To my mother.

Thank you for being my greatest inspiration!

PROJECT OUTPUTS

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Contents

LIST OF ABBREVIATIONS AND SYMBOLS

α1AT	α1-antitrypsin
α2M	α2-macroglobulin
AP	Acute Pancreatitis
AP-1	activator protein-1
APACHE II	Acute Physiology and Chronic Evaluation II
APC	Allophycocyanin
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
BUN	Blood Urea Nitrogen
BUV	BD Horizon Brilliant™ Ultraviolet
BV	Brilliant Violet™
CARS	Compensatory Anti-inflammatory Response Syndrome
CD	Cluster of Differentiation
CHBAH	Chris Hani Baragwaneth Academic Hospital
CRP	C-Reactive Protein
CECT	Computed Enhanced Computed Tomography
CT	Computed Tomography
CXCL4	Chemokine Ligand 4
CXCR3	Chemokine Receptor 3
DEPPD	N, N-Diethyl-para-phenylenediamine
DRV/R	Darunavir/Ritonavir
DOP	Day of Pain
EDTA	Ethylenediaminetetraacetic Acid
EFV	Efavirenz
ELISA	Enzyme-linked Immunosorbent Assay
ERCP	Endoscopic Retrograde Cholangiopancreatography
FACS	Fluorescence-Activated Cell Sorting
FACSDiva	Fluorescence-Activated Cell Sorting Diva Software
FITC	Fluorescein Isothiocyanate
HAART	Highly Active Antiretroviral Therapy
HMGB1	High-Mobility Group Box Protein 1
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen – Antigen D Related
HPB	Hepatopancreaticobiliary
ICU	Intensive Care Unit
IFN-γ	Interferon-Gamma
IL	Interleukin
IL-6	Interleukin-6
IL-6R	IL-6 receptor
IPN	Infected Pancreatic Necrosis
LC	Laparoscopic Cholecystectomy

LOS	Length of Hospital Stay
MAP	Mild Acute Pancreatitis
MARS	Mixed Antagonist Response Syndrome
MFI	Median Fluorescence Intensity
MPO	Myeloperoxidase
MSAP	Moderate Severe Acute Pancreatitis
MODS	Multiple Organ Dysfunction Syndrome
MSOF	Multiple System Organ Failure
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor Kappa B
NLR	Neutrophil Lymphocyte Ratio
NK	Natural Killer
NKT	Natural Killer T lymphocytes
Non-NRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
OF	Organ Failure
PSTI	Pancreatic Secretory Trypsin Inhibitor
PE	Phycoerythrin
PE-CF594	Phycoerythrin Cyanine based dyes
PerCP	Peridinin-Chlorophyll-Protein
RAC	Revised Atlanta Classification
RHO	Spearman's Coefficient or Spearman's Correlation
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RSD	Robust Standard Deviation
RVD	Retroviral Disease
SAHCS	Southern African HIV Clinicians Society
SAP	Severe Acute Pancreatitis
SDEN	Standard Deviation of The Electronic Noise
SIRS	Systemic Inflammatory Response Syndrome
SPSS	Statistical Package for the Social Sciences
STAT3	Signal Transducer and Activator of Transcription 3
TAP	Trypsinogen activation peptide
TNF-α	Tumour Necrosis Factor-Alpha
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Acute pancreatitis (AP) is the sudden onset of inflammation of the pancreas, which involves a cascade of complex immune responses (Shamoon et al., 2016; Kim et al., 2017). Alcohol abuse and gallstones are the most common aetiologies, accounting for approximately 80% of AP cases worldwide (Ding et al., 2020). The disease course of AP is not clearly understood; however, the conventional theory revolves around events that take place in pancreatic acinar cells (Criddle, 2016). The premature activation of enzymes (particularly trypsinogen) induces damage to pancreatic acinar cells, which leads to auto-digestion of the pancreas. As a result, the immune system becomes activated and a systemic inflammatory response syndrome (SIRS) develops, subsequently leading to the occurrence of single or multiple organ failure (OF) and in some cases mortality (Lee and Papachristou, 2020).

The 2012 Revised Atlanta Classification (RAC) defines three classes of AP severity based on clinical criteria. The categories are mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), and severe acute pancreatitis (SAP) (Banks et al., 2013). In South Africa, most units, including the CHBAH unit where this study was done, use the RAC guidelines to diagnose and stratify AP severity (Anderson, 2019; Cook et al., 2022). Furthermore, biochemical markers such as C-reactive protein (CRP) and creatinine are used to assist in AP diagnosis, prognosis, and initiation of therapy (Silva-Vaz et al., 2020). The disadvantages of these tests are that they are time-sensitive and inconsistently sensitive.

Preliminary studies have shown that severe forms of AP result from dysregulated immune cells and inflammatory molecules such as cytokines (Fonteh, Smith and Brand, 2018). Infiltration of immune cells (leukocytes) into the pancreas following AP onset has proven to be closely related to the severity and prognosis of the disease (Peng, Li and Yu, 2021). Cytokines such as interleukin-6 (IL-6) and IL-8 and chemokines such as chemokine ligand 4 (CXCL4) and chemokine ligand 2 (CXCL2) have been reported to be predictors of AP severity. However, their role in prognosticating AP has not been fully established. IL-6 is a cytokine of interest in AP due to its pleiotropic effects, such as stratifying severity and AP prognostication and possibly serving as a guide for management and therapeutic options (Kolber et al., 2018).

Worldwide, AP treatment is predominantly supportive (Wu and Banks, 2013). Most patients are managed in general surgical wards. Those experiencing organ dysfunction or other complications are managed in critical care units (MacGoey, Dickson, and Puxty, 2019). In South Africa, initial management options are clinical interventions that highly depend on biochemical tests results and the discretion of the clinician's assessment of the patient (Anderson et al., 2008). Interventions include; resuscitation, analgesia and supplemental oxygen in the presence of hypoxaemia (Anderson et al., 2008; MacGoey, Dickson, and Puxty, 2019). Early stratification of AP severity is crucial, especially within 24 hours of admission, because this period provides the opportunity for interventions aimed at organ support to allow recovery. Furthermore, patients who require transfer to centres of excellence can be identified early on. However, early and accurate stratification, monitoring and prediction of patient outcomes is still a great challenge due to the inconsistency of the currently available biochemical markers, scoring systems and the complex underlying pathophysiology of AP (Kaplan et al., 2017; Zhang et al., 2017).

Improved AP prognostication may provide improved guidance for management and potential therapeutic targets. Currently, there is no molecular marker or composite score that can precisely predict AP severity and subsequent complications at an early stage to inform late severity and potential complications. However, prior work in our laboratory indicated that IL-6 expression is higher in SAP than in MSAP patients (Nalisa et al., 2021). Furthermore, the monocyte frequency was also higher than that of lymphocytes in SAP patients compared to MSAP and MAP patients (Nalisa et al., 2021). Therefore, exploring the association of IL-6 expression with subsets of leukocytes known to produce IL-6 across different AP severity groups may be of value in the early prediction of AP severity and possible outcomes of patients. Furthermore, CXCL4 and ROS, which play vital roles in the AP inflammatory response, were assessed as potential prognostic markers.

1.2 Literature Review

1.2.1 Epidemiology, aetiologies, and risk factors for acute pancreatitis (AP)

Globally, the major challenge affecting the health sector is non-communicable diseases (NCDs). AP is an NCD recognised as a leading cause of hospitalisation among gastrointestinal disorders. There has been an increase in the incidence and prevalence of AP over the past few decades (Bertilsson and Kalaitzakis, 2015; Krishna et al., 2017). The median and interquartile

range (IQR) of the annual global incidence was reported as 76.2 (68.9 to 83.4) in 2017 per 100 000 population. This was an increase of 13.3% from 1990 to 2017 (Ouyang et al., 2020). Acute Pancreatitis cases have often been reported from western countries; however, recently, there have been reports from other countries (Carlos et al., 2019; Patel et al., 2022). South African studies have reported an AP incidence of approximately 80 in 100 000, which is within international estimates (Anderson et al., 2008; Nesvaderani et al., 2015). Inconsistency in reports of AP incidence and prevalence is mainly caused by differences in study settings, aims, methodologies, complications associated with accurate diagnoses and the use of different diagnostic criteria (Iannuzzi et al., 2022). Furthermore, the demographic distribution of AP occurrence also varies in different regions based on etiological factors and lifestyle patterns (Li et al., 2021).

Acute Pancreatitis has various aetiologic factors, such as metabolic disorders, surgery and autoimmunity, but the main ones are gallstones and alcohol abuse, which account for ~80% of AP cases (Ding et al., 2020). In many European countries, gallstones are the leading cause of AP (Roberts et al., 2017). In the past, African countries reported alcohol abuse as the predominant aetiology associated with AP (Mutebi, Abdallah, and Saidi, 2007; Anderson et al., 2008). South Africa has been reported to have one of the highest incidences of alcohol-associated AP globally, with an annual alcohol consumption level of approximately 16.6 L per drinker (Anderson et al., 2008; van Walbeek and Blecher, 2014). In a study by Anderson et al., (2008) that was conducted with a cohort of 282 AP patients,between 2001 and 2006 at Addington Hospital in Durban, South Africa, 62% of the patients had alcohol-associtaed AP. However, Thomson, Brand and Fonteh, (2018), showed that biliary-associated AP was the most common aetiology in a South African cohort. Furthermore, a recent study in South Africa from August 2018 to September 2019 reported an equal prevalence of biliary- and alcohol-associated AP (Nalisa et al., 2021). This observation is likely associated with increasing lifestyle changes that influence AP aetiological and risk factors (Koncz et al., 2020; Pang et al., 2018; Reid et al., 2017).

Acute Pancreatitis incidence does not differ based on gender and age. However, Yadav and colleagues (2011) reported that alcohol-associated AP has a peak incidence age range of 35 to 44 years in males and 25 to 34 years in females, respectively. Furthermore, Krishna et al., (2017) and Roberts et al., (2017) reported that most people in their early forties have AP associated with alcohol aetiology and those in their mid-sixties are usually diagnosed with

biliary pancreatitis. However, the changing dynamics in lifestyle diseases occurring in much younger populations might influence such findings in the future (Iannuzziet al., 2022). AP associated with alcohol intake is common in men, and AP associated with gallstones is common in women (Weiss, Laemmerhirt and Lerch, 2019). It is essential for the AP aetiology to be determined on admission or during the early course of the disease, as it influences the ability to select the best management strategies, therapy and prevention of the recurrence of the disease (Lauret, Rodríguez-Peláez and Rodrigo Sáez 2015; Leppäniemi et al., 2019).

According to the WHO (2020), approximately 70% of deaths worldwide are due to NCDs, such as diabetes, hypertension, cancers and AP. The South African statistic of deaths attributed to NCDs is 51% (WHO, 2020). In a study by Li and colleagues (2021), it was reported that the estimated mortality rate globally due to AP in 2019 was 1.4/100 000 (0.0014%). This was a decrease from 12% in 2003 (Fagenholz et al., 2007). Overall, the mortality rate of patients admitted to hospitals due to AP is estimated to range from <1% to 30% (Nesvaderani et al., 2015; Stoppacher, 2018). The lowest mortality rate (<1%) is associated with MAP since it is often self-limiting and resolves within the first week of AP onset, while the highest mortality rate (10-30%) is associated with SAP (Stoppacher, 2018).

Although some AP cases are idiopathic, other possible risk factors include endoscopic retrograde cholangiopancreatography (ERCP), adiposity, obesity and comorbid diseases such as human immunodeficiency virus (HIV), hypertriglyceridaemia, diabetes and the use of certain medications (steroids and oestrogens) (Pang et al., 2018). Diabetes mellitus is one of the most common comorbidities affecting approximately 422 million adults worldwide (WHO, 2016). Studies have revealed that type 2 diabetes mellitus increases the risk of AP by 1.5 to 3 times, especially in younger diabetic patients (Yang et al., 2013; Mikó et al., 2018). Additionally, Richardson and Park (2021) reported a type 3c diabetes mellitus (T3cDM), which occurs following an AP episode and has been reported in approximately 15% of AP patients within a year of being diagnosed with AP (Richardson and Park, 2021).

The incidence of AP is higher in HIV-positive populations compared to the general population due to complications resulting from HIV infection or anti-retroviral (ARV) therapy (Anderson and Thomson, 2017). A study by Nel and colleagues (2020) stated that, according to the Southern African HIV Clinicians Society (SAHCS) guidelines for ARV therapy, atazanavir/ritonavir (ATV/r), efavirenz (EFV), darunavir/ritonavir (DRV/r), and

lopinavir/ritonavir (LPV/r) were the prescribed therapeutic options for HIV-infected adults in 2020. However, on a global scale, highly active antiretroviral therapy (HAART), which includes these drugs, has been implicated in the development of AP in HIV-positive patients (Weissman et al., 2020).

1.2.2 Classification of acute pancreatitis (AP)

Based on a 2012 review of the 1992 Atlanta classification, AP severity is classified into three groups MAP, MSAP and SAP (Banks et al., 2013, Seppänen and Puolakkainen, 2020). In MAP, inflammation is localised to the pancreas, and there is neither OF) nor local/systemic complications, recovery occurs within the first week of AP onset (Banks et al., 2013). MSAP and SAP are similar, except that MSAP patients experience transitory OF (less than 48 hours) and possibly specified local complications while SAP patients experience persistent OF and various local complications (Banks et al., 2013; Phillip, Steiner and Algül, 2014; Ignatavicius et al., 2017; Liao et al., 2020). Local complications may include pseudocyst, pancreatic and peripancreatic necrosis (either sterile or infected) and pancreatic fluid collection (Banks et al., 2013). SAP is characterised by a clinical course in two phases, early and late AP (Figure 1.1); however, these phases depend on the type of treatment or clinical interventions provided initially after AP onset (Pagliari et al., 2019; Phillip et al., 2014). The early phase usually lasts for a week from AP onset and is characterised by a complex inflammatory reaction beginning with a systemic proinflammatory phase known as the SIRS (Phillip, 2014; Żorniak , Beyer and Mayerle, 2019; Seppänen and Puolakkainen, 2020). SIRS results from a complex cytokine cascade that involves the overcompensation of anti-inflammatory and pro-inflammatory cytokines leading to a compromised immune system (Chakraborty and Burns, 2022).

Subsequently, the SIRS phase is followed by a mixed inflammatory response known as the mixed antagonist response syndrome (MARS), followed by the compensatory anti-inflammatory response syndrome (CARS) known as the suppressed inflammatory response (Phillip, Steiner and Algül, 2014; Weitz et al., 2015). Downregulation of the immune system occurs in the CARS phase, which indirectly increases pancreatic and peripancreatic necrotic tissue susceptibility to infections (Heckler et al., 2021; Meher et al., 2015). This could be one of the reasons why infections are absent within the first few days of AP onset and occur later towards and beyond the end of the first week (Heckler et al., 2021). During the CARS phase, pathogens migrate through the damaged pancreas from the intestinal lumen into necrotic tissues

(Phillip, Steiner and Algül, 2014). This period is referred to as the second phase of AP and includes local and systemic complications, sepsis, persistent OF and death (Phillip, Steiner and Algül, 2014). Three organ systems are assessed to define OF: respiratory, cardiovascular and renal (Banks et al., 2013). OF is often defined if two or more of these three organ systems are dysfunctional based on the modified Marshall scoring system (Garg and Singh, 2019; Marshall et al., 1995).

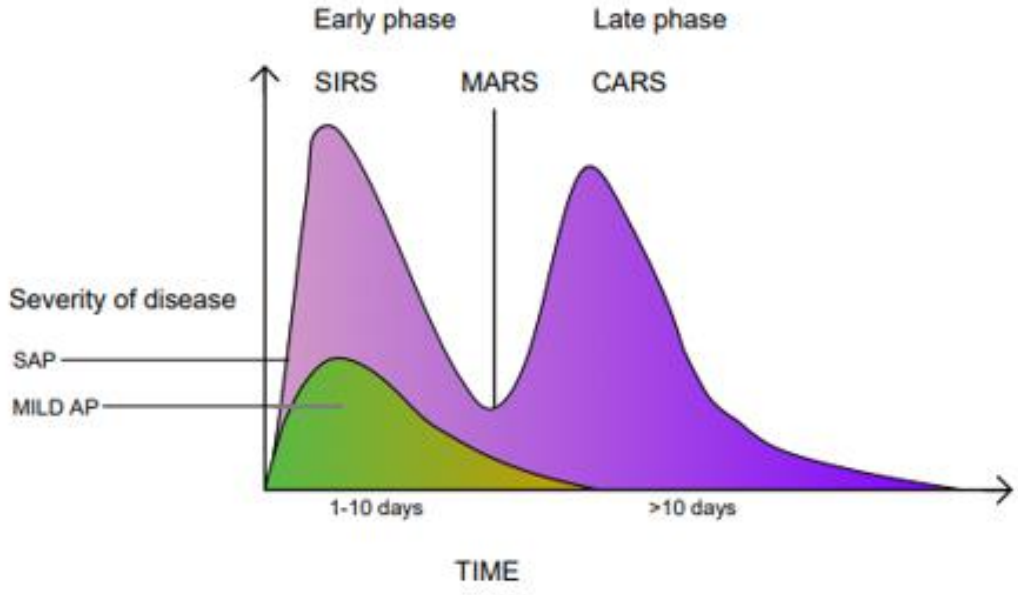

Figure 1.1: The clinical course of acute pancreatitis in mild and severe acute pancreatitis patients. The SIRS phase is present in both mild and severe patients, and the MARS phase manifests clinically as MSAP. However, the CARS phase is specific to severe patients and is highly dependent on the type of clinical interventions provided initially upon AP onset and hospital admission. **Notes:** AP: acute pancreatitis; SAP: severe acute pancreatitis SIRS: systemic inflammatory response syndrome; MARS: mixed antagonist response syndrome; CARS: compensatory anti-inflammatory response syndrome (Adapted from Meher et al., 2015).

1.2.3 Clinical course of acute pancreatitis (AP)

Despite various research done over centuries, the definite pathogenesis of AP is still elusive. The obstacles are that AP has a rapid course and that the pancreatic tissue is relatively inaccessible (Weitz et al., 2015). The most common pathophysiological theory of AP is centred on pancreatic acinar cell injury, which is a result of the intra-acinar autodigestion of the pancreas by pancreatic enzymes, leading to leukocyte activation, infiltration and altered acinar cell signalling (Dudeja, Dawra and Saluja., 2018; Derrick, Frandy and Wirawan., 2020). Early

events of AP also include the secretion of vascular and inflammatory mediators, reduction of digestive enzymes secretion by acinar cells and activation of cell death pathways (Gorelick and Thrower, 2009; Banks et al., 2013; Cenci et al., 2017). Premature activation of pancreatic enzymes stimulates the auto-digestion of pancreatic acinar cells, causing the secretion of various cytokines and inflammatory mediators such as IL-1, IL-6, IL-8 and tumour necrosis factor (TNF-α). This, directly or indirectly, activates an inflammatory cascade, stimulating organs such as the liver which synthesises the acute phase protein CRP, eventually causing acute respiratory distress syndrome (Dabrowski et al., 2014). Furthermore, it amplifies the inflammatory reaction and the extent of pancreatic injury.

According to a 2019 study by Szakács and colleagues, age and comorbidities modify the clinical outcomes of AP patients. This is because comorbidities increase with age and may subsequently increase the risk of AP or even induce a change in the disease course, which results in longer hospital stays and, in some cases, mortality (Márta et al., 2019). Older age has been flagged as a risk factor for severity and mortality in AP and the age range of >45 to <70 years is more associated with adverse clinical outcomes according to the various scoring systems (Moran et al., 2018). There is scant data evaluating the association or impact of comorbidities on the severity of AP. Obesity is known to be associated with many medical comorbidities and has been validated as a predictor of local and systemic complications and mortality in AP patients (Smeets et al., 2019). Intrapancreatic fat elevation is associated with increased body mass index (BMI), which increases visceral fat surrounding the pancreas (Khatua, El-Kurdi and Singh, 2017). According to the American Pancreatic Association and the International Association of Pancreatology, evidence-based guidelines suggest that using risk factors such as age, BMI and comorbidities is considered part of a three-pronged approach to predict outcomes in AP patients (Moran et al., 2018). Acute Pancreatitis complications can be local or remote, acute or delayed, and abdominal or systemic (Bansal et al., 2019). Pancreatic necrosis is regarded as one of the most crucial complications in AP patients and is also noted as an essential indicator of AP severity (Singh et al., 2017). Necrosis occurs in approximately 6%–20% of AP patients, with 90% of the affected patients prone to developing at least one OF (Garg and Singh, 2019). AP patients without necrosis have a 6% morbidity rate and no mortality. However, those with AP with complicated necrosis have a morbidity and mortality incidence rate of 82% and 23%, respectively (Singh, 2018).

Acute Pancreatitis diagnosis is based on typical clinical symptoms such as abdominal pain and nausea, physical examination, laboratory analysis and radiological data (Walkowska et al., 2022). Based on the practice guidelines published in 2006, AP diagnosis requires a minimum of two of the following features: abdominal pain, elevated serum amylase or lipase (three times more than the upper limit of normal) and AP evidence on contrast-enhanced computed tomography (CECT) (Banks et al., 2013; Nadhem and Salh, 2017). Almost every patient with AP experiences upper abdominal pain during the onset of the disease. Pancreatic enzymes derived from pancreatic acinar cells, such as amylase, lipase and the proenzyme trypsinogen, are the basis of laboratory diagnosis of AP (Rompianesi et al., 2017). Other laboratory tests performed include complete blood counts, blood urea nitrogen (BUN), creatine, electrolytes, liver function tests, and tests for inflammatory markers, such as C-reactive protein (CRP) and IL-6 (Ćeranić , Zorman and Skok, 2020). According to a 2012 study by Gomez and colleagues, the specificity and sensitivity of amylase tests in diagnosing AP were 78.6% and 99.1%, respectively. However, the elevation in pancreatic enzyme levels does not correlate with disease severity and should not be employed as a tool for assessing AP prognosis or severity (Basnayake and Ratnam, 2015). C-reactive protein levels >150 mg/dL at 48 hours are preferred for stratifying AP severity (Cenci et al., 2017).

In AP patients, adequate management is essential within the first two to three days of disease onset, as this can determine the future prognosis (Goodchild et al., 2019). It is also essential that the aetiology is determined and prognostic markers used for disease severity stratification are tested on admission (Wu and Banks, 2013). Both results assist in directing the need for definitive treatment and preventing recurrence (Leppäniemi et al., 2019). It has also been observed that delayed admission worsens prognosis in patients with SAP and early OF (McClave, 2019). To date, definite treatment for AP is still lacking. However, various treatment and management options are offered to patients as supportive care, including pain management, fluid therapy, organ support, parenteral nutrition or early enteral feeding and monitoring (Abu-El-Haija et al., 2018). AP treatment and management options and follow-up depend highly on AP aetiology (Leppäniemi et al., 2019). For instance, in biliary-associated AP, surgical procedures such as laparoscopic cholecystectomy (LC) and endoscopic retrograde cholangiopancreatography (ERCP) are done to evaluate and manage the disease (Walkowska et al., 2022). Furthermore, once patients are discharged, they are advised to do follow-up visits to prevent recurrence (Goodchild et al., 2019). Aggressive hydration is recommended to all patients except in cases where cardiovascular or renal comorbidities preclude it. Early

aggressive intravenous hydration is often beneficial within the first 12–24 hours of admission but may be less beneficial beyond that time (Crosignani et al., 2022).

Lately, the prognosis of SAP has improved because of early and aggressive conservative treatment in intensive care units (ICU) (Mihoc et al., 2021). In some cases, early ERCP is a suggested management strategy for biliary AP involving biliary obstruction (Saritaş and Üstündağ, 2021). Enteral feeding is the preferred route of nutrition in SAP patients and results in the reduction of infectious complications and the need for surgery, which decreases the chances of mortality (Jabłońska and Mrowiec, 2021). All these therapeutic options are primarily supportive and time-sensitive (Wu and Banks, 2013).

1.2.4 Prognostic makers used for the prediction of acute pancreatitis (AP) severity

In the past, much effort has been devoted to research involving laboratory parameters which can be used to stratify the severity of AP into three risk groups. The ideal laboratory tests for assessing AP severity and prognosis should be simple, accurate, inexpensive and easily accessible in urgent cases (Hassan et al., 2018). Several clinical studies have investigated the role of circulating cytokines as predictors and markers of AP severity (Farrel et al., 2021; Meher et al., 2015). Prediction of AP severity guides the management of the disease and improves the outcome (Li et al., 2017). Various biochemical molecules such as IL-6, CRP and procalcitonin have been identified as potential markers for early severity stratification (Jones et al., 2017).

Prognostic markers in the early assessment of severity in AP are considered crucial, especially on admission. This is regarded as a period of opportunity for establishing interventions to inhibit or minimise pancreatic necrosis and OF (Silva-Vaz et al., 2020). Nevertheless, available clinical scoring systems such as APACHE-II, Balthazar and Ranson scores have disadvantages. For instance, they are not easy to learn by heart, require more than 48 hours for complete AP stratification and depend on tests not widely available such as serial tomography and IL-6 tests (Borges et al., 2017). Introducing the measurement of immune biomarkers which increase the secretion of IL-6, reactive oxygen metabolites and platelet-activating factors in clinical settings may assist in circumventing shortcomings faced by clinicians in terms of prognostication and stratification of AP severity (Bhanou et al., 2018; Li et al., 2017; Meher et al., 2015).

1.2.4.1 IL-6 in acute pancreatitis (AP)

Over the past years, IL-6 has been flagged as an important inflammatory and severity marker in AP (Rao and Kunte, 2017; Chen et al., 2021). Acute Pancreatitis severity is associated with increased IL-6 levels following pancreatic acinar cell injury, and IL-6 levels have been shown to increase with increasing disease severity (Chen et al., 2021). IL-6 is expressed by various immunologically active cells, such as monocytes, leukocytes, neutrophils, endothelium and pancreatic acinar cells, as a response to stimuli (Rao and Kunte, 2017). Among these cells, only a few, predominately neutrophils, macrophages and hepatocytes, have IL-6 receptors (Kolber et al., 2018; Szatmary and Gukovsky, 2016). IL-6 stimulates the differentiation of cells such as B cells, macrophages, and Th17 cells to produce antibodies (Kolber et al., 2018). Interleukins in circulation usually degrade rapidly, with IL-6 having a 15.5-hour half-life (Liu et al., 2021). However, when testing for IL-6, it is permissible to store samples at –20° C for a maximum of six months before analysis, provided the blood samples are centrifuged and frozen within 6 hours after phlebotomy. IL-6 levels in plasma and serum have been reported to distinguish SAP from MAP on day one; however, maximum levels are observed at 72 hours following the onset of the disease (Kolber et al., 2018; Meher et al., 2015). Most patients with MAP have undetectable plasma or serum levels of IL-6; however, in SAP patients, the value of IL-6 is significantly high on the day of admission (Rao and Kunte, 2017). This makes plasma and serum IL-6 levels vital in reflecting the severity of an AP attack (Kolber et al., 2018; Luo et al., 2021).

High plasma IL-6 concentrations after 24 hours from admission are often associated with mortality (Manjunath and Kumar and Pradeep, 2017). Interestingly, simultaneously measuring IL-6 and lipase levels improves the accuracy of both the diagnosis and prognosis of SAP (Hassan et al., 2018). However, the level of IL-6 measured on day one cannot be used in comparison with the Ranson and APACHE II scores established on day two for diagnosis or severity stratification (Ferreira et al., 2015). IL-6 induces hepatocytes to produce CRP. Its serum concentration is often high on admission, reaching a peak at 72 hours following the onset of inflammation, which assists in the early prediction of AP severity (Liu et al., 2021, Meher et al., 2015). Inflammatory mediators upstream of CRP, such as IL-6 and other cytokines, often react faster in AP cases. Some preliminary results of these mediators look promising as early prognostic markers for stratifying AP severity (Do, 2015). Among various anti-inflammatory and pro-inflammatory cytokines, IL-6 is the most suitable in terms of sensitivity and specificity

for early stratification of SAP (Chen et al., 2021; Meher et al., 2015). It distinguishes SAP and MAP with 86% to 100% sensitivity and 71% to 100% specificity (Kılıc et al., 2017).

According to a 2000 study conducted by Czakó and colleagues on AP in rats, an elevation in serum IL-6 measurements was observed to precede severe pancreatic oedema and necrosis. IL-6 is a better earlier marker of AP severity in humans than CRP. Notably, a study by Jain et al. (2018) showed that assessing serum IL-6 increases the accuracy of SIRS in predicting SAP. Concerning complications, IL-6 has been found to predict remote OF, which is a fundamental aspect of SAP (Kolber et al., 2018). The disadvantages of an IL-6 assay are that the concentration of serum and plasma IL-6 decreases rapidly, and, in routine clinical practices, it is one of the most expensive and complex assays (Ćeranić, Zorman and Skok, 2020).

1.2.4.2 The role of C-reactive protein (CRP) in acute pancreatitis (AP)

In addition to IL-6, another widely accepted marker for stratifying AP severity is CRP. C-reactive protein is an acute-phase reactant produced by hepatocytes and is commonly high in inflammatory conditions (Sproston & Ashworth, 2018). It is the most frequently used marker because of its clinical availability and low cost. It is also a reliable parameter for diagnosing SAP early (Ćeranić et al., 2020). Vinish et al. (2017) demonstrated that measuring CRP levels on admission day or earlier than 48 hours does not reliably stratify AP severity. However, statistical significance is obtained when CRP is measured after 48 hours or on the third day after disease onset. It always peaks later than the interleukins (Sandberg and Borgströmet, 2002; Fisic et al., 2013; Vinish et al., 2017). A concentration greater than 150mg/dL is usually accepted as a predictor of AP severity (Stirling et al., 2017). At this cut-off level, CRP is characterised by sensitivity and specificity of 80–86% and 61–84, respectively (Meher et al., 2015). This becomes useful in diagnosing necrotising pancreatitis within the first 48 hours of AP onset (Fujiwara et al., 2021). Furthermore, the value of CRP lies in its ability to predict the recovery of acute pancreatic inflammation since follow-up CRP levels accurately reveal patients who are likely to develop complications or recover completely (Deherkar et al., 2019).

1.2.5 The role of immune cells in acute pancreatitis (AP)

1.2.5.1 The role of monocytes and macrophages in acute pancreatitis (AP)

Neutrophils and macrophages play a dual role in inflammation and stimulate repair following injury (Habtezion, 2015; Hu et al., 2020). Tissue injury associated with the activation of the

innate immune system and rapid infiltration of neutrophils is usually followed by macrophage recruitment (Habtezion, 2015; Wan et al., 2021. However, when a disturbance affects this balance, continuous inflammation occurs, and neutrophils release interferon-gamma (IFNγ), which leads to the recruitment of pro-inflammatory macrophages that impair pancreatic regeneration and promote de-differentiation of the pancreatic epithelium (Habtezion, 2015).

Monocytes are a vital type of white blood cell (WBC) found in the circulatory system (Zheng et al., 2019). Macrophage precursors enter the bloodstream from the bone marrow as promonocytes, which develop into monocytes and eventually migrate to tissues and undergo final differentiation into specific types of tissue-resident macrophages (Mosser and Edwards, 2008; Zheng et al., 2019). Monocytes and macrophages are critical inflammatory cells in the pathogenesis of AP, and the degree to which macrophages are activated can be a vital factor in determining AP severity (Hidalgo-Sastre et al., 2021; Wu et al., 2018; Zheng et al., 2013). Furthermore, macrophages are one of the major leukocyte populations in an inflamed pancreas (Peng, Li and Yu, 2021; Zheng et al., 2013). Macrophages can initiate and resolve inflammation, which makes them an interesting target for designing therapeutic strategies based on controlling the systemic effects of AP (Sahay et al., 2020).

The heterogeneity and plasticity of macrophages, which exhibit anti-inflammatory and pro-inflammatory properties, have indicated that macrophages do not only mediate inflammatory injury, they can also be induced to modify the sequential events that occur during AP development (Peng, Li and Yu, 2021; Zheng et al., 2013). According to Zheng and colleagues (2013), experimental strategies practised by Gloor and colleagues in 2000 to inhibit or eradicate macrophages using macrophage inhibitors such as liposome-encapsulated dichloromethylene diphosphonate and gadolinium chloride were shown to modulate SIRS. However, most studies that practiced macrophage inhibition, usually administer the inhibitors before AP induction, which is clinically irrelevant because most patients present macrophages after pancreatic injury.

1.2.5.2 Lymphocytes in acute pancreatitis (AP) disease severity

Lymphocytes are white blood cells responsible for the immune response (Shi et al., 2018). Based on their functions and surface molecules, they are classified into B lymphocytes (B cells), T lymphocytes (T cells), and natural killer (NK) cells (Shi et al., 2018). T cells include $CD4^+$ and $CD8^+$ lymphocytes and develop in the thymus gland. However, they become

activated in the periphery of various tissues and organs (Zhou et al., 2020). B cells develop in the foetal liver and mature in the bone marrow; their primary role is to produce antibodies (Zhou et al., 2020). According to Bhatia et al. (2020), $CD4^+$ T cells deplete more than $CD8^+$ T cells in MSAP and SAP patients due to increased infiltration from blood vessels to the inflamed pancreas. Increased T cell infiltration into the pancreas is promoted by the systemic increase of pro-inflammatory cytokines such as IL-6 secreted by pancreatic acinar and other innate immune cells (Bhatia et al., 2020). Based on the AP pathophysiology, T and B cells also produce cytokines that contribute to the inflammatory process by immune system dysregulation and, subsequently, OF (Singh and Garg, 2016; Fonteh, Smith and Brand, 2018). In MAP patients, as the disease resolves, the $CD4^+$ T cell frequency returns to its normal range; however, if the disease course progresses to a more severe form, CD4 levels remain low due to complications such as necrosis and tissue abscess (Bhatia et al., 2020).

Natural Killer cells are a subset of cytotoxic lymphocytes that play various roles in innate and adaptive immunity (Ding et al., 2020; Willinger, 2019). Natural Killer cells are large and granular and lack expression of T or B cell receptors. Natural Killer cells are characterised by phenotypic markers $CD3^-$ $CD16^+$/and/$CD56^+$; they are important cytotoxic lymphocytes in innate immunity and secrete TNF-α and IFN-γ to counteract viral infections (Rehermann, 2015). In humans, these CD3-negative cells are recognised by CD16 and CD56 surface markers and makeup 5–15% of peripheral blood mononuclear cells in healthy individuals (Dunai et al., 2022). Dabrowski et al. (2008) and Wei et al. (2019) reported a drastic decrease in peripheral NK cells in patients with SAP compared to MAP, suggesting a correlation between AP severity and NK cells. It has also been observed that low levels of NK cells at admission are associated with developing secondary infections in AP (Wang et al., 2017). Natural Killer T lymphocytes are a subtype of T cells characterised by properties of both T and NK cells, such as CD16 and CD56 expression and granzyme production (Ding et al., 2020). Natural Killer and NKT cells migrate into the pancreas after AP onset and indirectly promote inflammation (Ding et al., 2020).

1.2.5.3 Role of platelets in acute pancreatitis (AP)

Platelets are non-nucleated, disc-shaped cells produced from megakaryocytes (MKs) (Ribeiro, Migliari Branco, and Franklin., 2019). Platelets are vital in promoting neutrophil accumulation in AP (Wetterholm et al., 2016). Under normal circumstances, platelets in the circulatory system do not adhere to either blood vessel walls or leukocytes because of the anti-thrombotic

properties of the vascular endothelium (Carestia et al., 2016). However, in cases of vascular injury or after activation of the endothelium due to inflammatory conditions, platelets adhere to subendothelial molecules, such as collagen, triggering platelet activation (Carestia et al., 2016). Molecules that mediate immune responses by monocytes, macrophages, and neutrophils are released on platelet activation (Ribeiro, Migliari Branco, and Franklin., 2019). A group called chemokines are among the molecules released by activated platelets. Chemokines are a large group of small peptides that activate G-protein coupled receptors and play a role in leukocyte recruitment to inflammatory sites and lymphoid areas (Lämmermann and Kastenmüller., 2019). Chemokines are classified based on their conserved amino-terminal cysteine residues, with the CXC class having two cysteine residues with any amino acid between them (Lämmermann and Kastenmüller., 2019).

Secreted chemokines often facilitate the recruitment of leukocytes to inflammation areas. CXC chemokines such as CXCL1 and CXCL2 stimulate extravascular neutrophil recruitment (Wetterholm et al., 2016). The CXCR2 and CXCL interaction is crucial in inflammatory diseases and cancer (Cheng et al., 2019). In addition, CXCR2 is vital in supporting the infiltration of neutrophils in the pancreas (Bhatia and Hegde, 2007). CXCL4 is a member of the CXC family and it is abundant in platelet α-granules and is released on activation of platelet aggregation (Guo et al., 2017). CXCL4 facilitates chemotaxis for neutrophils and monocytes via binding to the C-X-C motif of the chemokine receptor 3 (CXCR3) (Yue et al., 2018). CXCL4 is also involved in the platelet-dependent accumulation of neutrophils by the generation of CXCL2 in AP (Wetterholm et al., 2016). However, CXCL4 lacks the Glu-Leu-Arg (ELR) sequence required for binding chemotactic CXCRs to neutrophils (Wetterholm et al., 2016). Studying the role of CXCL4 has been challenging due to the absence of well-defined CXCL4 receptors (Wetterholm et al., 2016).

1.2.6 The role of cytokines and reactive oxygen species in acute pancreatitis (AP)

According to Gomez-Cambronero et al. (2002), pancreatic injury induces at least two pathways, the cytokine storm and oxidative stress (OS). Although an association between pancreatic injury and the uncontrolled systemic response in AP has not been fully established, clinical and experimental evidence reports that pro-inflammatory cytokines and oxidative stress play a role in the development of local and systemic complications associated with severe forms of AP (Pădureanu et al., 2022; Gomez-Cambronero et al., 2002). Generally, oxygen

radicals are known to promote inflammation in various inflammatory diseases (Pădureanu et al., 2022). OS is defined as an imbalance between antioxidant defence systems and the production of reactive oxygen species (ROS) (Pădureanu et al., 2022). ROS are defined as highly reactive molecules with a single unpaired electron in their outermost electron shell (Sies, 2017). There are two ROS groups, free oxygen radicals, such as superoxide and non-radicals, such as hydrogen peroxide (H_2O_2) (Sies, 2017). Preliminary studies have investigated the role of OS in AP, and it has been shown that ROS are not direct triggers of AP development. However, they play a role in AP pathogenesis by mediating pancreatic tissue damage (Swentek, Chung and Ichii, 2021; Johnson., 2007).

ROS are produced in pancreatic acinar cells, where they usually affect the patterns of cell death (Criddle, 2016). However, due to an inflammatory response, neutrophils in circulation can also produce ROS, which indirectly influences the development of pancreatic injury and, subsequently, AP complications (Criddle, 2016). Furthermore, the role of ROS in AP has been shown by the depletion of pancreatic glutathione and increased lipid peroxidation (Pădureanu et al., 2022). Moreover, circulating xanthine oxidase produced by a damaged pancreas can act as a source of systemic oxidative stress contributing to organ dysfunction (Zhou et al., 2016). In summary, ROS regulate the inflammatory cascade activation, recruiting inflammatory cells and causing tissue damage (Forrester et al., 2018; Yu and Kim, 2014). A distinctive feature of the AP inflammatory response is the induction of cytokine expression, which is regulated by several signalling molecules such as ROS. Figure 1.2 is a flow diagram showing how OS induces inflammation in AP

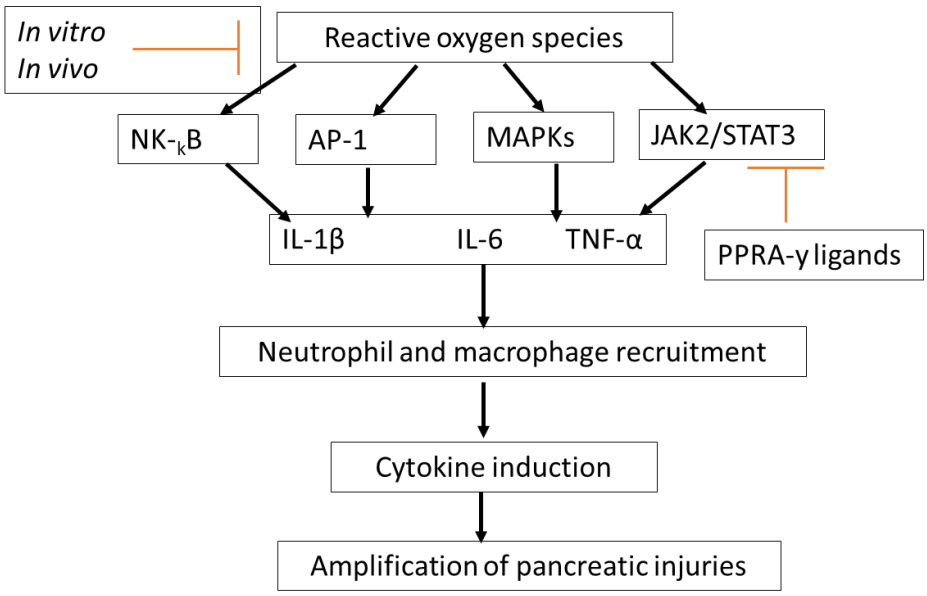

Figure 1.2: Flow diagram of how oxidative stress induces inflammation in acute pancreatitis. Reactive oxygen species activate redox-sensitive transcription factors NF-κB and AP-1 as well as inflammatory mediators MAPKs. MAPKs and JAK2/signal transducer and activator of transcription 3 (STAT3), which subsequently induces the expression of cytokines such as IL-6, IL-8, IL-1β, and TNF-α in the pancreas. Induced cytokines then recruit macrophages and neutrophils in the injured pancreatic tissues. Cytokine and chemokine expression becomes upregulated, leading to the amplification of pancreatic injuries (adapted from Yu and Kim, 2014). **Notes:** NF-κB: nuclear factor-κB; AP-1: activator protein-1; MAPKs: mitogen-activated protein kinases; JAK2: Janus kinase 2; STAT3: signal transducer and activator of transcription 3; IL: interleukin; TNF- α: tumour necrosis factor-alpha.

1.3 Rationale

The conventional theory regarding the pathophysiology of AP involves the premature release and activation of intracellular acinar proenzymes by trypsin (Leal and Almeida, 2019). This activity then induces acinar cell injury, leading to an intra-pancreatic inflammatory process (Watanabe, Kudo and Strober, 2017). However, knowledge regarding the immunopathogenesis of this disease is still scanty due to the underlying complexities associated with its pathophysiology. The absence of a clear understanding of this disease course and associated complications introduces difficulties in stratifying AP severity early, prognosticating the disease and improving management strategies.

Preliminary studies have highlighted the importance of early AP severity stratification, emphasising how it will decrease the financial burden, morbidity and mortality of AP patients in healthcare facilities (Leal and Almeida, 2019). Nonetheless, AP Stratification is not a once-off task; it requires reassessment. It is advisable that it is done immediately on admission, after 24 and 48 hours, and seven days post-admission (Leal and Almeida, 2019). For better

assessment and stratification, AP predictive markers, such as IL-6, CRP, and nuclear factor kappa B (NF-κB), have been flagged as potential AP prognostic markers (Jakkampudi et al., 2016; Meher et al., 2015; Silva-Vaz et al., 2020). However, these markers provide acceptable prognostic accuracy when measured 48 or 72 hours following AP onset (Do, 2015; Meher et al., 2015; Stirling et al., 2017). Other studies have also stated that mediators of pancreatic inflammation, such as CXCL4 and ROS, can also serve as AP prognostic markers (Yu and Kim, 2014; Noel et al., 2016). It is noteworthy that existing diagnostic and prognostic methods for AP still rely on clinical assessments without considering the most definite markers of immune dysregulation.

Although there are various molecular markers, composite scores and research interventions, a reliable method to predict and monitor the severity of AP still does not exist in general clinical practice. It is from this background that there is a need to investigate immune markers, such as IL-6, alongside associated cells, to further improve on its prognostic value in monitoring AP severity and predicting possible complications early and during the course of the disease or duration of hospital stay. This is important as it concerns MSAP and SAP patients, in particular, whose management is influenced by early stratification challenges. IL-6 has been extensively studied in the early and late AP phases of AP independently. However, studies that monitor the expression of IL-6 over the early to late severe AP phases in the same cohort of patients are relatively scanty.

Previous work done by our research group in a cohort of South African AP patients reported a high expression of IL-6 compared in SAP compared to MSAP and MAP patient groups (Kay, Smith, and Brand, 2017; Nalisa et al., 2021; Thomson, Brand, and Fonteh, 2018). Furthermore, a higher expression of monocytes than NK cells was observed in SAP compared to MSAP patients in the study by Nalisa et al. (2021). Therefore, this study aimed to explore a link between intracellular and circulating IL-6 expression with subsets of lymphocytes and myeloid cells, such as NK cells and monocytes, that are known to produce IL-6. Furthermore, these findings were correlated with different AP severity groups over the two AP phases (SIRS and CARS). Additionally, the levels of other inflammatory mediators in AP, such as platelets, CXCL4 and ROS, were also assessed as potential markers for monitoring disease severity to inform management. Furthermore, the correlation was evaluated for all the experimental results to explore links among these immune markers as potential targets for AP management.

1.4 Hypothesis

Inflammatory mediators such as IL-6, CXCL4, associated white blood cells (lymphocytes, monocytes and platelets), and ROS predict severity and adverse outcomes in patients with AP.

1.5 Overall Aim

To determine the intracellular abundance of IL-6 and CXCL4 in white blood cells and platelets as well as the effect of ROS in MSAP and SAP patients for early stratification and potentially improved care.

1.6 Specific Objectives

1.6.1 To explore a link between intracellular IL-6 expression in lymphocytes and myeloid cells and circulating IL-6 levels in the two SAP phases.

1.6.2 To determine the intracellular abundance of CXCL4 protein in platelets and circulating CXCL4 levels in the plasma of AP patients with MSAP and SAP.

1.6.3 To determine the ROS expression in AP patients.

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

CHAPTER TWO: MATERIALS AND METHODS

2.1 Ethical Considerations

Before sampling, all recruited participants were duly informed about the study and written informed consent was requested from them. Ethical clearance for this study was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical) (Clearance Certificate M200757). Appendix A, B, C, D and E are patient and control group information and consent forms.

2.2 Methodology

2.2.1 Study sites

This was a prospective study where AP patient sampling was done at a tertiary care hospital setting, and sampling for the control group was done from participants at the University of the Witwatersrand (WITS). With the aid of qualified clinicians, patients diagnosed with AP using the 2012 RAC system were recruited from the Hepatopancreatobiliary (HPB) unit of the Chris Hani Baragwanath Academic Hospital (CHBAH), Johannesburg, South Africa, from December 2020 to January 2022. Diagnosed patients were further classified into three severity groups, MAP, MSAP and SAP, using the RAC guidelines.

2.2.2 Inclusion and exclusion criteria of study participants

The inclusion criteria for the study for AP patients were:

(i) patients of African descent,
(ii) patients 18 years and above,
(iii) patients with no other inflammatory conditions, and
(iv) patients admitted and diagnosed with AP according to the RAC guidelines within 72 hours of disease onset.

Patients who presented to the hospital post 72 hours of disease onset were also recruited, and samples were collected for the other days of the sampling criteria, taking note of the sample collection days that had been missed due to the patients not having presented to the hospital. Patients were excluded if they had undergone therapy with immunomodulatory medications within one month of current admission regarding the day of the current onset of AP or if they had any OF before their current episode of pancreatitis, and if they were diagnosed with other

chronic medical conditions associated with inflammation (including auto-immune diseases). This was an immune response aimed study; hence the day of disease onset was more vital than the day of admission in the inclusion criteria. The inclusion criteria for the control group were individuals of African descent, 18 years and older, with no known history of pancreatic diseases or chronic inflammatory conditions.

2.2.3 Sampling strategy

Based on convenience sampling, 24 AP patients were recruited. The group consisted of 17 MAP, 4 MSAP and 3 SAP from the HPB unit at CHBAH. Additionally, eight healthy volunteers, age and sex-matched to recruited AP patients, were recruited from the WITS Faculty of Health Sciences to serve as the control group. This was a pilot study, so no sample size calculations were made. Two types of blood collection tubes were used, one 10 mL purple top BD vacutainer® ethylenediaminetetraacetic acid (EDTA) tube (BD Biosciences, New Jersey, USA) and one 10 mL red top BD vacutainer® clot activator tube (BD Biosciences, New Jersey, USA). The EDTA tube was used to collect whole blood from which plasma was isolated, and the clot activator tube was used to collect blood from which serum was isolated. Approximately 16 mL of blood was collected on Days (D) 3, 5, 7, 9, 13 and 15 post-onset of epigastric pain. Of the 16mL, 10mL was collected into the EDTA tube and 6mL into the clot activator tube. On average, for the current study cohort, patients often presented at CHBAH approximately post 72 hours of epigastric pain (D3 post onset of symptoms). Demographic data was captured after obtaining informed consent, and clinical data, such as stratification of severity, CRP levels, platelet count, WBC levels, and patients' outcomes, including ICU admission, were obtained from the patient hospital files as captured by the consulting clinician.

2.2.4 Overall blood sampling, processing, and research methodologies

Whole blood, plasma, and serum were processed from the 24 AP patients and stored in the Department of Surgery laboratory at WITS within six hours of phlebotomy. All blood samples were stored vertically at room temperature until they were processed to prevent haemolysis and to enable gravity separation. Assays were only performed on patients with more than three sampling points to allow longitudinal analysis. All the MSAP (n=4) and SAP (n=3) patients were included in all the assays. However, only 5 MAP patients were included in the IL-6 ICS, and from these, only 4 MAP patients were included for the CXCL4 ICS, ELISA and ROS assays. The other MAP samples (n=12) were not included in any assays for accuracy in

reporting. Due to the MAP group having the most patients, results would have been biased towards the MAP group. Furthermore, the current study objectives were focused on patients who progress to a second AP phase, and those are the severe group of patients. Hence, all severe patients were included in all assays, and a selection was made in the MAP group. Whole blood was analysed by ICS for both IL-6 and CXCL4, and plasma was used for analysing circulating for IL-6 and CXCL4 using two different ELISA Kits. ROS studies were done on both serum and plasma samples (Figure 2.1).

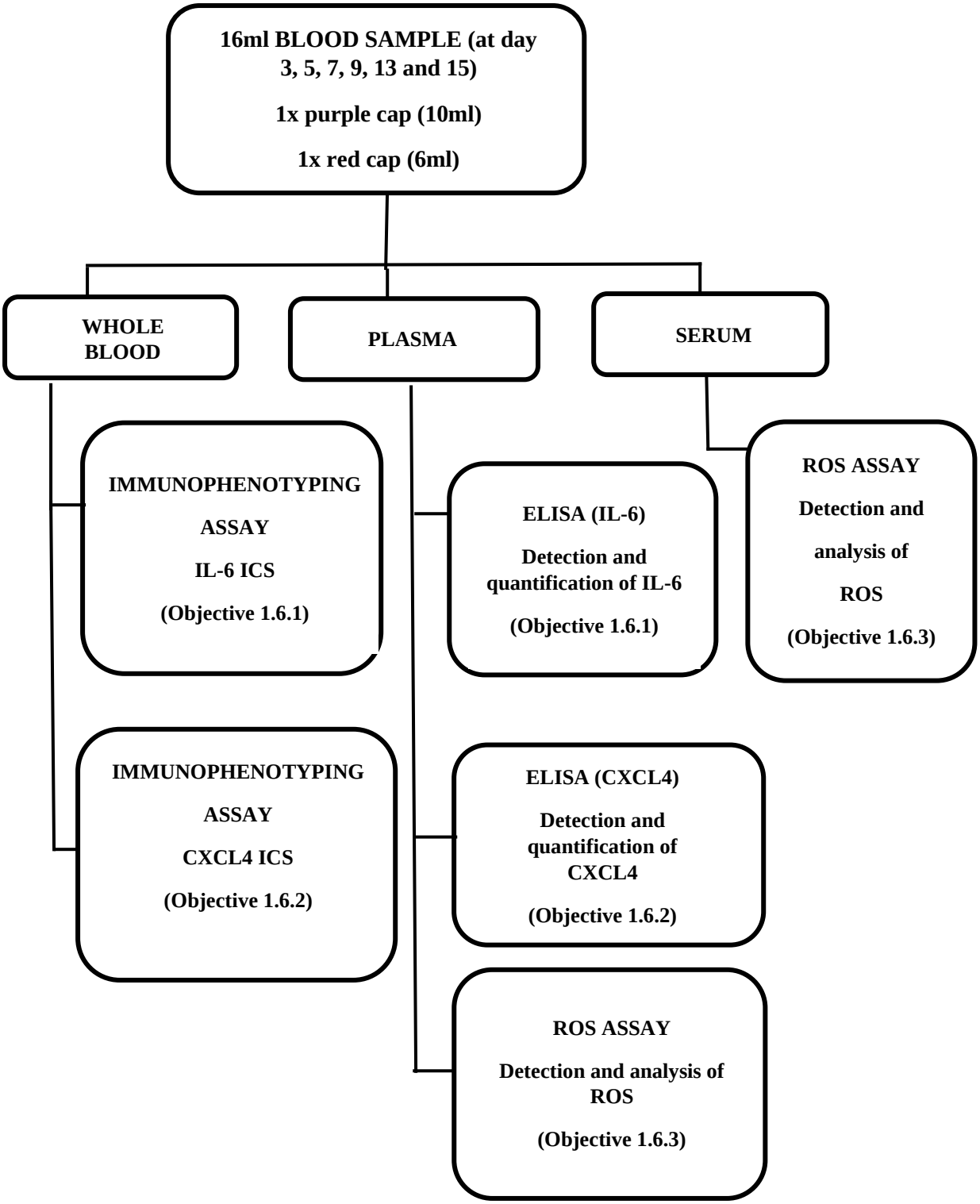

Figure 2.1: Flow diagram showing the summary of methods used to answer study objectives. **Notes:** IL-6: Interleukin-6; CXCL4: Chemokine ligand 4; ELISA: Enzyme-linked immunosorbent assay; ROS: Reactive oxidative species.

2.2.4.1 Whole blood processing and storage

One hundred microlitres of whole blood from the EDTA tube was transferred into a sterile 2 mL corning cryogenic vial (Corning Incorporated, New York, USA), making a total of six aliquots for each sample at each sampling point. This was followed by adding 1000 µl of 1X BD FACS Lysing Solution (BD Biosciences, New Jersey, USA) to the vial to lyse red blood cells and fix the white blood cells. The mixture was then incubated for 15 minutes at room temperature to fix the cells. The cryogenic vials containing the fixed cells were stored at -80º C until they were required for immunophenotyping.

2.2.4.2 Plasma isolation and storage

Plasma was isolated from the remaining whole blood in the EDTA tube in 2.2.2.1. The blood in the EDTA tube was allowed to stand at room temperature for 30 minutes for the red blood cells and supernatant to separate by gravity separation. The supernatant (the plasma portion) was transferred into a clean 15 mL Falcon tube (Thermo Fisher Scientific, Massachusetts, USA). The plasma was centrifuged at room temperature for 30 minutes at 300 x *g*, and 200 µl of the plasma was transferred into single-use sterile 0.6 mL microcentrifuge tubes (Thermo Fisher Scientific, Massachusetts, USA) using a single channel pipette to a total of 10 replicates. The aliquots of plasma were stored at -80º C until needed for ROS and ELISA assays.

2.2.4.3 Serum isolation and storage

The BD clot activator tube (BD Biosciences, New Jersey, USA) with 6 mL of blood was incubated upright for 30 minutes at room temperature to allow the blood to clot. Subsequently, the tube was centrifuged for 10 minutes at 300 x *g*. After centrifugation, 200 µl of the supernatant or serum component was carefully aspirated into sterile 0.6mL microcentrifuge tubes using a single channel pipette to 10 replicates. Aliquots were stored at –80° C until they were required for the ROS assay.

2.2.5 Immunophenotyping and intracellular cytokine staining

Flow cytometry is a laser-based technique that enables the detection and quantification of physical and chemical characteristics of single cells or particles in a heterogenous fluid mixture (Mckinnon, 2018). Immunophenotyping using flow cytometry is a laboratory technique that evaluates individual cells in suspension based on the presence of specific markers known as

antigens (Bhola et al., 2020; de Tute, 2011). These antigens are defined as protein structures found on the surface or within cells. Specific groupings of these markers are unique to particular cell types or lineages and also in determining stages of cell maturation (Craig and Foon, 2008; McKinnon, 2018). Most detectable antigens by immunophenotyping are identified by clusters of differentiation or cluster designation (CD) number (Mckinnon, 2018). Immunophenotyping uses the reaction of fluorescently labelled antibodies with cell antigens to detect and determine specific cell types in beads, samples of blood cells, lymph nodes or marrow cells (Bhola et al., 2020; de Tute, 2011).

The ICS is a combination of immunophenotyping and cytokine analysis concerning cytokines found in specific cell types, such as lymphocytes and monocytes (Mandala et al., 2021; Nomura, Maino and Maecker, 2008). Intracellular staining is used to label markers not expressed on the cell surface (Gong et al., 2022; Nomura, Maino and Maecker, 2008). This includes internalised molecules and cytokines in cells usually not expressed on the cell surface (Lovelace and Maecker, 2018). ICS requires that the cell membrane is permeabilised before staining so that the fluorescently labelled antibody can access the marker of interest and ensure increased sensitivity and specificity in quantification (Lovelace and Maecker, 2018). In this study, the BD Perm/Wash™ Buffer (BD Biosciences, New Jersey, USA) was used as a permeabilising agent. Before sample acquisition, the ICS protocol was standardised and optimised to produce accurate and reproducible outcomes. Standardisation was achieved by performing the following steps

1) Cytometer set up and tracking (CS&T) for instrument set up in terms of checking if detectors are well positioned and monitoring the optimal performance of the flow cytometer,

2) Antibody titrations to determine ideal staining concentration,

3) Photomultiplier tube (PMT) voltage or application settings to exclude background and ensure linearity,

4) Compensation set up to subtract spillover of signals channels different from the primary,

5) Setting up eight peak beads for consistency when collecting samples longitudinally and

6) Fluorescence minus one (FMO) control, a gating control.

2.2.5.1 Multicolour panels on intracellular determination of IL-6 and CXCL4 in the different AP severities

The ICS panels for IL-6 and CXCL4 were established based on the brightness of fluorochromes, antigen density, co-expression and the probability of fluorochrome spillover of targeted immune cells and their subsets. A nine-colour and four-colour panel were designed to characterise the heterogeneous cell populations present and responsible for secreting IL-6 and CXCL4, respectively, in the three AP severity groups, MAP, MSAP and SAP. The final multicolour panel contained nine antigen markers specific for monitoring the subsets and status of lymphocytes, monocytes, granulocytes and the presence of intracellular IL-6. In the case of CXCL4, the final multicolour panel contained four antigen markers specific for identifying platelets and monitoring their activation status concerning CXCL4 secretion.

2.2.5.2 Antibody titration

2.2.5.2.1 IL-6 antibody titration

The nine fluorochrome-conjugated anti-human antibodies used for designing the IL-6 ICS panel (Table 2.1) were obtained from BD Biosciences (BD Biosciences, New Jersey, USA), except CD66b, CD19, CD16 and CD56, which were obtained from BioLegend (BioLegend, San Diego, California). Before immunophenotyping, all the antibodies were optimised by titration to stain the various cell populations optimally without excess background to poor resolution. Lymphocyte populations were identified using CD3 BD Horizon Brilliant™ Ultraviolet 496 (BUV496); CD56 Phycoerythrin Cyanine 7 (PE-Cy7); CD16 Allophycocyanin (APC); CD19 Alexa Fluor 700 (AF700) and CD57 Fluorescein isothiocyanate (FITC). Monocyte populations were stained using CD16 APC, CD14 Phycoerythrin (PE), and human leukocyte D-related BD Horizon Brilliant™ Violet 650 (HLA-DR BV650). Granulocyte populations were stained using CD16 APC and CD66b Peridinin-Chlorophyll-protein cyanine 5.5 (PerCPCy5.5). Lymphocyte and monocyte populations were further intracellularly stained with IL-6 Brilliant™ Violet 421 (BV421).

The nine antibodies were individually titrated using anticoagulated whole blood obtained from HC samples at seven 2-fold serial dilutions of the antibodies (5, 2.5, 1.25, 0.625, 0.3, 0.15 and 0.1 µl). Cryovials containing the fixed and frozen white blood cells from 2.2.2.1 were retrieved from the - 80° C freezer and thawed in a water bath at 37° C for 2 minutes. Once the cells had

thawed, approximately 820 µl were transferred into eight 5 mL Corning Falcon tubes (Corning Incorporated, New York, USA). One was labelled as unstained, and seven labelled with different antibody concentrations based on serial dilution. The Falcon tubes containing cells were then placed in a centrifuge, washed at 600 x *g* for 5 minutes, and the supernatant was discarded. The cells were resuspended in 1 mL of 1X phosphate buffered saline (PBS) (Sigma-Merck, Missouri, USA) and washed once at 600 x *g* for 5 minutes. The supernatant was discarded, and cells were permeabilised by adding 500 µl of 1X BD Perm/Wash™ Buffer (BD Biosciences, New Jersey, USA). The permeabilised cells were incubated at room temperature for 10-15 minutes, followed by a wash step at 600 x g for 5 minutes. The pellet was resuspended in a mixture of 50 µl of stain buffer (1X PBS, 5% FBS, and 0.1% NaN_3) and 1 µl of the FC Blocker (BD Biosciences, New Jersey, USA) to block non-specific binding of antibodies to the Fc receptor-expressing cells, such as myeloid and B cells. The FC blocker also reduces background noise, improving the accuracy of results. Different antibody concentrations were added to the Falcon tubes, except the unstained tube. Eventually, each Falcon tube had the same volume of cells but different antibody concentrations. The contents in each Falcon tube were briefly vortexed and incubated at room temperature for 30 minutes in the dark. The stained cells were resuspended by adding 1000 µl of 1X BD Perm/Wash™ buffer (BD Biosciences, New Jersey, USA) and washed at 600 x *g* for 10 minutes. Afterwards, stained white blood cells were resuspended in 300 µl staining buffer before flow cytometric analysis. A total of 100 000 events were acquired on the BD LSR II Fortessa^{TM} (BD Biosciences, New Jersey, USA). The data were analysed in terms of the stain index (SI), which refers to the difference between the Median Fluorescence Intensity (MFI) of positive and negative populations divided by two times the standard deviation of the negative population. From these calculations, a plot of antibody volume versus SI was used to select the optimal volume to be used, which was the volume corresponding to the highest SI value. Optimised titred volumes for each antibody are presented in Table 2.1.

Table 2.1: Optimised multicolour IL-6 ICS panel used to analyse and characterise leukocytes in AP patients.

Specificity	Fluorochrome	Clone	Targeted cell type	Optimised titre volume (µl)
CD3	BUV496	UCHT1	Lymphocytes	1.0
HLA-DR	BV605	G46-6	Monocytes and CD8 cells	1.5
CD56	PE-Cy7	HIB19	NK cells, $CD4^+$ T cells and $CD8^+$ T cells	1.25
CD66b	PerCP-Cy5.5	G10F5	Granulocytes	1.5
IL-6	BV421	MQ2-13A5	Lymphocytes and Monocytes	2.5
CD14	PE	MφP9	Monocytes	2
CD19	AF 700	HIB19	B Lymphocytes	1.25
CD57	FITC	NK-1	Mature NK cells	1.5
CD16	APC	3G8	Granulocytes, monocytes, and NK cells	2.5

Notes: BUV: BD Horizon Brilliant™ Ultraviolet; BV: Brilliant Violet™; PE-CY7: Phycoerythrin Cyanine 7; PerCP-Cy: Peridinin-Chlorophyll-protein cyanine; PE: Phycoerythrin Cyanide dye™; AF: Alexa Flour; FITC: Fluorescein isothiocyanate; HLA DR: human leukocyte D related.

2.2.5.2.2. CXCL4 antibody titration

The four fluorochrome-conjugated anti-human antibodies used for designing the CXCL4 ICS panel are shown in Table 2.2. All the antibodies were obtained from BD Biosciences (BD Biosciences, New Jersey, USA), except CXCL4, which was from R&D Systems (R&D Systems, Minnesota, USA). Before immunophenotyping, all the antibodies were optimised by titration to stain the platelet population optimally using CD41a BUV496 and to determine the activation status of platelets using CD62p BV421 and PAC-1 FITC. CXCL4 AF647 was used for intracellular staining of the platelets. The antibody titration protocol used was similar to the one stated in 2.2.5.2.1.

Table 2.2: Optimised multicolour CXCL4 ICS panel used to analyse and characterise platelets in AP patients.

Specificity	Fluorochrome	Clone	Targeted cell type	Optimised titre volume (µl)
CD41a	BUV496	HIP8	Platelets	1.25
CD62p	BV421	AK-4	Activation marker	2.5
PAC-1	FITC	PAC-1	Activation marker	2.5
CXCL4	AF647	170138	Platelets	2.5

Notes: BUV: BD Horizon Brilliant™ Ultraviolet; BV: Brilliant Violet™; AF: Alexa Flour; FITC: Fluorescein isothiocyanate.

2.2.5.3 Cytometer set up and tracking

Instrument set up and tracking was done using one drop of BD Cytometer Setup and Tracking beads (CS&T) beads (BD Biosciences, New Jersey, USA) diluted in 350 µl of BD FACSFlow™ (BD Biosciences, New Jersey, USA) and following the manufacturer's protocol (BD™ Cytometer Setup and Tracking Beads, 2007).

2.2.5.4 Photomultiplier tube (PMT) voltages optimization

The principle behind optimising PMT voltages is to ensure optimal instrument performance throughout an experiment, especially experiments performed more than once. Additionally, this setup eliminates electronic noise from cell debris. Voltages of all antibody fluorochromes were optimised for the IL-6 and CXCL4 assays to ensure consistent results. Voltage optimisation established negative and positive populations of target parameters and their linearity. The following voltages were set and used to acquire data for IL-6 ICS

- Forward side scatter, which is the measurement of cell size (FSC) 380v,
- Side scatter, the measurement of complexity/granularity of cells (SSC) 345v,
- BUV496 400v,
- PE 520v,
- APC 650v,
- AF700 550v,
- BV650 530v,
- PE-Cy7 656v,
- FITC 525v,

- PerCP-Cy5-5 560v,
- BV421 390v.

Approximately 100 000 events were recorded per sample at a threshold setting of 20 000. Furthermore, the following voltages were set and used to acquire data for CXCL4 ICS

- FSC 465v,
- SSC 287v,
- BUV496 360v,
- BV421 370v,
- FITC 560v,
- AF647 750v.

Approximately 100 000 events were recorded per sample at a threshold setting of 500. Application settings for the IL-6 and CXCL4 ICS panels were created and saved to maintain consistency of the generated results using the BD FACSDiva version 6 software (BD Biosciences, New Jersey, USA).

2.2.5.5 Compensation controls and calculations

Titred antibody volumes were used for compensation controls. In multicolour flow cytometry, compensation is a process that corrects fluorescence spillover between or among different fluorochromes. Applying compensation enables a correction in spillover to avoid false positives or negatives. Compensation settings were done using single stained Anti-Mouse Ig, κ/Negative Control Compensation Particles Set[3] (BD Biosciences, New Jersey, USA). One drop of negative and positive beads (Anti-Mouse Ig, κ/Negative Control Compensation Particles Set[3]; (BD Biosciences, New Jersey, USA) were added into 5 mL Corning Falcon tubes (Corning Incorporated, New York, USA) containing 50 µl of 1X PBS (Sigma-Merck, Missouri, USA). The optimised pre-titrated volumes of antibodies were added to each Falcon tube in the dark and incubated for 30 minutes. The stained beads were washed with 2 mL 1X PBS at 600 x g for 5 minutes. The supernatant was decanted, and the pellet resuspended in 300 µl staining buffer. A total of 10 000 events were acquired and recorded using the compensation menu in the FACSDiva version 6 software (BD Biosciences, New Jersey, USA) on the BD LSR II Fortessa™ (BD Biosciences, New Jersey, USA) flow cytometer. Spillover was automatically calculated, subtracted, and applied to experimental samples.

2.2.5.6. 8 peak beads

Samples were analysed once but not in a single experiment, so Rainbow Calibration Particles (8 peaks), 3.0 - 3.4 µm (BD Biosciences, New Jersey, United States), were used as a control to determine the linearity in fluorescence detection channels for each experiment. The assay was performed by adding one drop of Rainbow Calibration Particles (8 peaks) (Lot number: 9121914) into a 5 mL Corning Falcon tube (Corning Incorporated, New York, USA) containing 500µl 1X PBS (Sigma-Merck, Missouri, USA). The Falcon tube was briefly vortexed once for five seconds and acquired on low using the FACSDiva version 6 software (BD Biosciences, New Jersey, USA) in the BD LSR II Fortessa ™ (BD Biosciences, New Jersey, USA) flow cytometer. The peak that appeared clearly from the eighth one for all antibodies was chosen, and the MFI range was calculated. The 8 peak beads serve both as an experimental and instrument control.

2.2.5.7 Fluorescence minus one controls

The FMO controls help confirm the sensitivity of channels individually and ensure the specificity of each marker at the relevant channel (Cossarizza et al., 2019). The panel of an experiment guides the FMO controls. When FMO controls are being set, each Corning Falcon tube (Corning Incorporated, New York, USA) of the controls includes all the antibody conjugates in an experiment except the one being controlled for. These controls provide measurements of the fluorescence spread from the other staining parameters into the channel of interest; however, they do not provide a measure for nonspecific binding (Cossarizza et al., 2019). FMOs also serve as a gating control (Lee et al., 2019).

2.2.6 Measurement of circulating IL-6 expression in acute pancreatitis

IL-6 was measured from plasma samples from 5 HC and 11 AP; 3 SAP, 4 MSAP and 4 MAP patients considering all three severities within the two SAP phases. IL-6 expression was detected and quantified using the commercially available RAB0306 Human IL-6 ELISA kit (Sigma Aldrich, St Louis, Missouri, USA). All the reagents from the ELISA kit and plasma samples cryopreserved in Section 2.2.2.2 were brought to room temperature 15 minutes before the assay began. After the plasma had thawed, a two-fold dilution was done using the assay/sample diluent buffer A (Sigma Aldrich, St Louis, Missouri, USA). The standard samples, wash buffer, assay diluent buffers, horseradish peroxidase (HRP)-Streptavidin and

biotinylated human IL-6 detection antibody were prepared as working solutions according to the manufacturer's protocol (Sigma Aldrich, St Louis, Missouri, USA). Eight standards, including the blank control, were prepared using 1:3 serial dilutions. The first standard had 40 µl of the IL-6 standard (Sigma Aldrich, St Louis, Missouri, USA) and 440 µl of assay/sample diluent buffer A (Sigma Aldrich, St Louis, Missouri, USA). Subsequently, 200 µl was transferred from each 2 mL microcentrifuge tubes (Thermo Fisher Scientific, Massachusetts, USA) tube into 2 mL microcentrifuge tubes with 400 µl assay diluent buffer A, excluding the blank control. After preparation of standards and plasma, 100 µl of the standards and plasma were added in duplicate to a 96-well IL-6 antibody-coated plate (Sigma Aldrich, St Louis, Missouri, USA) and incubated overnight at 4° C with gentle shaking. The next day the solution in the plate was discarded, and the plate was washed with the wash buffer (Sigma Aldrich, St Louis, Missouri, USA) four times. After washing, 100 µl of 1 X prepared detection antibody (Sigma Aldrich, St Louis, Missouri, USA) was added to each well. The plate was incubated for one hour at room temperature with gentle shaking, rewashed, and 100 µl of the prepared HRP-Streptavidin solution (Sigma Aldrich, St Louis, Missouri, USA) added to each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. The incubation period was followed by another wash; 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) one-step substrate reagent (Sigma Aldrich, St Louis, Missouri, USA) was added to each well, followed by an incubation period of 30 minutes at room temperature in the dark with gentle shaking. Lastly, 50 µl of stop solution (Sigma Aldrich, St Louis, Missouri, USA) was added to each well. The absorbance was measured at 450 nm using the Multiskan SKY Remote Smart Start plate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). A standard curve was generated using GraphPad Prism™ version 5 (California, USA) and Microsoft Excel version 7 (Washington, USA) based on the absorbance values obtained and associated known concentrations of the standards. The concentration of IL-6 in plasma was then determined based on the standard curve.

2.2.7 Measurement of circulating CXCL4 expression in acute pancreatitis

CXCL4 was also measured from plasma samples from 5 HC and 11 AP, 3 SAP, 4 MSAP and 4 MAP patients considering all three severities within the two SAP phases. The expression levels of CXCL4 were detected and quantified using the commercially available DPF40 Human CXCL4/PF4 Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota, USA). All the reagents from the ELISA kit and plasma samples cryopreserved in Section 2.2.2.2 were

brought to room temperature 15 minutes before the assay. Thawed plasma was diluted 10-fold using the calibrator diluent RD6-13 (R&D Systems, Minneapolis, Minnesota, USA). The wash buffer, standard samples and substrate solution were prepared into working solutions according to the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota, USA). Eight standards, including the blank control, were prepared using 1:2 serial dilutions. The first standard contained 50 µl of the CXCL4 standard (R&D Systems, Minneapolis, Minnesota, USA) and 450 µl of calibrator diluent RD6-13. Subsequently, 200 µl was transferred from each tube into tubes with 200 µl calibrator diluent RD6-13, excluding the blank control, which only had 200 µl calibrator diluent RD6-13. Each well had 100 µl assay diluent RD1-15 (R&D Systems, Minneapolis, Minnesota, USA) added, then 50 µl of the prepared standards and plasma were added in duplicate to a 96-well plate (R&D Systems, Minneapolis, Minnesota, USA) followed by two hours incubation at room temperature with gentle shaking. After two hours, the solution in the plate was discarded, and the plate was washed four times with wash buffer (R&D Systems, Minneapolis, Minnesota, USA). After washing, 200 µl of the Human PF4 Conjugate (R&D Systems, Minneapolis, Minnesota, USA) was added to each well, and the plate was incubated for two hours at room temperature with gentle shaking. The wells were then washed four times, and 200 µl of Substrate Solution (R&D Systems, Minneapolis, Minnesota, USA) was added to each well, followed by incubation for 30 minutes at room temperature in the dark. Lastly, 50 µl of stop solution (R&D Systems, Minneapolis, Minnesota, USA) was added to each well, and the absorbance was measured at 450 and 570 nm for correction of optical imperfections, using the Multiskan SKY Remote Smart Start plate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). A standard curve was generated in Microsoft Excel version 7 (Washington, USA) using the standard absorbance values obtained and associated known concentrations. The concentration of CXCL4 in the samples was then determined using the linear standard curve equation.

2.2.8 Analysis of reactive oxygen species in acute pancreatitis

The ROS study was done using serum and plasma. However, spectrophotometric assays for ROS using serum are more stable than plasma. The methodology used for both samples was the same, except the dilution ratio in serum was 1:20 and 1:10 in plasma. Results for both assays are described and reported in Chapter 3.

Serum samples from 5 HC and 11 AP patients, 4 MAP, 4 MSAP and 3 SAP sampled over D3-D15, were diluted 1:10 in 0.1M Sodium acetate buffer (pH 4.8) and 140 µl of 0.1M Sodium

acetate buffer was added into each well of the 96-well plate. Subsequently, 5 µl of diluted serum samples from the HC, AP patients and diluted standard samples were added in duplicate to the 96-well plate. N, N-Diethyl-para-phenylenediamine (DEPPD), which is an oxidised stable radical cation that can be measured spectrophotometrically, was prepared immediately before use by dissolving in 0.1M Sodium acetate buffer (pH 4.8, 100 µg/ml). After that, 5.56 g of iron sulphate was dissolved in 960 mL 0.1M Sodium acetate buffer and mixed with the prepared DEPPD at a ratio of 1:25. The DEPPD and iron sulphate mixture was then added to each well (100µl), and a colour reaction depicting the number of hydroperoxyl compounds and hydrogen peroxide equivalents was observed. The absorbance of the wells was recorded at 505 nm at 25°C every 15 seconds for 30 repeats using an FL 600 Microplate reader (BioTek, Winooski, Vermont, USA). A standard curve was generated in Microsoft Excel version 7 (Washington, USA) based on obtained absorbance values and associated known concentrations. The concentration of ROS in the samples was then determined using the linear standard curve equation, and the acquired sample ROS concentration data were further analysed using GraphPad Prism™ version 5 (California, USA).

2.2.9 Statistical data analysis

Statistical analysis for the ICS data for both IL-6-associated immune cells and CXCL4 association with platelets was done using Microsoft Excel (version 7) software and the Statistical Package for the Social Sciences (SPSS) (version 27). Spearman's rank test was used to calculate the correlation coefficient (rho) between variables, and results were expressed as rho, 95% and 99% confidence intervals. ELISA and ROS data were analysed using GraphPad Prism™ software version 5 (GraphPad Software Incorporated, California, USA). The Shapiro-Wilk test was used to test for normality. Quantitative data for non-normally distributed variables were reported as the median (lower-upper quartile). The mean ± standard deviation for normally distributed variables was reported as assessed with Shapiro-Wilk's test. Experimental data from the current study was found to be non-parametric; therefore, the Kruskal Wallis test was used to determine significant differences among the three AP severity groups and HCs and $p<0.05$ was considered statistically significant. Subsequently, Dunn's Multiple Comparison Test was used for post hoc analysis. Results were expressed as medians and IQRs. Spearman's rank test was used to calculate rho using SPSS (version 27) to identify links among all the experimental data. Results were expressed as rho, 95% and 99% confidence intervals.

CHAPTER THREE: RESULTS

3.1 Demographics, Patient Characteristics and Clinical Data

Twenty four AP patients from CHBAH, Johannesburg, South Africa were included in the current study based on the inclusion criteria. Table 3.1 provides an overview of the patient sample codes, some demographics and associated sampling dates. A Summarry of the clinical characteristics, including aetiology, AP severity, comorbidities, clinical interventions and outcomes is presented in Table 3.2. The most common comorbidity in the patient group was HIV, accounting for 20.8% (5/24). Of the five HIV-positive patients (1 MSAP and 4 MAP), 3/5 were newly diagnosed with HIV during the sampling period. The other two HIV-positive patients were already on the Efavirenz/Emtricitabine/Tenofovir (TDF/EFV/FTC) treatment. This regimen is a combination of non-nucleoside reverse transcriptase inhibitor (non-NRTI) (efavirenz) and NRTI (emtricitabine, tenofovir DF) (Everson et al., 2018). Only one MAP patient had two comorbidities, HIV and hypertension and only 2/24 (8.3%) SAP patients were admitted into the ICU (Table 3.2). Samples could not be collected for all patients at every time point due to late presentation or early discharge (Table 3.1).

Table 3.1: Sample codes, days on which samples were collected per patient, gender, age, weight, height, and severity.

Patient Sample code	Gender	Age	Height (m)	Weight (kg)	Severity	Day 3	Day 5	Day 7	Day 9	Day 13	Day 15
CHAP039	F	29	n/a	n/a	Mild		✓				
CHAP040	M	41	1.72	76	Mild		✓	✓	✓	✓	✓
CHAP041	M	28	1.62	86.5	Mild	✓	✓	✓	✓		
CHAP042	F	55	1.5	72	Mild		✓	✓	✓	✓	✓
CHAP044	F	43	n/a	n/a	Mild	✓					
CHAP045	F	31	1.52	90	Moderate		✓	✓	✓	✓	
CHAP046	F	30	1.3	83	Severe	✓	✓	✓	✓	✓	✓
CHAP047	F	35	1.31	82	Mild		✓				
CHAP048	M	49	1.7	115	Severe		✓	✓	✓	✓	✓
CHAP049	F	39	1.31	62.1	Mild		✓	✓			
CHAP050	F	29	1.62	93	Mild	✓	✓	✓	✓	✓	
CHAP051	M	34	1.4	88	Mild	✓					
CHAP052	M	47	n/a	n/a	Severe			✓	✓	✓	
CHAP053	F	35	1.34	71.2	Mild	✓	✓	✓	✓	✓	
CHAP054	F	33	1.22	69.3	Mild		✓				

CHAP055	F	55	n/a	86	Mild	✓	✓					
CHAP056	F	52	1.4	70	Mild				✓			
CHAP057	M	54	1.53	57.9	Moderate	✓	✓	✓	✓	✓	✓	
CHAP058	M	51	1.43	79	Moderate			✓	✓	✓		
CHAP059	M	31	1.51	81	Mild		✓	✓				
CHAP060	M	46	1.5	77	Moderate				✓	✓	✓	
CHAP061	M	31	1.42	69	Mild			✓				
CHAP062	F	28	1.33	72	Mild			✓				
CHAP063	F	47	1.63	84	Mild		✓					

Notes: The table displays the sample code CHAP: Chris Hani Acute Pancreatitis, and the tick (✓) sign indicates the days on which blood samples were collected from patients. M: male, F: female, m: metres, kg: kilograms.

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The median age of the 24 recruited patients was 37 years, and the IQR was 31 to 48 years (Table 3). The gender distribution was 41.7% (10/24) male and 58.3% (14/24) female. The severity distribution was 17 (71%) MAP, 4 (17%) MSAP, and 3 (12%) SAP (Figure 3.1). The most common aetiology was biliary-induced pancreatitis (14, 58%) followed by alcohol (9, 38%) and, lastly, post-endoscopic retrograde cholangiopancreatography (ERCP) (1, 4%) associated pancreatitis (Figure 3.2).

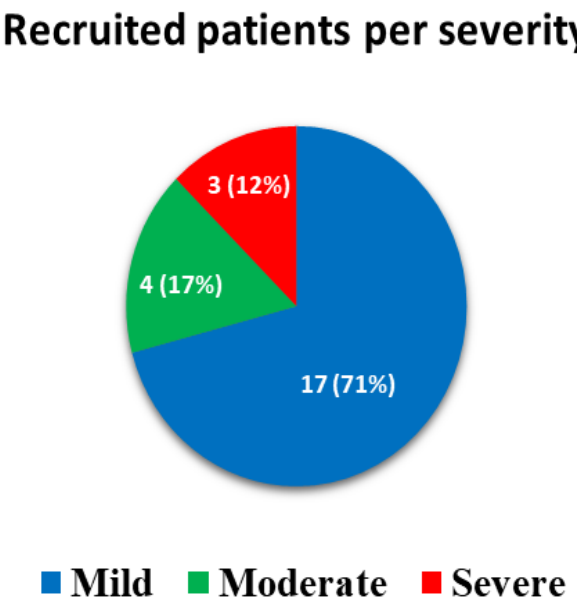


Figure 3.1: The distribution of recruited acute pancreatitis patients per risk category. Most patients were from the MAP group (71%). **Notes:** MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

Table 3.2: Patient characteristics and clinical parameters assessed

Parameter	Value (n=24)
Demographics	
Age in years [median (IQR)]	37 (31-48)
Male [n, (%)]	10 (41.7)
Female [n, (%)]	14 (58.3)
BMI kg/m^2 [median, (IQR)], all patients	36 (33.6-42.8)
MAP [median, (IQR)]	35.7 (32.4-42.8)
MSAP [median, (IQR)]	36.4 (29.5-38.8)
SAP [median, (IQR)]	44.5 (39.7-49.1)
Age in years [median (IQR)]	37 (28-55)
Aetiology	
Biliary [n, (%)]	14(58)
Alcohol [n, (%)]	9 (38)
ERCP [n, (%)]	1(4)
Severity	
Mild [n, (%)]	17 (71)
Moderate [n, (%)]	4 (17)
Severe [n, (%)]	3 (12)
Clinical outcomes ∞	
Median length of hospital stay (LOS, days)	
MAP [median; IQR]	5 (4-6)
MSAP [median; IQR]	14 (13-15)
SAP [median; IQR] ∞	11 (9-14)
Total Number of ICU admissions [n, %]	2 (8.3)
MAP group (n)	0
MSAP group (n)	0
SAP group (n)	2
Death in hospital [n, (%)]	2 (8.3)
MAP group (n)	0
MSAP group (n)	0
SAP group (n)	2
Surgical procedures∞	
Total number of patients that had procedures [n, %]	10 (41.7)
Total laparoscopic cholecystectomy [n, %]	9 (37.5)
MAP group (n)	7
MSAP group (n)	1
SAP group (n)	1
Endoscopic retrograde cholangiopancreatography [n, %]	6 (25)
MAP group (n)	3
MSAP group (n)	1
SAP group (n) ∞	2
Comorbidities§	
Total number that had comorbidity [n, %]	7 (29.2)
Total number that had two or more comorbidities [n, %]	1 (4.1)
HIV-positive [n, %]	5 (20.8)
Hypertension [n, %]	2 (8.3)
Diabetes [n, %]	0
Other [n, %]	0

Notes: §: An indication that some patients have more than one comorbidity; ∞: Indicates missing data within a group, LOS: length of stay; ICU: Intensive Care Unit; BMI: Body Mass Index; IQR: interquartile range; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.1.1 Patient characteristics within the different acute pancreatitis severity groups

The overall study population and certain aetiologies appeared to be associated with specific severity groups and demographic characteristics (Figure 3.2). In the MAP group, most patients had biliary-associated AP (12/17), followed by alcohol-associated AP (4/17); the least prevalent aetiology in this severity group was post-ERCP-induced AP (1/17). In the MSAP and SAP groups, alcohol was the most common aetiology (3/4) and (2/ 3), respectively. Biliary-associated AP was more common in females (10/14) than males (4/14). The frequency of alcohol-associated AP was more predominant in males (8 /9) than in females (1/9).

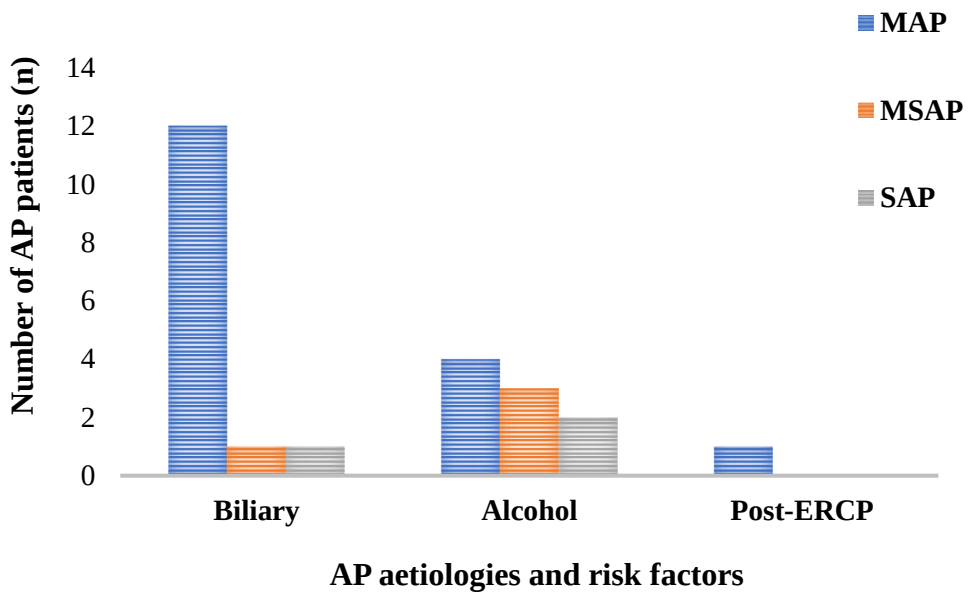


Figure 3.2: The number of recruited patients per aetiology within respective AP severities. The majority of the mild patients (12/17) had biliary-associated AP, and most MSAP (3/4) and SAP (2/3) had alcohol-associated AP. **Notes:** ERCP: Endoscopic retrograde cholangiopancreatography; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n= 17 MAP, 4 MSAP and 3 SAP.

The median age of MAP, MSAP and SAP patients was 35, 48.5 and 47, respectively (Figure 3.3).

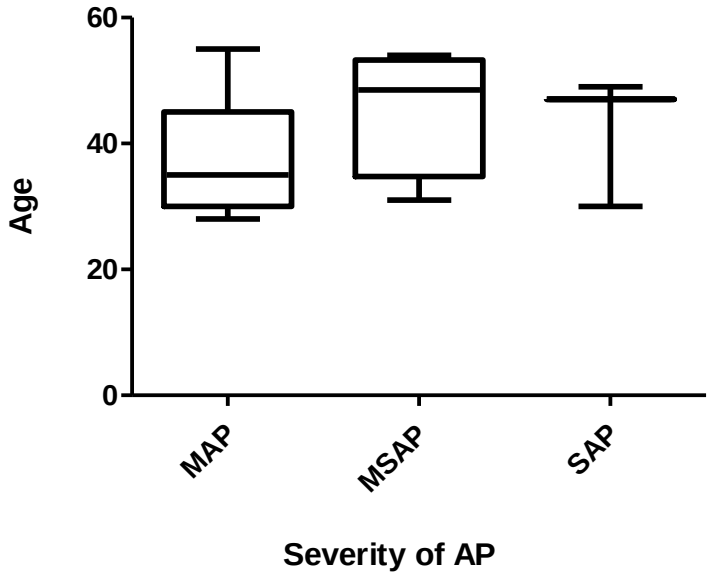


Figure 3.3: The age of AP patients per severity, n=17 (71%) for MAP patients, n=4 (17%) for MSAP and n=3 (12%) for SAP patients. **Notes:** AP: acute pancreatitis; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.1.2 Assessment of clinical parameters with AP severity

Clinical parameters such as CRP, creatine, WBC and platelet count were documented from the patient files according to the clinician's notes into the datasheet in Appendix F. The clinical data were recorded from the day of patient admission and each day of sample collection. Documented clinical parameters of the patients were compared across severity groups using the Kruskal-Wallis test at a 0.05 level of significance. Table 3.3 shows no statistically significant differences in the LOS based on AP severity (p=0.894) or in WBC, platelets, and creatine levels. However, a statistically significant difference was only found between CRP and AP severity (p=0.019), with CRP increasing with increasing AP severity.

Table 3.3: Comparison of clinical outcomes/parameters across severity (mean ± SD)

	LOS (Days)	CRP (mg/L)	WBC ($x10^9$/L)	Platelets ($x10^9$/L)	Creatine (µmol/L)
MAP	11.333(±6.351)	31.7(±39.615)	9.662(±4.779)	320.2(±162.37)	68.455(±13.456)
MSAP	12(±2.646)	75.667(±45.004)	11.458(±3.901)	320.25(±152.93)	60.000(±3.606)
SAP	14(±8.485)	263(±144.81)	25.685(±24.247)	274.5(±47.376)	59.333(±44.23)
P-value	0.894	**0.019***	0.4896	0.8056	0.6755

*Kruskal Wallis is significant at the 0.05 level (2-tailed). **Notes:** LOS: length of hospital stay; CRP: C-reactive protein; WBC: white blood cells; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.2 Intracellular IL-6 Levels in Innate and Adaptive Cells in AP Patients

The data for cells associated with the expression of IL-6 was obtained by ICS flow cytometry. Data were analysed for 12 AP patients, 5 MAP, 4 MSAP, and 3 SAP. Only one patient in each severity group had D3 to D15 data; other patients had data varying from D5 to D15. Six age- and sex-matched volunteers were considered for healthy controls. The targeted cell populations and subpopulations analysed are listed in Table 2.1. in Chapter 2 and include T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$), granulocytes ($CD66b^+$, $CD16^+$), NK cells ($CD16^+$, $CD56^+$, and $CD57^+$), monocytes ($CD14^+$, $CD16^+$) and IL-6^+.

3.2.1 Gating strategy and representative dot plots for ICS

In principle, gating refers to the sequential identification and segregation of a target population of interest based on a panel of known markers visualised by fluorescence in a specified emission spectrum (Verschoor et al., 2015). A gating strategy is a selection of an area on the scatter plot (generated during an experiment) that separates cells that will be further analysed from those that will be excluded. Gating is essential because it ensures that the data to be analysed is acquired on the accurately targeted population. This assay used FlowJo V10.8.1 (Oregon, USA) for gating.

Representative gating strategies for IL-6 are shown in Figures 3.4, 3.5 and 3.6 and show gating for leukocytes, lymphocytes, monocytes and IL-6. Gated cells were further analysed, and results were reported as median and IQR percentages of NK cells, NKT cells, monocytes, and associated IL-6 expression (Table 3.4 and 3.5). Furthermore, the neutrophil-lymphocyte ratio (NLR,) which is considered a potential marker in determining AP severity and probability of ICU admission, was also assessed and reported as median and IQR percentages of parent lymphocyte and neutrophil populations (Table 3.6).

The initial gate for IL-6 ICS analysis was the time gate, which was a plot of time on the x-axis against CD3 BUV496 and showed the consistency of the flow of cells during acquisition (Figure 3.4). From the time gate, singlets were gated using forward scatter height (FSC-H) and FSC area (FSC-A) to exclude doublets or clumped cells which may interfere with the physical and fluorescent characteristics of single cells, resulting in false data. WBCs, including lymphocytes, monocytes and granulocytes, were gated from the singlet population based on their size and granularity, using side scatter area (SSC-A) and FSC-A (Figure 3.4).

The lymphocyte populations were discriminated using CD3 BUV496 and included T and B lymphocytes and NK cells (Figure 3.5). B cells were categorised using CD19 AF700, and subpopulations of NK cells were categorised using CD16 APC, CD56 PECy7 and CD57 BB515. Monocyte populations were categorised using CD14PE, CD16 APC, CD56 PECy7, and HLA-DRBV650. Monocytes were further gated into various subpopulations, which included classical ($CD14^{+}CD16^{-}$), non-classical ($CD14^{-}CD16^{+}$), intermediate ($CD14^{+}CD16^{+}$), and other monocytes subpopulations that were $CD14^{+}HLADR^{+/-}$ (Figure 3.5). The neutrophil subpopulation of granulocytes was categorised using CD66b PerCP-CY5.5. Populations of WBCs were further discriminated into IL-6 BV421 and HLA-DR for intracellular IL-6 analysisassay (Figure 3.6, Table 2.1 and Appendix H). Every subpopulation was represented as a percentage of the parent populations.

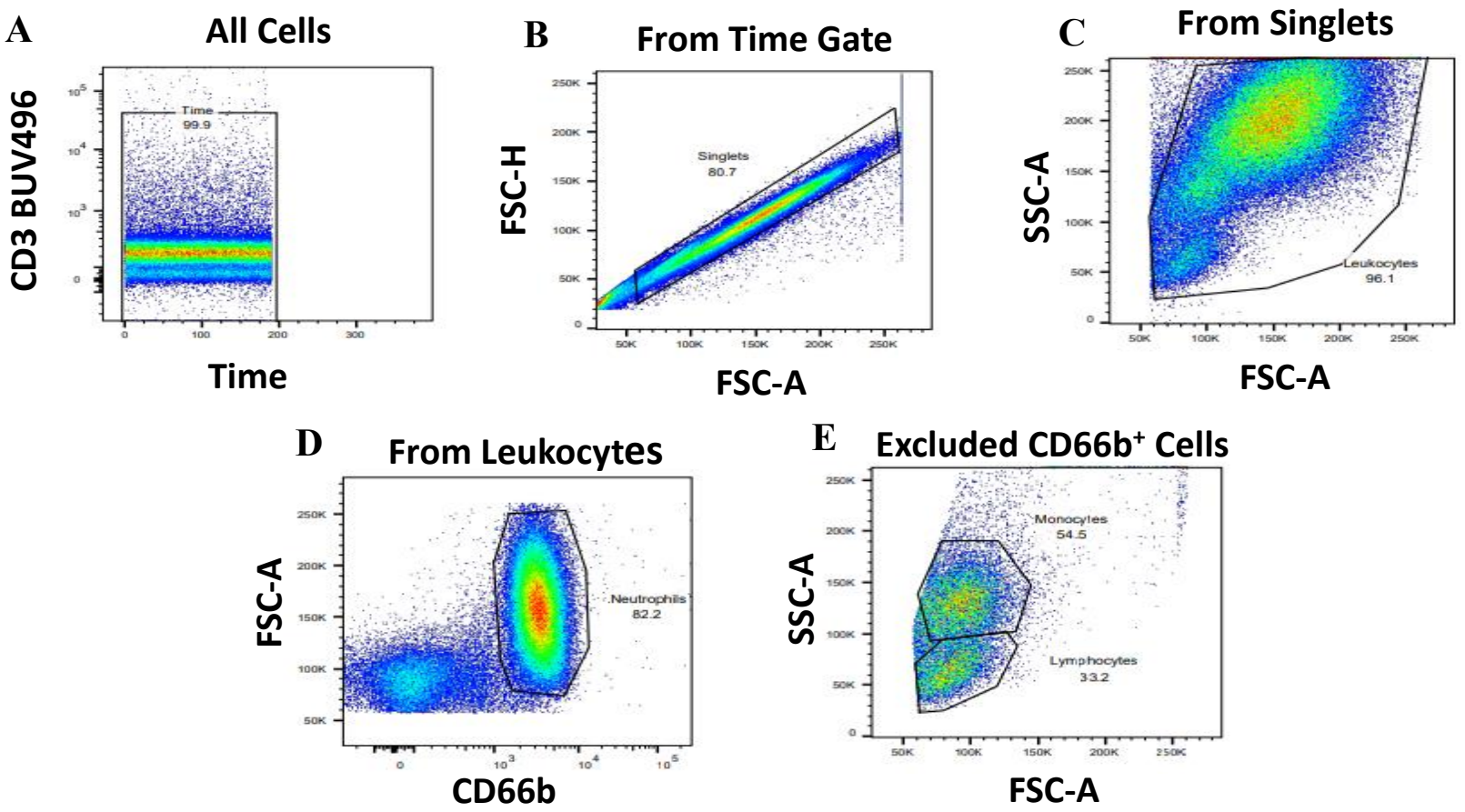


Figure 3.4: Gating strategy used for leukocytes (A-E) using an MSAP patient sample on D9. The initial gate, A: Time gate, showing the consistency in the flow of cells during acquisition. B: Singlet gate, which excludes doublets using FSC-H and FSC-A. C: In C, leukocytes were gated collectively based on granularity and size using FSC-A and SSC-A, respectively. Thereafter, the biggest and most granular leukocytes (granulocytes/neutrophils) were discriminated in D using FSC-A and CD66b. Subsequently, lymphocytes (smallest and least granular) and monocytes (bigger and more granular) leukocyte subpopulations were gated in E using SSC-A and FSC-A. **Notes:** Full fluorochrome names are provided in Table 2.1. in Chapter 2. CD: cluster of differentiation; FSC-H: forward scatter height; FSC-A: forward scatter area; SSC-A: side scatter area.

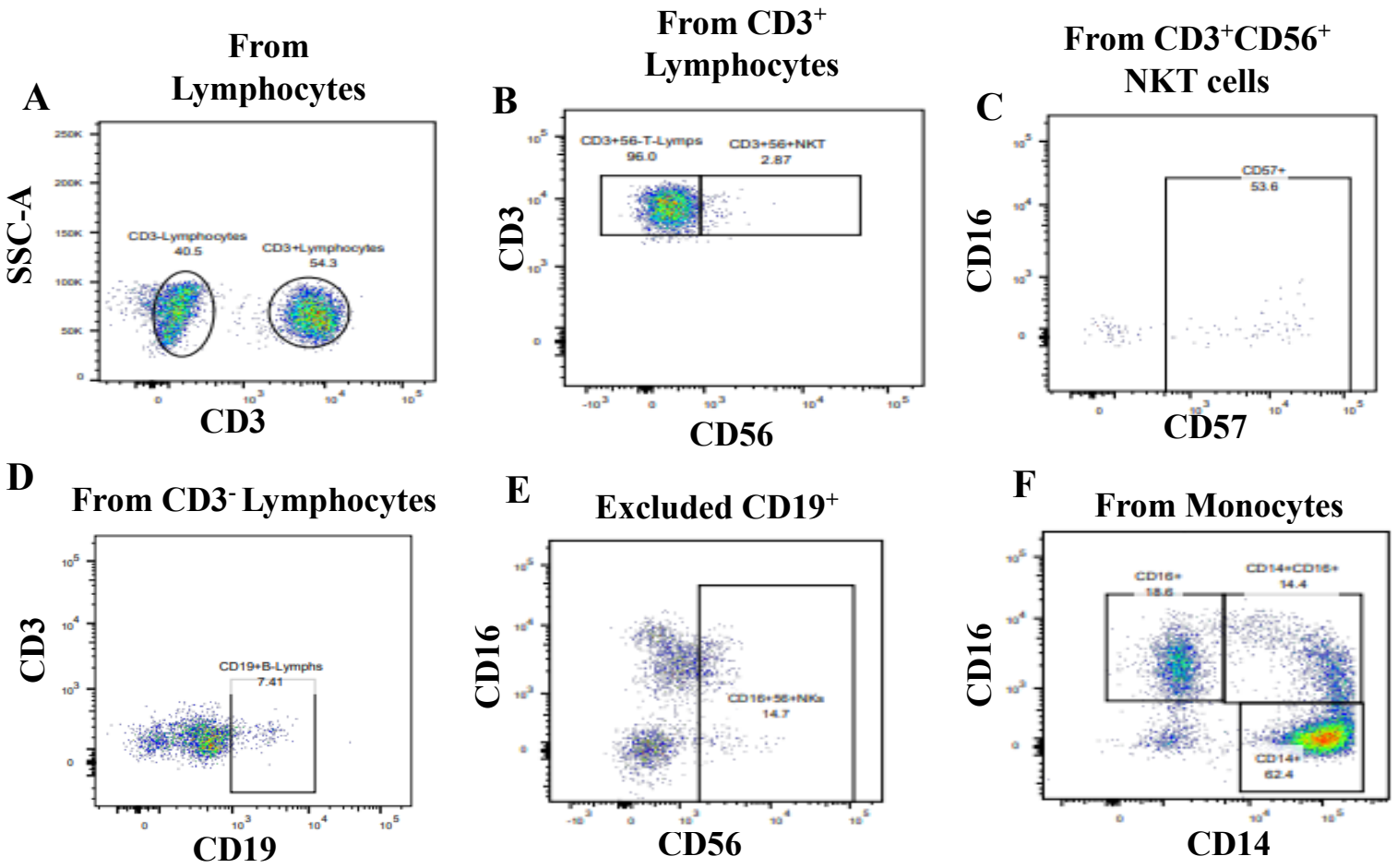


Figure 3.5: Gating strategy for lymphocytes and monocytes (A-F) using an MSAP patient sample on D9. Dot plot A: T lymphocytes were gated from the lymphocyte population from Figure 3.4 using SSC-A and CD3. B: From CD3+ lymphocytes, $CD3^+CD56^+$ NK T lymphocytes were gated. In C, mature $CD3^+CD56^+$ NKTs were gated using CD57. D: $CD3^-$ lymphocytes (B lymphocytes) were gated using CD19. E: lymphocytes that were neither $CD3^-$ nor $CD3^+$ were further gated into NK cells using CD16 and CD56. In F, monocytes were gated into nonclassical monocytes ($CD16^+CD14^-$), intermediate ($CD16^+CD14^+$) and Classical ($CD16^-CD14^+$). Table 2.1 shows the full fluorochrome names. **Notes:** CD: cluster of differentiation; SSC-A: side scatter area.

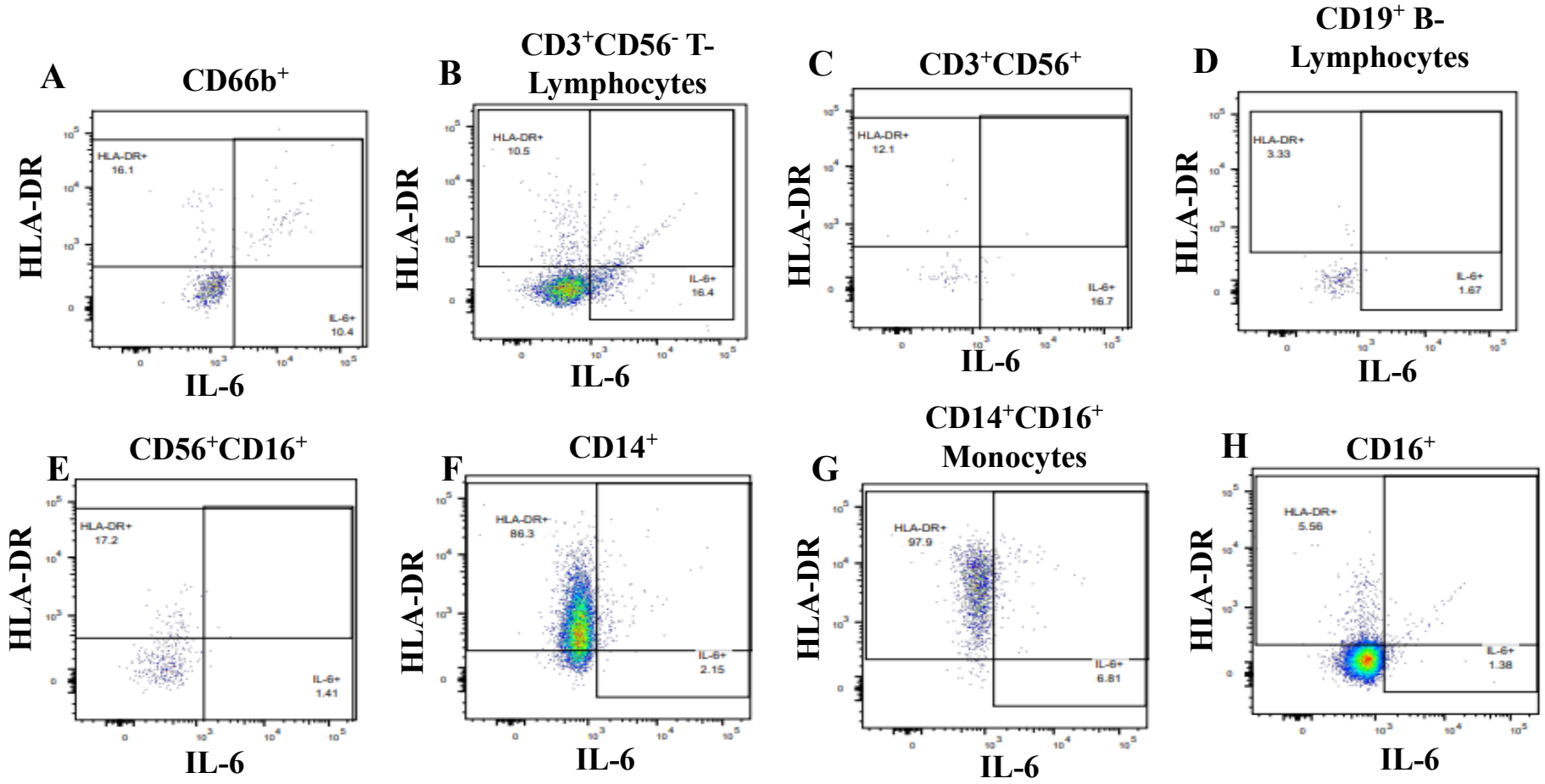


Figure 3.6: Intracellular IL-6/HLA-DR gating strategy for leukocytes from subpopulations in Figure 3.5 (A-H) using an MSAP patient sample on D9. A: IL-6 and HLA-DR were gated from the neutrophil population. B: From $CD3^+$ $CD56^+$ lymphocytes, IL-6 and HLA-DR were gated. In C, IL-6 and HLA-DR were gated from mature $CD3^+CD56^+$ NKT cells. D: IL-6 and HLA-DR were gated from $CD19^+$ B- lymphocytes. E: IL-6 and HLA-DR were gated from $CD56^+CD16^+$ NK cells. In F, classical monocytes ($CD14^+$) were gated for intracellular IL-6 and HLA-DR. G: IL-6 and HLA-DR were gated from intermediate monocytes ($CD14^+CD16^+$), and in H, IL-6 and HLA-DR were gated from non-classical monocytes. **Notes:** CD: cluster of differentiation; HLA-DR: Human Leukocyte Antigen – Antigen D Related; IL-6: Interleukin-6.

3.2.1 NK and NKT cells associated with IL-6 secretion

3.2.1.1 NK cell populations and severity: NK subsets were gated into two classes; $CD16^{+}CD56^{+}$ NKs and mature $CD16^{+}CD56^{+}$ $CD57^{+}$ NKs, which were further assessed for the intracellular abundance of IL-6 (Figure 3.5 E and Table 3.4). These were Most of the NKs cells were mature ($CD57^{+}$), and among the AP patients, the MSAP group had the highest NKs cells in the early phase (D3-D7) compared to the MAP and SAP groups (Table 3.4). The highest expression level in terms of the median and IQR was 67.5% (67.5%-67.5%) on D3, while D5 and D7 were 44.2% (41.7%-46.7%) and 42.4% (39.0%-51.9%), respectively. In the late phase (D9-D15), SAP patients had the highest expression of NKs on D9 and D15, with the medians and IQR being 47.1% (26.4%-51.9%) and 40.7% (21.4%-60.0%), respectively. Overall, MAP patients had the least expression of NKs, ranging from 27.8% (22.2%-37.0%;) to 39.7% (27.4%-41.7%) across the two phases (Table 3.5).

In terms of intracellular IL-6 expression from NK cells, MAP patients had the most expression in the early phase, with the highest IL-6 median and IQR expression of 1.29% (0.9%-2.2%) on D3, depicted in Table 3.4. The MSAP group of patients had the most IL-6 expression associated with NKs in the late phase, with an expression median and IQR of 0.8% (0.3%-2.1%) on D9 to 1.7% (1.4%-2.0%). SAP patients had the least percentage of intracellular IL-6 expression associated with NKs, with an IL-6 median and IQR of 0.1% (0.1%-0.2%) to 0.5% (0.3%-0.6%) across the two AP phases. Similarly, the HC group had low intracellular IL-6 in NKs, with a median and IQR expression of 0.5% (0.3%-0.6%). Overall, IL-6 was expressed more in the early than the late phase by NKs in all three severity groups. In terms of the median fluorescent intensity (MFI), which is the average of the fluorescence intensity of all the signals from the cells of interest that were measured, no trend was observed that could distinguish intracellular IL-6 expression across the three severity groups over time (D3-D15). Overall, there was no evident trend in the expression levels on IL-6 intracellularly from D3-D15.

3.2.1.2 NKT cell populations and severity: NKT subsets were gated into two classes, as shown in Figures 3.5 B and C, CD3+CD56+ NKTs and mature CD3+CD56+CD57+ NKTs. Most NKT cells were mature (CD57+), and SAP patients had the highest percentage of NKT cells in the early phase compared to the MSAP and MAP groups. The medians and IQR were 61.6% (61.6%-61.6%), 68.0% (61.6%-74.3%) and 62.4% (54.5%-69.1%) for D3, D5 and D7, respectively (Table 3.4). Overall, the MAP patient group had the least expression of NKT cells compared to the severe AP groups across the two phases (Table 3.4).

Regarding intracellular IL-6 expression, MAP and SAP patients had the most expression in the early (D3-D7) and late phase (D9-D13). The highest IL-6 expression in the MAP group was on D3 and D7, with the medians and IQR being 11.7% (10.9%-12.4%) and 11.6% (8.3%-16.0%). However, in the late phase, specifically D9, the SAP group had the highest intracellular IL-6, 10.3% (5.9%-12.0%) (Table 3.4). The least IL-6 expression associated with NKT cells was observed in the HC group with a median and IQR of 5.0% (2.9%-7.8%), followed by the MSAP group of patients with a median and IQR of 2.5% (2.3%-2.8%) to 15.6% (12.2%-19.0%) across the two AP phases (Table 3.4).

The MFI is the average fluorescence intensity of all the cells of interest that have been measured. Severe patients had the highest IL-6 MFI across the two phases. The SAP IL-6 MFI median and IQR were 1797 (1758-1835) to 2716 (2535-5899). The MSAP and MAP groups had lower IL-6 MFI when compared to the SAP group. Furthermore, a decrease in IL-6 MFI was observed between the MAP and MSAP groups from D3-D15 (Table 3.4).

Table 3.4: Total percentage of Natural Killer cells, their sub-populations, and the intracellular abundance of IL-6 per AP severity groups and days of pain

% NK cells (CD3-)						%NKT cells (CD3+)			
Severity group	**No of patients**	**CD16+ CD56+**	**CD16+ CD56+CD57+**	**IL-6 NKs**	**MFI IL-6**	**CD16+ CD56+**	**CD16+ CD56+CD57+**	**IL-6 NKTs**	**MFI IL-6**
Healthy control	n=6	8.1 (5.7-9.5)	39.8 (37.5-51.0)	0.5 (0.3-0.6)	1487 (1444-1757)	6.2 (2.9-7.6)	54.3 (33.2-81.3)	5.0 (2.9-7.8)	1895 (1719-895)
Day 3									
MAP	n=4	4.0 (3.6-4.8)	27.8 (22.2-37.0)	1.29 (0.9-2.2)	1472 (1434-1523)	2.6 (2.1-3.0)	44.9 (43.8-46.9)	11.,7 (10.9-12.4)	1892 (172-1992)
MSAP	n=1	5.8 (5.8-5.8)	67.5 (67.5-67.5)	1.2 (1.2-1.2)	1485 (1485-148)	0.7 (0.7-0.7)	66.7 (66.7-66.7)	4.8 (4.8-4.8)	2076 (2076-2076)
SAP	n=1	6.0 (6.0-6.0)	12.3 (12.3-12.3)	0.5 (0.5-0.5)	1359(1359-1359)	2.4 (2.4-2.4)	61.6(61.6-61.6)	5.2 (5.2-5.2)	2250 (2250-2250)
Day 5									
MAP	n=5	2.2 (1.7-2.7)	36.5 (32.9-49.2)	1.0 (0.9-2.0)	1419 (1362-1708)	2.1 (2.0-3.1)	56.2 (46.7-56.4)	9.5 (7.6-9.8)	1937 (1916-1994)
MSAP	n=2	4.7(4.5-4.7)	44.2 (41.7-46.7)	2.3 (1.4-3.2)	1596 (1446-1745)	2.0 (1.4-2.7)	49.3 (47.6-50.9)	15.6 (12.2-19.0)	2775 (2161-3388)
SAP	n=2	7.1 (10.3-13.6)	42.6 (24.8-60.3)	0.5 (0.3-0.6)	1723 (1638-1807)	1.5 (1.4-1.5)	68.0 (61.6-74.3)	3.8 (3.7-3.8)	2519 (2469-2569)
Day 7									
MAP	n=5	2.8 (2.9-3.2)	38.3 (38.1-50.2)	1.7 (1.1-3.9)	1649 (1327-1758)	3.2 (2.7-3.3)	35.9 (34.0-51.3)	11.6 (8.3-16.0)	1946 (1899-2843)
MSAP	n=3	3.7 (4.0-8.4)	42.4 (39.0-54.2).	0.0 (0-0.4)	0.0 (0.0-781)	1.3 (1.1-1.4)	41.9 (33.2-57.4)	5.4 (4.9-6.0)	2104 (1904-2315)
SAP	n=3	3.7 (3.7-12.4)	24.6 (14.3-51.9)	0.4 (0.3-1.4)	1544 (1529-2067)	2.8 (2.4-3.0)	62.4 (54.5-69.1)	4.8 (2.7-11.3)	1924 (1828-2542)
Day 9									

MAP	n=5	2.7 (3.4-9.2)	39.7 (27.4-41.7)	1.0 (0.3-14.7)	1437 (1383-1528)	5.0 (3.1-6.2)	39.1 (26.0-58.5)	9.4 (8.5-10.0)	1849 (1777-2271)
MSAP	n=4	5.9 (6.7-8.4)	24.4 (19.3-38.8)	0.8 (0.3-2.1)	1436 (1366-1658)	1.8 (1.5-4.9)	54.8 (46.5-59.8)	4.2 (3.2-6.0)	1907 (1855-2052)
SAP	n=3	2.1(2.3-11.3)	47.1(26.4-51.9)	0.5 (0.3-1.4)	1304 (652-1610)	3.9 (2,8-4.5)	49.2 (45.6-57.3)	10.3 (5.9-12.0)	2317 (2279-2667)
Day 13									
MAP	n=5	2.8 (3.7-9.1)	33.7 (32.9-38.5)	0.9 (0.7-1.7)	1571 (1459-1582)	4.0 (3.0-4.7)	56.5 (37.1-65.6)	12.1 (11.2-16.8)	1789 (1638-2067)
MSAP	n=4	9.8 (11.8-12.5)	33.7 (14.9-55.2)	1.3 (0.8-1.6)	1358 (1331-1442)	2.7 (2.4-5.7)	46.8 (37.2-52.6)	5.4 (4.6-6.5)	2072 (1966-2204)
SAP	n=3	7.0 (10.4-15.1)	19.7 (12.3-52.0)	0.5 (0.4-0.8)	1356 (1343-1726)	2.4 (2.1-3.2)	52.4 (46.1-62.2)	6.7 (4.0-10.0)	2716 (2535-5899)
Day 15									
MAP	n=2	2.9 (4.2-5.4)	28.6 (26.4-30.7)	0.7 (0.5-0,9)	1379 (1360-1397)	3.2 (2.7-3.7)	41.7 (34.8-48.6)	9.2 (7.7-10.7)	1714 (1599-1828)
MSAP	n=2	12.6 (13.7-14.9)	27.7 (25.3-30.0)	1.7 (1.4-2.0)	1557 (1493-1622)	8.0 (5.3-10.6)	49.2 (47.1-51.3)	2.5 (2.3-2.8)	1861 (1794-1927)
SAP	n=2	9.0 (11.8-14.7)	40.7 (21.4-60.0)	0.1 (0.1-0.2)	2026 (1948-2103)	2.6 (2.4-2.7)	47.3 (43.6-50.9)	8.7(6.2-11.2)	1797 (1758-1835)

Notes: All numbers are represented as median and IQR percentages (%), except the MFI (Median fluorescence intensity). This table shows data rounded to one decimal place. CD: cluster of differentiation; IQR: interquartile range MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis; n: number of AP patients; NK: natural killer cells; NKT: natural killer T lymphocytes. These cells were gated, as represented in Figures 3.4-3.6.

3.2.2 Monocyte populations and AP severity

Monocytes were gated into three populations, classical monocytes ($CD14^{+}CD16^{-}$), intermediate monocytes ($CD14^{+}CD16^{+}$), and non-classical monocytes ($CD14^{-}CD16^{+}$) (Figure 3.5 F). Classical monocytes were the most expressed subpopulation in severe AP groups from D5-D13, with the MSAP group median and IQR ranging from 52.4% (44.4%–59.9%) to 74.8% (72.4%–77.1%) and SAP ranging from 42.8% (39.2%–42.9%) to 68.8% (66.8%–70.9%). MSAP and SAP patient groups showed a trend of decreasing classical monocytes from the early to late AP phase, D3-D15 (Table 3.5). Overall, the MAP group generally had the least classical monocytes, with a median and IQR from 48.4% (46.7–61.2) to 74.8% (69.9%–76.5%) (Table 3.5). No trend was observed in HLA-DR expression in monocytes across all severity groups over time from D3-D15.

Regarding intracellular IL-6 expression in monocytes, no trend was evident in all three severity categories over time (D3-D15). However, based on the IL-6 MFI, classical and non-classical monocytes in the MSAP patients seem to be secreting IL-6, with non-classical monocytes having the highest MFI of 15553 (9289-21818) on D5 (Table 3.5).

Table 3.5: Total percentage of monocytes, their sub-populations, and the intracellular abundance of IL-6 per AP severity groups and days of pain

% Monocyte subsets												
Severity group	**No. of patients**	**Percentage of Monocytes in parent population**	**$CD14^+$ $CD16^-$**	**HLA-$DR^+IL\text{-}6^+$ ($CD14^+$ $CD16^-$)**	**$IL\text{-}6^+$ ($CD14^+$ $CD16^{)}$)**	**MFI $IL\text{-}6^+$ ($CD14^+$ $CD16^-$)**	**$CD14^-$ $CD16^+$**	**HLA-$DR^+IL\text{-}6^+$ ($CD16^{+)}$**	**$IL\text{-}6^+$ ($CD16^+$)**	**MFI $IL\text{-}6^+$ ($CD16^+$)**	**$CD14^+$ $CD16^+$**	**$IL\text{-}6^+$ ($CD14^+$ $CD16^+$)**
Healthy control	n=6	7.1 (6.2-8.4)	64.0 (47.9-69.0)	2.0 (1.1-4.5)	0.0 (0.0-0.0)	1779 (1642-2098)	13.7 (12.0-15.6)	1.2 (0.9-1.8)	1.8 (1.0-2.9)	2037 (1974-2431)	8.7 (6.1-10.5)	0.0 (0.0-0.0)
Day 3												
MAP	n=4	7.2 (6.7-7.7)	74.8 (69.9-76.5)	3.6 (1.7-6.1)	0.6 (0.3-1.2)	1849 (1840-1915)	9.7 (9.2-12.7)	0.5 (0.3-1.5)	1.9 (1.0-2.9)	2517 (2475-2602)	8.8 (7.5-10.7)	0.0 (0.0-0.0)
MSAP	n=1	7.4 (7.4-7.4)	70.0 (70.0-70.0)	0.9 (0.9-0.9)	0.1 (0.1-0.1)	2012 (2012-2012)	5.6 (5.6-5.6)	1.6 (1.6-1.6)	0.5 (0.5-0.5)	2648 (2648-2648)	16.8 (16.8-16.8)	0.0 (0.0-0.0)
SAP	n=1	6.9 (6.9-6.9	71.3 (71.3-71.3	0.3 (0.3-0.3)	0.1 (0.1-0.1)	1742 (1742-1742)	10.7 (10.7-10.7)	0.4 (0.4-0.4)	1.4 1.4-1.4)	1754(1754-1754)	12.6 (12.6-12.6)	0.0 (0.0-0.0) 0
Day 5												
MAP	n=5	6.8 (6.6-7.8)	62.6 (56.6-63.6)	3.6 (1.7-6.1)	0.2 (0.0-0.4)	1972 (1920_1999)	10.0 (8.1-12.0)	0.5 (0.5-1.0)	0.9 (0.8-2.2)	2655 (2210-2817)	10.8 (5.6-11.9)	0.0 (0.0-0.0)
MSAP	n=2	11.2 (9.9-12.5)	74.8 (72.4-77.1)	1.4 (0.7-2.7)	0.1 (0.1-0.2)	2287(2279-2294)	6.0 (5.8-6.3)	0.7 (0.6-.0.8)	1.2 (0.8-1.6)	15553 (9289-21818)	12.4 (11.4-13.3)	0.0 (0.0-0.0)

SAP	n=2	9.3 (9.1-9.5)	68.8 (66.8-70.9)	0.3 (0.2-0.3)	0.0 (0.0-0.0)	2627 (2200-3054)	10.1 (8.9-11.2)	0.1 (0.1-0.10	0.6 (0.5-0.7)	2181 (2164-2198)	18.0 (14.8-21.1)	0.0 (0.0-0.1)
Day 7												
MAP	n=5	9.4 (9.4-10.4)	50.0 (46.0-66.9)	114.2 (11.9-16.9)	0.0 (0.0-2.0)	2048 (1950-2090)	7.3 (6.0-17.5)	3.1(1.0-6.7)	1.3 (1.0-2.7)	2648 (2307-2922)	13.0 (7.5-18.3)	0.0 (0.0-0.1)
MSAP	n=3	12.4 (10.3-13.7)	71.6 (68.9-73.9)	0.1 (0.1-0.2)	0.0 (0.0-0.1)	2338 (2233-6678)	6.1 (5.9-6.7)	0.1(0.1-0.3)	0.4 (0.3-0.6)	2716 (2512-3973)	13.9 (9.9-14.1)	0.0 (0.0-0.0)
SAP	n=3	8.5 (8.0-9.2)	61.9 (55.6-62.8)	0.7 (0.5-1.0)	0.1 (0.1-0.2)	2392 (2234-2408)	13.5 (11.5-19.4)	0.3(0.2-0.6)	0.8 (0.5-0.8)	1891 (1838-2329)	19.2 (13.6-25.2)	0.0 (0.0-0.0)
Day 9												
MAP	n=5	9.4(6.8-10.8)	56.2 (42.1-72.0)	3.3 (1.6-38.4)	0.1 (0.1-1.2)	1977 (1920-2128)	8.9 (4.6-11.2)	3.0 (2.2-11.9)	1.8 (0.6-3.3)	2302 (2225-2328)	4.5 (2.5-12.0)	0.0 (0.0-0.5)
MSAP	n=4	9.3(8.0-11.3)	70.0 (59.1-75.1)	0.4 (0.3-0.5)	0.0 (0.0-0.1)	2219 (2137-2503)	7.5(6.3-9.3)	0.9 (0.2-1.6)	0.4 (0.3-1.4)	2574 (2416-3271)	8.8 (5.8-13.6)	0.0 (0.0-0.0)
SAP	n=3	5.6 (5.4-7.1)	60.8 (54.4-66.	1.0 (0.9=1.7)	0.1 (0.0-0.1)	2171(1901-2239)	11.9(9.0-25.7)	0.9 (0.6-1.3)	0.3 (0.2-0.5)	2067 1975-2794)	13.4 (9.3-16.8)	0.0(0.0-0.8)
Day 13												
MAP	n=5	8.1(6.3-10.0)	48.4 (46.7-61.2)	4.6 (2.4-4.8)	0.1(0.1-0.2)	1981(1933-2099)	13.4 (12.0-14.8)	0.9 (0.3-1.0)	0.8 (0.4-3.0)	2542 (2435-2619)	4.6(4.1-20.4)	0.0 (0.0-0.1)
MSAP	n=4	9.5(8.5-9.9)	52.4 (44.4-59.9)	0.4 (0.4-0.7)	0.1(0.0-0.1)	2368 (2241-2518)	11.4 (9.4-12.6)	0.6 (0.3-1.0)	0.7 (0.4-1.4)	2503 (2388-2632)	10.3 (7.2-15.0)	0. 0(0.0-0.1)

SAP	n=3	9.2 (7.4-11.0)	42.8 (39.2-42.9)	0.8 (0.7-2.0)	0.0 (0.0-0.0)	1686 (1668-2242)	19.1 (18.5-19.2)	1.3 (0.9-1.8)	0.5(0.5-0.8)	1959(1821-1966)	23.6(17.8-26.7)	0. 0(0.0-0.0)
Day 15												
MAP	n=2	11.0(9.4-12.5)	69.8(68.2-71.4)	1.1(1.0-1.2)	0.0(0.0-0.0)	2080(1990-2171)	8.1(7.9-8.2)	0.6(0.5-0.8)	1.5(1.0-1.9)	2215(2213-2218)	12.7(10.5-14.9)	0.0(0.0-0.0)
MSAP	n=2	9.7(7.6-11.7)	53.3(51.8-54.8)	0.4(0.3-0.4)	0.0(0.0-0.0)	2658(2592-2725)	14.4(10.6-18.3)	0.4(0.3-0.5)	2.1(1.1-3.0)	3620(3022-4218)	6.8(5.7-7.9)	0.0(0.0-0.0)
SAP	n=2	8.8(8.6-9.0)	50.7(48.3-53.1)	1.0(0.8-1.2)	0.0(0.0-0.0)	3114(2579-3648)	21.5(17.1-25.9)	0.7(0.6-0.8)	0.490.4-0.5)	3108(2485-3730)	14.1(9.8-18.4)	0.0(0.0-0.0)

Notes: All numbers are represented as median and IQR percentages (%), except the MFI (Median fluorescence intensity). This table shows data rounded to one decimal place. CD: cluster of differentiation; HLA-DR: human leukocyte antigen D- related; MFI: Median Fluorescence Intensity; n: number of patients; 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 Figures 3.4. to 3.6.

3.2.3 Evaluating the neutrophil-lymphocyte ratio

The total population of neutrophils was gated from leukocytes using FSC-A and CD66b PerCP-Cy-5-5 to evaluate the usefulness of neutrophil-lymphocyte ratio (NLR) as an early predictive marker of SAP and OF in AP (Figure 3.4 D). Monocytes and lymphocytes were gated from the excluded $CD66b^+$ cells using FSC-A and FSC-H (Figure 3.4 E). The gated lymphocyte population in Figure 3.4 E was considered the total lymphocyte population and was used for calculating the neutrophil-lymphocyte ratio (NLR). The NLR was calculated and evaluated for 11 AP patients; n= 4 MAP, 4 MSAP and 3 SAP in comparison to six HCs (Table 3.6). The normal NLR range is between 0.78 and 3.53 (Forget et al., 2017).

The average NLR for the six HCs was 0.7, which was lower than that for AP patients. Severe patients had the highest NLR across the two AP phases, with the MSAP group ranging from 2.9-13.5 and the SAP group ranging from 1.6-11.2 across the two phases (Table 3.6). D3 had the highest NLR for both MSAP and SAP groups, 13.5 and 11.2, respectively (Table 3.6). D15 had the least NLR, and overall, a decreasing trend in NLR was observed from D3-D15 in all three severity groups. (Table 3.6).

Table 3.6: Total neutrophils and lymphocyte populations and the NLR. The severe groups of patients had the highest NLR from D3-D15, and a decreasing trend was evident from D3-D15 across the three severity groups.

Severity /Day	No of patients/HCs	Neutrophils CD66b+	Total Lymphocytes	NLR
		%		
HCs	n=6	36.5 (30.4-38.9)	51.7 (44.8-53.9)	1.4
		DAY 3		
MAP	n = 4	72.8 (70.1-75.6)	19.0 (15.3-21.3)	3.8
MSAP	n = 1	83.4 (83.4-83.4)	6.2 (6.2-6.2)	13.5
SAP	n = 1	83.9 (83.9-83.9)	7.5 (7.5-7.5)	11.2
		DAY 5		
MAP	n = 5	58.4 (56.7-60.8)	31.0 (30.8-33.6)	1.9
MSAP	n = 2	76.7 (77.7-77.7)	9.6 (9.2-9.9)	8.0
SAP	n = 2	75.4 (73.7-77.0)	13.1 (11.5-14.6)	5.8
		DAY 7		
MAP	n = 5	52.2 (49.6-60.4)	38.2 (24.8-38.6)	1.4
MSAP	n = 3	72.0 (72.6-72.6)	16.4 (12.5-16.7)	4.4
SAP	n = 3	80.5 (68.9-80.7)	7.8 (7.7-18.7)	10.3
		DAY 9		
MAP	n = 5	50.3 (47.1-64.2)	36.8 (26.6-43.0)	1.4
MSAP	n = 4	68.9 (74.1-74.1)	13.7 (13.3-19.4)	5.0
SAP	n = 3	82.4 (72.4-84.4)	8.7 (6.9-17.0)	9.5
		DAY 13		
MAP	n = 5	64.3 (55.5-71.6)	24.2 (19.2-33.5)	2.7
MSAP	n = 4	64.8 (70.6-70.6)	20.9 (15.4-27.4)	3.1
SAP	n = 3	56.1 (52.3-70.6)	27.3 (16.8-32.8)	2.1
		DAY 15		
MAP	n = 5	53.1 (44.9-61.3)	33.8 (27.7-39.8)	1.6
MSAP	n = 2	63.8 (64.1-64.1)	22.2 (20.7-23.8)	2.9
SAP	n = 2	52.7 (48.8-56.5)	34.0 (30.8-37.3)	1.6

Notes: All numbers are represented as median and IQR percentages (%), except the NLR, which is rounded to one decimal place HCs: Healthy controls; NLR: Neutrophil-lymphocyte ratio; n: number of patients/participants; CD: cluster of differentiation; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis

3.2.4 Association of intracellular CXCL4 with activated platelets

For this aspect of work, intracellular CXCL4 was assessed from 11 of the 24 recruited AP patients consisting of 4 MAP, 4 MSAP, and 3 SAP. Only one patient in each severity group had D3 to D15 data; others had data that varied from D5 to D15. Figure 3.7 shows a representation of the CXCL4 ICS gating strategy. The targeted cells were platelets with careful consideration of their activation status, using the platelet surface marker CD41a and platelet activation markers CD62p and PAC-1. CXCL4 was stained intracellularly. The markers used to target platelets and intracellular CXCL4 are listed in Table 2.2, and a detailed four-colour panel is in Appendix I.

The initial gate for CXCL4 ICS analysis was the time gate showing consistency of the flow of cells during acquisition. From the time gate, putative platelets were gated using low FSC-A and SSC-A properties. This gate included debris due to size of platelets (Figure 3.7 B and C). Based on the putative platelet gate, a singlet gate was introduced to exclude doublets using FSC width (FSC-W) and FSC area (FSC-A). A platelet population was gated from the singlet population using CD41a BUV496, followed by gating activated platelets using CD62p BV421 (Figure 3.7 E). From the $CD62p^+$ population, $PAC\text{-}1^+$, intracellular $PAC1^+CXCL4^+$ and $CXCL4^+$ were gated to determine the intracellular abundance of CXCL4 in activated platelets (Figure 3.7 F).

The activation status of platelets was measured based on the percentage of $CD41a^+CD62p^+$ and $CD41a^+PAC\text{-}1^+$ platelets. No trend in terms of the total platelet count ($CD41a^+$) was observed based on severity across time (Table 3.7). However, more activated ($CD62p^+$) platelets were noted in the SAP group of patients across the two AP phases. The median and IQR of $CD41a^+CD62p^+$ SAP cells ranged from 80.4% (80.4%-80.4%) on D3 to 97.6% (96.9%-98.2%) on D15. Intracellular CXCL4 was expressed more in the MAP group, followed by the SAP group and the MSAP group over time (D3-D15), in $CD41a^+CD62p^+$ platelets specifically. The MAP patients had a CXCL4 median and IQR range of 30.4% (17.2%-31.3%) on D3 to 23.4% (16.3-26.9) on D9, with an evident decreasing trend from D3-D9 (Table 3.7). HCs had one of the highest percentages of activated platelets, 95.6% (93.7%-98.1%). However, HCs had a relatively low CXCL4 expression, 6.7% (4.7%-7.9%), compared to AP patients (Table 3.7). It should be noted that, in both HCs and AP patients, platelets had nil PAC-1 expression; therefore, further analysis was not conducted for the $CD41a^+PAC\text{-}1^+$population (Table 3.7).

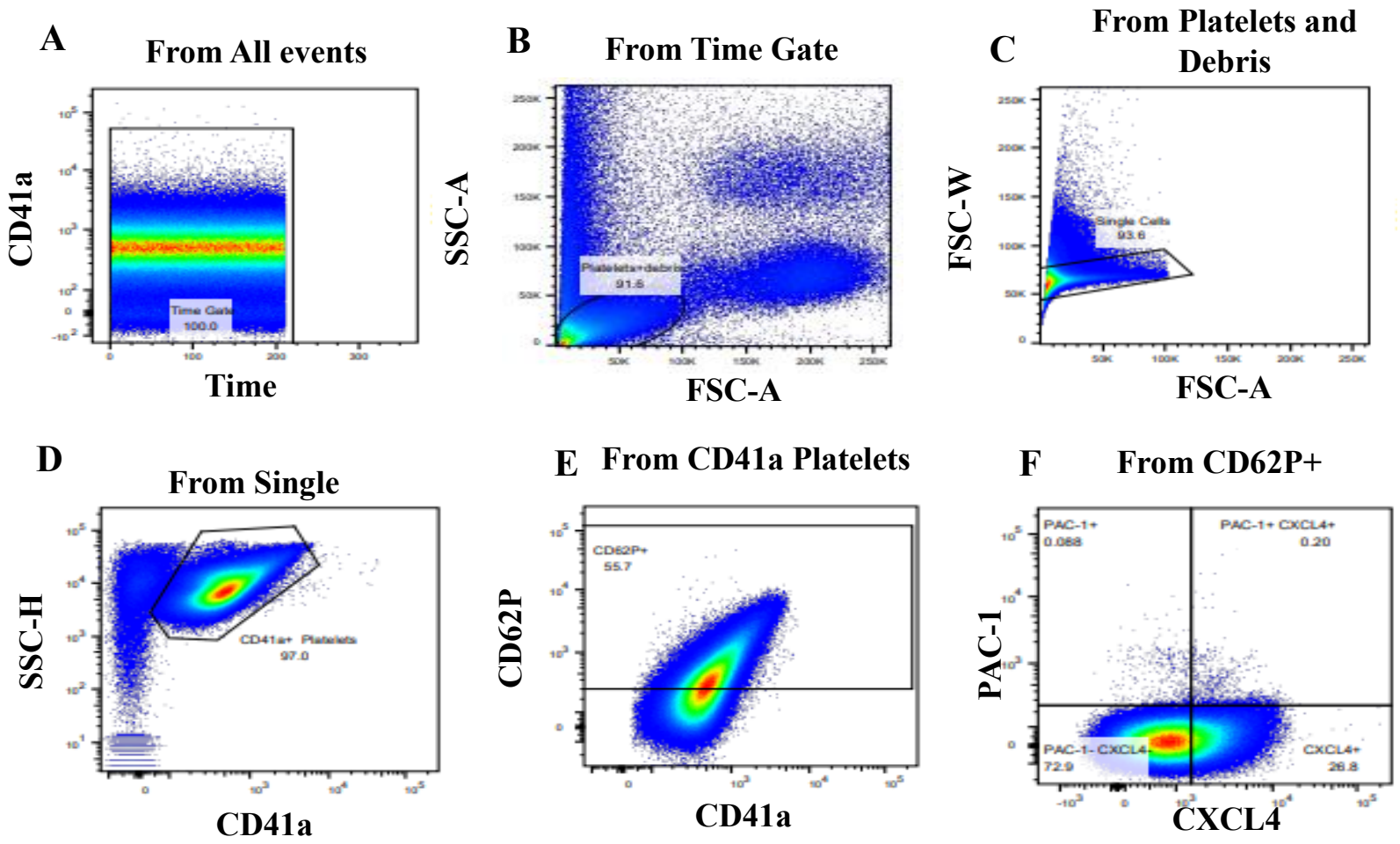


Figure 3.7: Representative scatter plots depicting platelet spread and intracellular CXCL4 gating strategy from whole blood, using a stained D9 MSAP sample (A-F). A: time gate, showing the consistency in the flow of cells during acquisition. B: gating of putative platelets based on forward and side scatter properties. Because platelets are small in size and have less granularity, they often aggregate at the bottom left corner of a scatter plot. C: Singlet gate using forward scatter area and width, excluding aggregated cells, to avoid inaccurate data analysis. D: Platelet population based on $CD41a^+$ cells. E: Activated platelets ($CD62p^+$) from the $CD41a^+$ population. F: Intracellular CXCL4 from activated platelets ($CD62p^+$ and $PAC\text{-}1^+$). **Notes:** CD: cluster of differentiation; CXCL4: chemokine ligand 4

Table 3.7: Total percentage of platelets, their sub-populations, and the intracellular abundance of CXCL4 per AP severity groups and days of pain

		% PLATELETS				
Severity group	**No of patients**			**$CD41^+CD62p^+$**		**$CD41^+CD62p^+CXCL4^+$**
		$CD41a^+$	**CD41a+CD62p+**	**$CXCL4^+$**	**$CD41^+CD62p^+$ PAC-1^+**	**PAC-1^+**
Healthy control	n=6	97.8 (95.7-98.5)	95.6 (93.7-98.1)	6.7 (4.7-7.9)	0.1 (0.0-0.1)	0.1 (0.0-0.1)
DAY 3						
MAP	n=3	97.0 (89.8-97.3)	79.0 (71.8-87.5)	30.4 (17.2-31.3)	0.1 (0.1-0.1)	0.2 (0.2-0.3)
MSAP	n=1	80.1 (80.1-80.1)	78.8 (78.8-78.8)	9.5 (9.5-9.5)	0.1 (0.1-0.1)	0.5 (0.5-0.5)
SAP	n=1	95.0 (95.0-95.0)	80.4 (80.4-80.4)	14.3 (14.3-14.3)	0.1 (0.1-0.1)	0.1 (0.1-0.1)
DAY 5						
MAP	n=4	95.5 (93.5-96.2)	89.3 (76.9-95.1)	25.0 (11.8-35.9)	0.1 (0.1-0.1)	0.2 (0.2-0.3)
MSAP	n=2	90.5 (88.4-92.5)	83.9 (77.1-90.6)	10.8 (7.7-13.8)	0.4 (0.2-0.5)	2.3 (1.3-3.3)
SAP	n=2	92.8 (91.6-93.9)	93.6 (93.4-93.)	9.0 (6.8-11.3)	0.1 (0.1-0.1)	0.1 (0.1-0.1)
DAY 7						
MAP	n=4	96.0 (93.8-96.)	78.7 (76.8-83.7)	24.2 (17.5-27.5)	0.1 (0.1-0.2)	0.3 (0.2-0.5)
MSAP	n=3	94.8 (92.3-95.3)	96.4 (95.9-97.1)	5.0 (4.0-5.4)	0.1 (0.1-0.1)	0.1 (0.1-0.1)
SAP	n=3	89.0 (88.3-91.9)	93.8 (93.4-95.6)	12.2 (7.1-12.3)	0.2 (0.1-0.3)	0.6 (0.3-1.0)
DAY 9						
MAP	n=4	96.5 (94.0-97.1)	58.3 (55.3-69.7)	23.4 (16.3-26.9)	0.2 (0.1-0.4)	0.3 (0.3-1.2)
MSAP	n=4	94.0 (91.4-96.4)	95.3 (94.2-96.8)	5.1 (4.1-5.8)	0.2(0.1-0.3)	0.2 (0.1-0.5)
SAP	n=3	91.4 (77.8-94.2)	90.1(88.6-94.1)	9.9.9(5.7-15.9)	0.2(0.1-0.2)	0.4 (0.2-0.6)

DAY 13						
MAP	n=3	90.9 (46.8-93.7)	95.1(84.8-95.6)	26.3 (15.6-30.0)	0.1 (0.1-0.9)	0.2 (0.2-1.2)
MSAP	n=4	98.1 (96.7-98.7)	95.7 (94.4-96.8)	2.6(1.8-3.4)	0.1 (0.1-0.1)	0.1(0.0-0.1)
SAP	n=3	90.1 (87.2-93.9)	94.1(93.0-96.1)	8.5 (6.0-8.7)	0.2 (0.2-0.4)	0.4 (0.3-0.5)
DAY 15						
MAP	n=2	90.6 (87.8-93.4)	68.6 (59.0-78.1)	27.9 (25.9-29.8)	0.0 (0.0-0.1)	0.2 (0.2-0.2)
MSAP	n=2	98.6 (97.9-99.2)	97.4 (96.9-97.8)	1.6 (1.4-1.9)	0.1 (0.1-0.1)	0.1 (0.0-0.1)
SAP	n=2	97.6 (96.9-98.2)	96.9 (96.6-97.3)	4.5 (3.4-5.6)	0.1 (0.1-0.1)	0.1 (0.1-0.1)

Notes: CD: cluster of differentiation; CXCL4: Chemokine ligand 4; n: number of patients; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.3 Circulating Cytokines and Reactive Oxygen Species as Potential Markers for Acute Pancreatitis Prognostication

Initial events during AP onset occur within the pancreatic acinar cells (Criddle, 2016). Subsequently, acinar cells, in combination with other cells, such as immune cells, act as inflammatory mediators by synthesising and releasing cytokines, chemokines and adhesion molecules (Escobar et al., 2012). Cytokines and chemokines play a crucial role in AP initiation and progression (Do, 2015). This section reports on how circulating IL-6, CXCL4 and ROS together with other immune cells can be useful in stratifying AP severity, monitoring and predicting the outcome of AP patients.

3.3.1 Interleukin-6 expression in plasma from acute pancreatitis (AP) patients

Eleven AP patient samples consisting of 4 MAP, 4 MSAP and 3 SAP were used to detect and quantify IL-6. Visible trends were observed over time and among the three severity groups (Figure 3.8). IL-6 concentrations in the SAP group appeared to be increasing with time from the early AP (D3-D7) to the late AP phase (D9-D15), while in MSAP and MAP patients, IL-6 concentrations generally decreased over this period (Figure 3.8). The HC IL-6 concentration, taken at one time point, was generally lower than the patient levels (Figure 3.8). A comparison of IL-6 concentrations within the three severity groups and HCs (n=5) showed increasing IL-6 levels with increasing AP severity (SAP>MSAP>MAP), especially in the late phase (Figure 3.8). In SAP patients, the median and IQR peaked on D9 at 712.5 pg/mL (195.9 pg/mL-829.6 pg/mL) of the late phase. In MSAP patients, it peaked at 655.6 pg/mL (319.0 pg/mL-992.1 pg/mL) on D5 of the early phase. Overall, IL-6 concentrations in MAP patients and the control group were lower (Figure 3.8). There were no statistically significant differences across severities and over time ($p>0.05$).

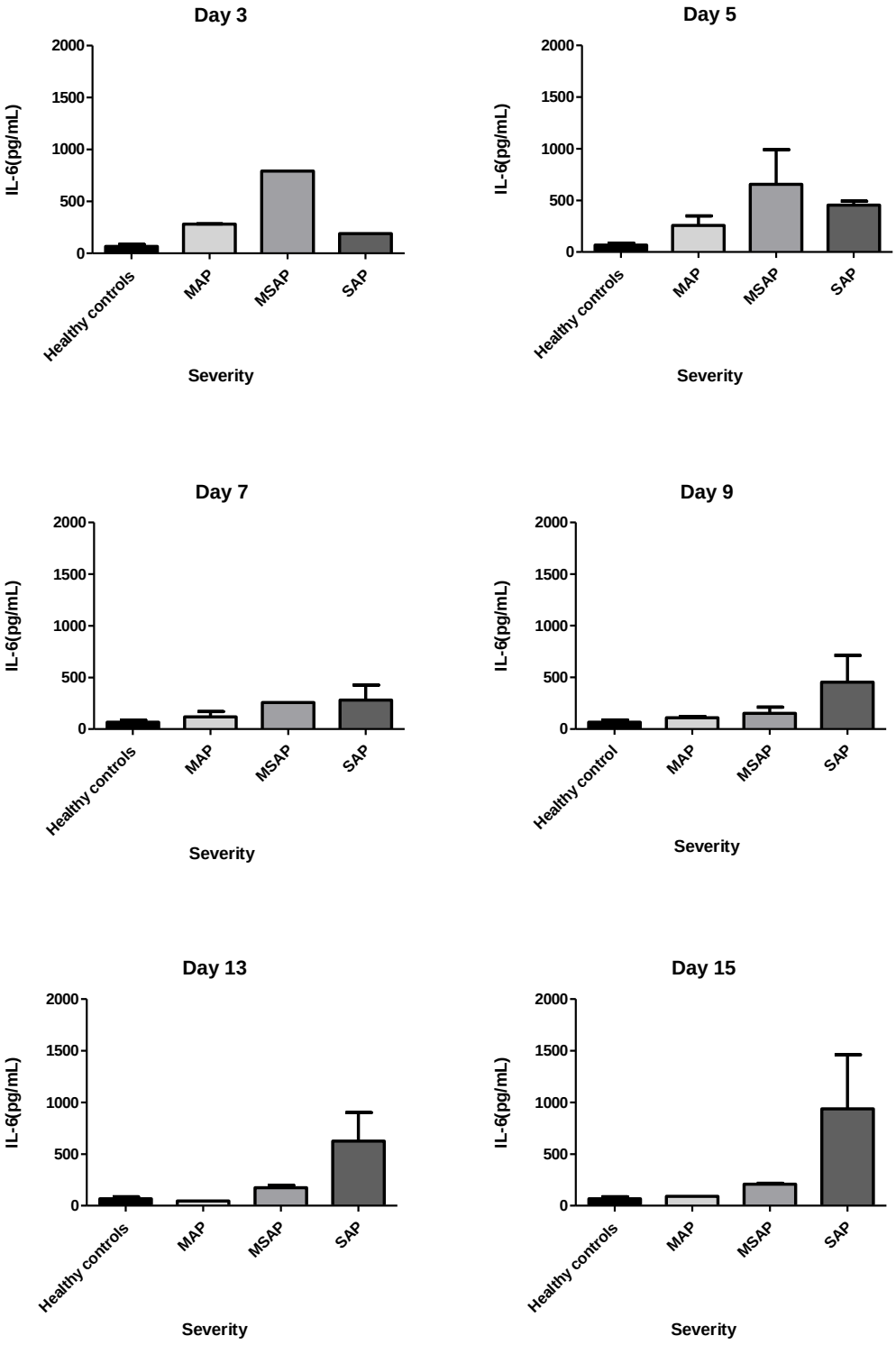


Figure 3.8: Plasma IL-6 expression using the RAB0306 Human IL-6 ELISA kit. The data shows the pattern of IL-6 expression over time from D3-D15 across the three severity groups and HCs. IL-6 concentrations in the SAP group appeared to be increasing with time from D3-D15; however, in the MSAP and MAP groups, IL-6 decreased over time. IL-6 was expressed more in the late phase of the SAP group than in MSAP, MAP and HCs. **Notes:** IL: interleukin; HC: healthy control; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n=4 MAP, 4 MSAP, 3 SAP and 5 HC.

3.3.1.1 Association of NKs and IL-6 using Spearman correlation matrix: In an attempt to measure the strength and direction of the association between NK cells, intracellular and plasma IL-6, as guided by the study objectives and previous work done in the research group, Spearman's rank test was used to calculate the correlation coefficient (rho). Findings of the Spearman correlations for NKs, circulating and intracellular IL-6 are presented in Table 3.8, and the interpretation for the Spearman's correlation rankings are in Appendix G. Based on the findings, weak positive correlations were found between:

(i) Natural killer cells ($CD56^+CD16^+$) and MFI of IL-6 (p=0.019)

(ii) IL-6 NKs and MFI of IL-6 (p=0.015)

(iii) IL-6 NKs and MFI of HLA-DR^+ NKs (p=0.013)

(iv) Days of pain and NKs ($CD56^+CD16^+$) (p=0.070)

Table 3.8: Spearman correlation matrix for NKs, circulating and intracellular IL-6

	Days	**IL-6 (pg/mL)**	**% $CD3^-$ $CD56^+CD16^+$ NKs**	**% $CD56^+CD16^+$ $CD57^+$NKs**	**% IL-6^+ NKs**	**MFI IL-6**	**% HLA-DR^+ NKs**	**MFI HLA-DR^+ NKs**
Days	--							
IL-6 (pg/mL)	-0.066	--						
% $CD3^-$ $CD56^+CD16^+$ NKs	**0.258**	0.026	--					
% $CD56^+CD16^+$ $CD57^+$NKs	-0.161	-0.265	0.097	--				
% IL-6^+ NKs	-0.116	-0.036	0.000	-0.181	--			
MFI IL-6	0.033	0.190	**0.331***	-0.066	**0.342***	--		
% HLA-DR^+ NKs	0.222	0.171	-0.214	-0.106	-0.014	0.065	--	
MFI HLA-DR^+ NKs	0.089	-0.115	-0.034	-.453**	**0.349***	0.008	-0.067	--

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

3.3.1.2 Association of NKTs and IL-6 using Spearman correlation matrix: In an attempt to measure the strength and direction of the association between NKTs cells, intracellular and plasma IL-6, Spearman's rank test was used to calculate the correlation coefficient (rho). Findings of the Spearman correlations for NKTs, circulating and intracellular IL-6 are presented in Table 3.9, and the interpretation for the Spearman's correlation rankings are in Appendix G. Based on the findings, weak positive correlations were found between

i. $CD3^+CD56^+CD57^+$ NKT-Lymphocytes and MFI IL-6 NKTs (p=0.137)
ii. IL-6 NKs and MFI HLA-DR^+ (p=0.042)
iii. HLA-DR^+ NKT-Lymphocytes and MFI HLA-DR^+ NKT-Lymphocytes (p=0.006)
iv. Days of pain and $CD3^+CD56^+$ NKT-Lymphocytes (p=0.014)
v. Days of pain and HLA-DR^+ NKT-Lymphocytes (p=0.013)

Table 3.9: Spearman correlation matrix for NKTs, circulating and intracellular IL-6

	Days	**IL-6 (pg/mL)**	**% CD3+ CD56+ NKTs**	**% $CD3^+CD56^+$ $CD57^+$ NKTs**	**% IL-6^+ NKTs**	**MFI IL-6^+ NKTs**	**% HLA-DR^+ NKTs**	**MFI HLA-DR^+ NKTs**
Days	--							
IL-6 (pg/mL)	-0.082	--						
% $CD3^+CD56^+$ NKTs	**0.346***	-.334*	--					
% $CD3^+CD56^+CD57^+$ NKTs	-0.150	-0.166	0.135	--				
% IL-6^+ NKTs	-0.122	-0.053	-0.093	-.403**	--			
MFI IL-6^+ NKTs	-0.111	0.177	-0.161	**0.237**	0.016	--		
% HLA-DR^+ NKTs	**0.350***	-0.003	0.030	-0.237	-0.097	-0.096	--	
MFI HLA-DR^+ NKTs	0.008	0.078	-0.063	-.442**	**0.289***	-0.087	**0.386****	--

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

3.3.2 Circulating chemokine ligand (CXCL4) expression in AP patients

Among the three severity groups, visible trends were observed overtime of the 11 (4 MAP, 4 MSAP and 3 SAP) patients tested for circulating CXCL4 (Figure 3.9). The CXCL4 levels were higher in the MAP and MSAP groups than in the SAP group in the early phase. However, as the patients progressed to the late phase (from D9), CXCL4 expression increased in the SAP group and gradually decreased in the MSAP and MAP groups (Figures 3.9 and 3.10). Similar to IL-6, CXCL4 remained the highest expressed from D9 in the SAP, with a median and IQR of 1395.1ng/mL (520.5ng/mL -1444ng/mL) compared to MSAP and MAP groups that had medians of 883.6ng/mL (708.6ng/mL-1466ng/mL) and 1161ng/mL (757.7ng/mL-1382ng/mL), respectively (Figure 3.10). Overall, the trend between CXCL4 expression, the three AP severity groups and HCs was that, in the early phase, MSAP and MAP had higher CXCL4 expression than the SAP group, and in the late phase, this was inverse. Healthy controls generally had the least CXCL4 expression compared to the AP patients (Figures 3.9 and 3.10). There was no significant difference among the AP patients and HCs $p>0.05$.

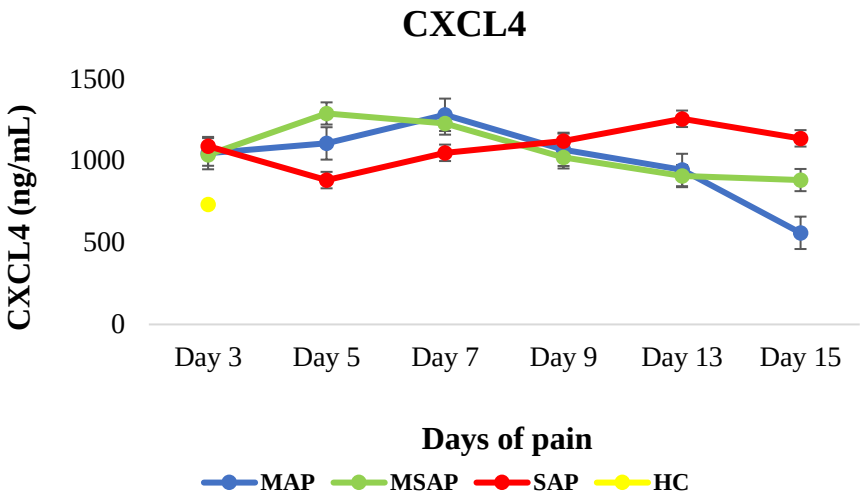


Figure 3.9: Chemokine ligand 4 analysis using the DPF40 Human CXCL4/PF4 Quantikine ELISA kit. The graph above shows the pattern of IL-6 expression over time (D3-D15) across the three severity groups and HCs. CXCL4 expression was higher in the MAP and MSAP groups than in the SAP group in the early phase, specifically D5 and D7. However, in the late phase, SAP CXCL4 was the highest and levels peaked on D13, which was more than that of the MAP and MSAP group of patients. **Notes:** CXCL4: Chemokine ligand 4; HC: healthy control; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n=4 MAP, 4 MSAP, 3 SAP and 5 HCs.

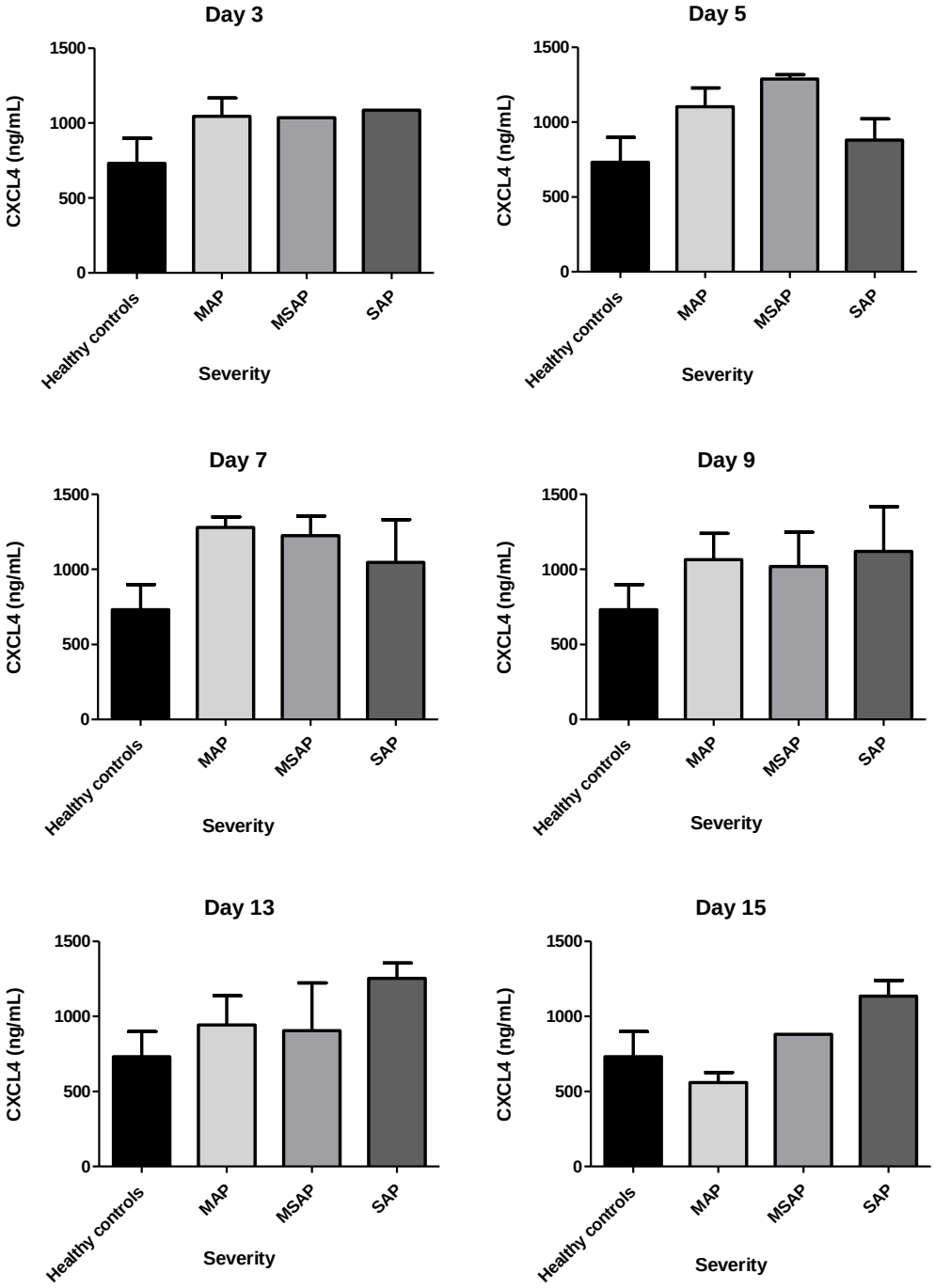
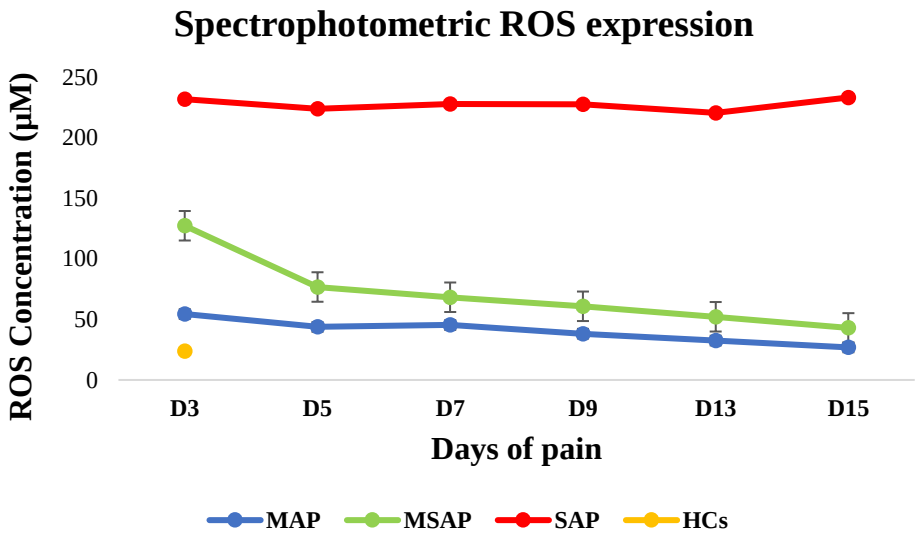


Figure 3.10: CXCL4 analysis using the DPF40 Human CXCL4/PF4 Quantikine ELISA kit, comparing CXCL4 expression across the three AP severity groups and HCs. Data in the above graphs represent the mean and standard deviations. No trend was observed in the early to late phase of the MAP and MSAP group. However, CXCL4 peaked on D13 of the late phase in the SAP patient group. There was no significant difference between the three AP severity groups and HCs ($p>0.05$). **Notes:** AP: acute pancreatitis; CXCL4: Chemokine ligand 4 HCs: healthy controls; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n=4 MAP, 4 MSAP, 3 SAP and 5 HCs.

3.3.3 The expression of ROS in acute pancreatitis (AP) patients

Based on the assessment of the 11 (4 MAP, 4 MSAP and 3 SAP) patients, visible trends were observed over time and among the three severity groups (Figures 3.11, 3.12 and 3.13). ROS was consistently high in the SAP group from the early to late AP phase, specifically D3 to D15, when ROS was measured in serum. In plasma, ROS expression was high in the SAP group on D5 to D13 compared to the HCs and the MSAP and MAP patient groups (Figures 3.11, 3.12 and 3.13). HCs, MSAP and MAP ROS levels were generally lower from the early to the late phase compared to the SAP patient group (Figure 3.11). Across severities, SAP patients expressed the highest level of ROS compared to MSAP, MAP and HCs (Figures 3.12 and 3.13). Overall, no statistically significant difference was observed over time for the individual groups ($p>0.05$) (Figure 3.11). However, across severities, between SAP patients and HCs, statistically significant differences were observed on D5 and D7 in serum samples ($p=0.0120$ and $p=0.0045$, respectively). Furthermore, on D7 in plasma samples, statistically significant differences were observed between SAP patients and HCs ($p=0.0066$) (Figure 3.12). In the late AP phase, statistically significant differences were observed from serum samples between SAP and HCs on D9 and D13, ($p=0.0050$) and ($p=0.0071$), respectively. In plasma samples, a statistically significant difference was found between MSAP patients and HCs ($p=0.0049$) (Figure 3.13).

A



B

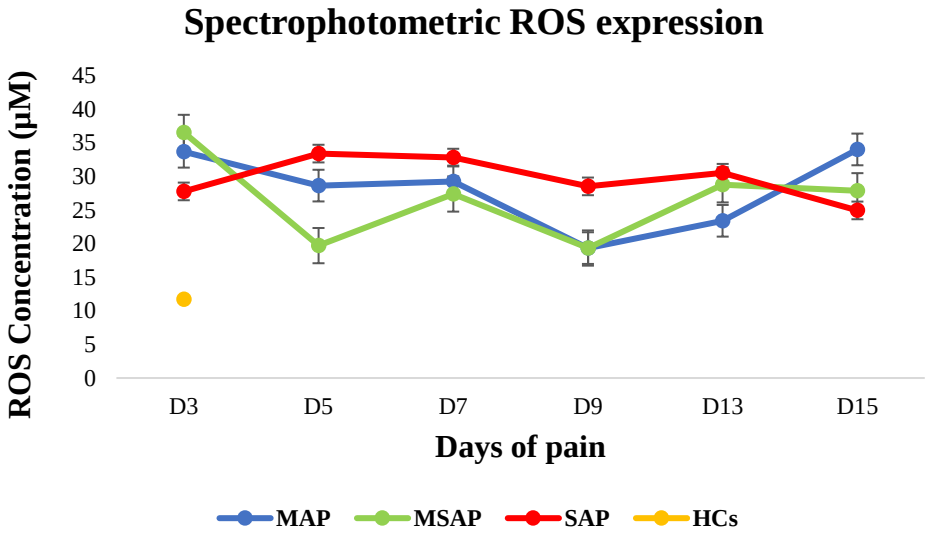


Figure 3.11: Line graphs showing the pattern of spectrophotometric ROS expression in serum (A) and plasma (B) among the three severity groups and HCs over time from the early to the late phase of AP. No statistically significant difference was observed among these AP risk groups over time. Overall, in A, the SAP group of patients had the highest ROS expression from D3-D15. In B, SAP patients had the highest expression from D5-D13. **Notes**: AP: acute pancreatitis; HC: healthy control; ROS: reactive oxidative species; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n=4 MAP, 4 MSAP, 3 SAP and 5 HCs

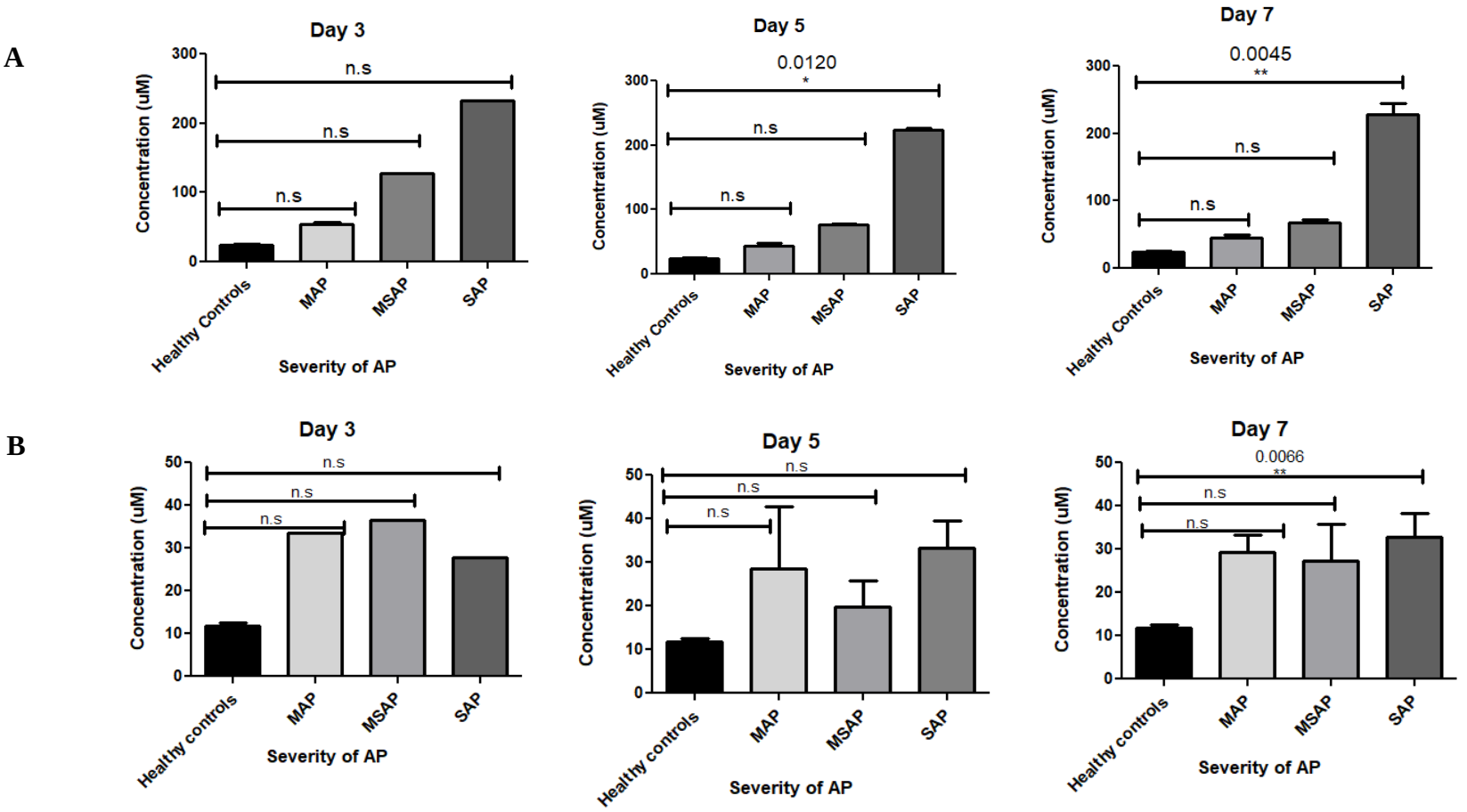


Figure 3.12: Bar graphs showing the expression levels of ROS in the early phase (D3-D7) from serum (A) and plasma (B) of AP patients. The data presented shows the mean and standard deviation. Statistically significant differences between SAP patients and HCs were observed on D5 (A) and D7 (A and B), A. (p=0.0120), (p=0.0045) and B (p=0.0066), respectively. Overall, SAP patients in A had the highest expression of ROS in comparison to MSAP, MAP and HCs. In B, SAP had the highest expression from D3-D5. **Notes**: AP: Acute Pancreatitis; ROS: reactive oxidative species; HCs: healthy controls; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n=4 MAP, 4 MSAP, 3 SAP and 5 HCs

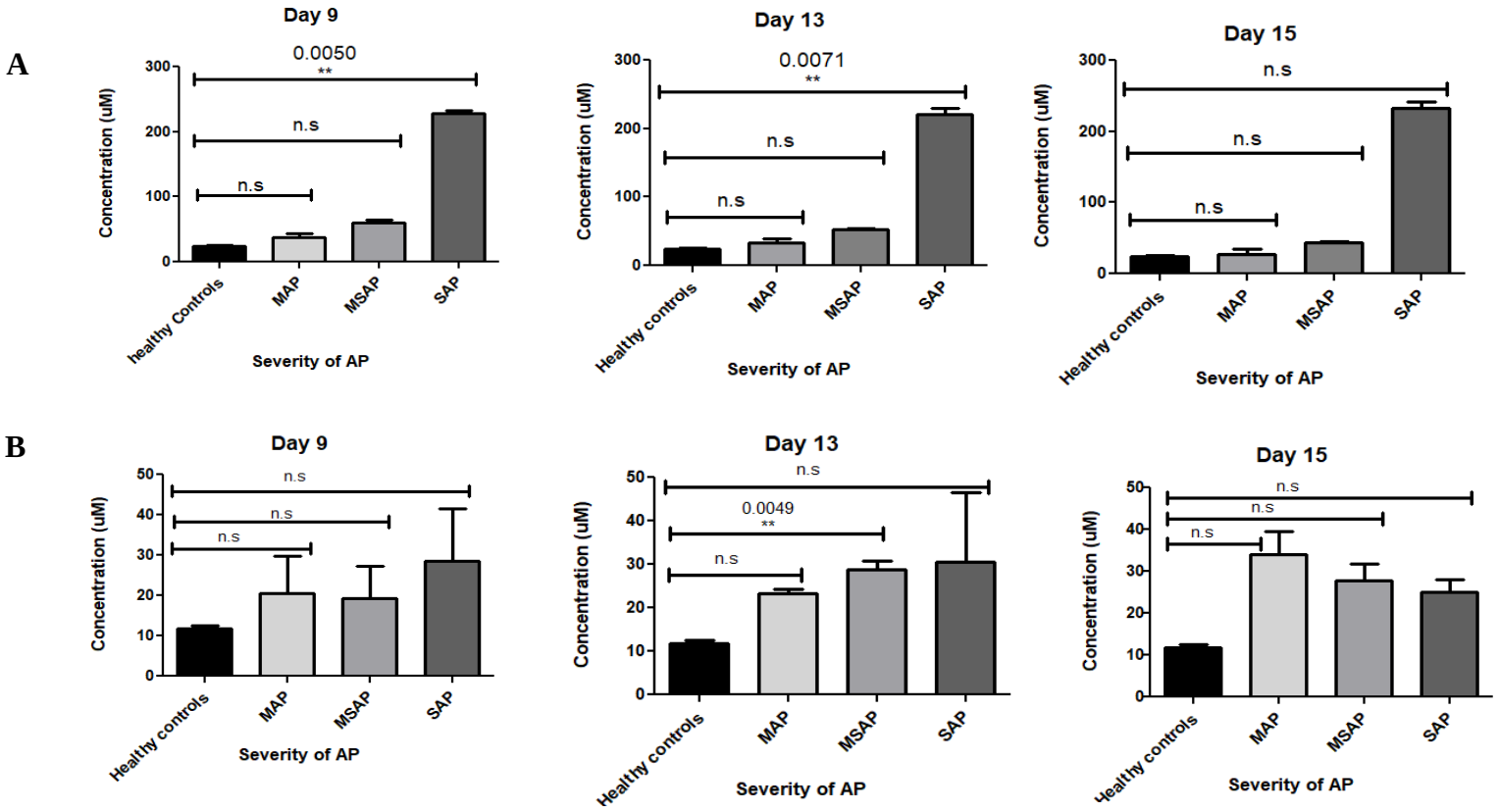


Figure 3.13: Bar graphs showing the expression levels of ROS in the late phase (D9-D15) from serum (A) and plasma (B) of AP patients. The data presented shows the mean and standard deviation. Severe patients in A had the highest ROS expression from D9-D15. In B, SAP patients had the highest ROS expression on D9 and D13. Statistically significant differences were observed on D9 (A) and D13 (A and B), between SAP patients and HCs A (p=0.0050), (p=0.0071) and between MSAP patients and HCs B (p=0.0049), respectively. **Notes:** AP: Acute Pancreatitis; ROS: reactive oxidative species; HCs: healthy controls; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n=4 MAP, 4 MSAP, 3 SAP and 5 HCs

3.3.4 Association of overall experimental data

A Spearman correlation test was used to determine whether a linear relationship exists between leukocytes, platelets and cytokines (IL-6 and CXCL4) in an attempt to understand the mechanisms of leukocytes and platelet activation and cytokine and chemokine secretion during the course of AP. Findings of the Spearman's correlations for all the experimental data are presented in Table 3.10, and the interpretation of Spearman's correlation rankings are in Appendix G. Based on the findings, weak positive correlations were found between

(i) Plasma IL-6 and plasma ROS ($p=0.094$)

(ii) MFI NKs and percentage of intracellular IL-6^+ in NKs ($p=0.058$)

(iii) MFI IL-6^+ NKT Lymphocytes and plasma IL-6 ($p=0.127$))

(iv) MFI IL-6^+ NKT Lymphocytes and circulating CXCL4 ($p=0.031$)

(v) MFI IL-6^+ NKT Lymphocytes and percentage of intracellular CXCL4 ($p=0.012$)

Table 3.10: Spearman correlation matrix for all the experimental data

			IL-6 (pg/mL)	CXCL4 (ng/mL)	ROS (µM)	% IL-6$^+$ NKs	MFI IL-6	% IL-6+ NKT-Lymphocytes	MFI IL-6$^+$ NKT-Lymphocytes	%CXCL4
Spearman's rho	**IL-6 (pg/mL)**	Rho	--							
		Sig. (2-tailed)								
	CXCL4 (ng/mL)	Rho	**0.256**	--						
		Sig. (2-tailed)	0.059							
	ROS (µM)	Rho	**0.228**	0.083	--					
		Sig. (2-tailed)	0.094	0.545						
	% IL-6$^+$ NKs	Rho	-0.031	0.006	0.042	--				
		Sig. (2-tailed)	0.824	0.963	0.761					
	MFI IL-6+ NKs	Rho	0.127	0.025	0.054	**0.258**	--			
		Sig. (2-tailed)	0.355	0.855	0.694	0.058				
	% IL-6$^+$ NKT-Lymphocytes	Rho	0.197	0.160	0.124	0.090	-0.075	--		
		Sig. (2-tailed)	0.149	0.244	0.368	0.513	0.584			
	MFI IL-6$^+$ NKT-Lymphocytes	Rho	**0.252**	**0.291***	0.067	-0.024	-0.003	-0.106	--	
		Sig. (2-tailed)	0.063	**0.031**	0.629	0.863	0.981	0.443		
	%CXCL4	Rho	0.128	-0.160	-0.015	0.159	0.068	**0.338***	0.116	--
		Sig. (2-tailed)	0.350	0.245	0.915	0.246	0.624	**0.012**	0.400	

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

CHAPTER FOUR: DISCUSSION

Clinical and demographic data of 24 AP patients across the two AP phases was explored in this pilot study. The role of leukocytes and platelets associated with the production of intracellular and circulating IL-6 and CXCL4 and the levels of ROS as a measure of oxidative stress in AP was explored in 12/24 AP patients (5 MAP, 4 MSAP and 3 SAP). In 2017, Anderson and Thomson reported an international AP incidence rate of 80 per 100 000 persons. AP is an NCD still poorly understood in African settings (Hofman, 2014; Hlafa, Sibanda and Hompashe, 2019). Therefore, it is necessary to investigate and understand its prevalence in various populations to improve healthcare system management guidelines. On a global scale, NCDs are now deemed a health sector challenge with worst-case scenarios experienced in low- and middle-income countries. According to the WHO (2020), NCDs, such as diabetes, heart diseases and cancer, account for 70% of deaths worldwide. According to Hofman (2014), it has been predicted that, by 2030, low- and middle-income countries will be prone to have a five-fold increase in deaths due to NCDs. Like most sub-Saharan African countries, the South African economy is burdened by the increasing incidence of NCDs and prevalent communicable diseases such as HIV/AIDS (Hofman, 2014; Mudie et al., 2019).

4.1 Demographics, Clinical characteristics and Outcomes

Over the past 20 years, the prevalence and hospitalisation rate associated with AP has been increasing, especially in the western world (Iannuzzi et al., 2022). In the United States, the estimated cost per person for hospitalisation is over $30 000 (Petrov and Yadav, 2019). In most studies, the prevalence of AP is reported based on the western world because there is still scanty epidemiological research from African, Asian and Latin American populations (Iannuzzi et al., 2022). In South Africa, studies have reported that the prevalence of AP is within international estimates, which is about 80 in 100 000 (Anderson et al., 2008; Nesvaderani et al., 2015). Epidemiological studies have reported that the risk of AP is high in adults between the ages of 35 and 44 years, with more males than females affected (Alam et al., 2021). However, it should be noted that both age and gender profiles vary in different studies due to differences in study settings, objectives and inclusion criteria. Furthermore, the incidence of the different aetiologies also varies across regions. Alcohol as a cause is more common in men, and gallstones have a slightly higher prevalence in females (Drake et al., 2021). In the current

study, the recruitment rate was inconsistent due to unforeseen circumstances, where sampling had to be suspended on and off due to COVID-19 restrictions in South Africa. However, demographic and clinical data were recorded from the 24 AP patients sampled from December 2020 to January 2022. Demographic data, such as age, is considered an AP risk factor (Márta et al., 2019; Roberts et al., 2017) and, in the present study, the median age for AP patients was 37 years, which is within the age range described by Alam et al., (2021). However, the proportion of males was less than that of females, probably due to the influence of the most common aetiology (gallstones, 58%) and risk factors (obesity, BMI>30 kg/m^2) which are common in females. In the AP cohort in this study, HIV was the most common comorbidity. These results are not unexpected, given the high prevalence of HIV in South Africa, with approximately 7.2 million reported HIV seropositive cases in 2019 (Satoh and Boyers, 2019). About 21% (n=5) of the sampled AP patients in the current study were HIV positive. Three of the five were newly diagnosed, and the other two were already on (ARVs) treatment. These results were in line with previous findings highlighting how ARVs and alcohol abuse are major AP risk factors in South Africa (Anderson and Thomson, 2017). Another comorbidity in the studied cohort of AP patients was hypertension. It is noteworthy that AP risk factors and aetiologies are highly dependent on the lifestyle patterns of a given population (Nesvaderani et al., 2015; Pang et al., 2018). Globally, gallstones are the most common aetiology associated with AP, with a two-fold occurrence compared to alcohol-associated AP (Bálint et al., 2020). However, discrepancies in reporting prevalent AP aetiologies still occur in studies due to differences in demographics and lifestyle patterns in various geographical locations (Matta et al., 2020).

According to a 2010 retrospective evaluation study by Chamisa , Mokoena, and Luvhengo, at Kalafong Hospital, Pretoria, South Africa, from 1988-2007, alcohol abuse was the most common risk factor and aetiology associated with AP. Furthermore, Anderson and colleagues (2008) reported similar findings based on a study conducted from 2001 to 2006 at Addington Hospital in Durban, South Africa. Although alcohol has been reported as the predominant aetiology in these two South African studies, there was higher biliary-associated AP (n=14, 58%) in the current study than alcohol-associated AP (n=9, 38%). These findings agreed with those reported by Thomson, Brand, and Fonteh, (2018), where biliary-associated AP was predominant over alcohol and ERCP-associated AP. Furthermore, in a study conducted from August 2018 to September 2019 at CHBAH and Charlotte Maxeke Johannesburg Academic

Hospital in South Africa, Nalisa et al (2021) reported a 1:1 ratio of alcohol- and biliary-associated AP. Biliary-associated AP is more prevalent in females than males, which could be why we report more biliary-associated cases since our study cohort comprised mainly females (n=14, 58%). Notably, all biliary-associated AP patients (n=14/24, 58%) comprising 12 females and two males in the current study had a BMI>30 kg/m^2, considered obese. These results were in accordance with what Khatua and colleagues (2017) observed, that most biliary-associated AP patients fall into the obese category. This is because in obese people, stones may form in the gall bladder, serving as an AP risk factor (Khatua , El-Kurdi, and Singh, 2017).

The LOS of patients with AP directly affects the costs and resources in hospital settings, and it has been established that LOS correlates with AP severity (Bollen, 2016; Singh et al., 2017). According to Wu and Banks (2013) and Singh et al. (2017), the mean LOS in the USA for AP patients ranges between 5-8 days, regardless of disease severity. The average LOS of the sampled AP patients in the current study was 5.7 (range 2-18 days) in MAP and 14 (range 13-15 days) in MSAP. In the SAP group, 2/3 patients died during their hospital stay, and LOS data for both patients were unavailable; however, 1/3 patients who recovered had a LOS of 16 days. From the studied patients, it can be suggested that comorbidities influence the LOS, because most MAP patients that were still hsopitalised during the second week of AP onset had comorbidities although they had no OF. These results agreed with the findings by Bateman (2018), where an average hospital stay of 10.3 days (range 6-19 days) was reported for MAP cases and 21.4 days (range 12-42 days) in SAP cases. Additionally, Banday et al. (2015) reported a mean LOS of 1.5, 6.9 and 14.2 in MAP, MSAP and SAP patients, respectively. In contrast, a South African study by Thomson, Brand, and Fonteh, (2018) reported a mean LOS for MAP/MSAP and SAP groups of 12 and 22 days, respectively, where the SAP LOS was more compared to what was observed in the current study.

In the current study, the LOS was higher in SAP patients than in MSAP and MAP, due to elevated inflammatory mediators and subsequent tissue damage in SAP patients warranting many more clinical interventions for management, which takes time and requires specialist care (Bollen, 2016; Chanda, 2017). Although the SAP patients had longer LOS in this study, there was no significant difference between LOS and severity, probably due to the small sample size. Clinical interventions are implemented to halt complications, longer LOS and AP-associated mortality. Based on RAC guidelines, MAP patients are not expected to experience

local complications or any form of OF (Banks et al., 2013). Similarly, in the studied cohort of patients, only MSAP and SAP patients had transient and persistent OF, respectively. Furthermore, 50% (2/4) MSAP patients had transient OF in the late phase of AP, and 67% (2/3) SAP patients had persistent OF in the late phase too. Regarding the two MSAP patients who experienced transient OF, one was diagnosed with COVID-19 during the second week of hospitalisation, which may have contributed to transient OF. The other MSAP patient presented late to the hospital, on D7 post onset of AP. The patient had not received any early clinical interventions following AP onset, which may have contributed to transient OF in the late AP phase. According to Garg and Singh (2019), late-phase OF is attributed to infected pancreatic necrosis (IPN) induced sepsis. Although MAP patients did not experience any OF, surgical interventions were common in this patient group. Overall, 42% of patients (10/24) had surgical interventions, with the MAP group accounting for most of the procedures; 78% of MAP patients had a laparoscopic cholecystectomy (LC), and 50% had ERCP. Laparoscopic cholecystectomy aims to prevent future AP attacks without altering the course of the index attack (Jee et al., 2018; Räty et al., 2015). The ERCP has a very limited role in treating biliary AP. Endoscopic Retrograde Cholangiopancreatography is an essential component of treatment in patients who have cholangitis; however, if patients do not have cholangitis, it has little role in the actual treatment of AP (Lee et al., 2018).

The AP severity has a major influence on mortality, hence the need to assess the rate of in-hospital mortality. Banks et al. (2013) reported a direct link between severe forms of AP and in-hospital mortality. We report an 8% (2/24) mortality in the current study cohort, which is consistant with the international rate of 10% (Yadav and Lowenfels, 2006; Anderson and Thomson, 2017). Furthermore, the reported study findings were also higher than similar AP studies done in South Africa, which reported a 5.7% mortality (Anderson and Thomson, 2017; Yadav and Lowenfels, 2006). In a study by Mossad et al. (2017), a link between mortality and hospital size was reported, stating that larger hospitals have the highest mortality rate and vice versa. However, this is biased because more severe cases are referred to tertiary hospitals, and the mortality rate is, therefore, skewed. CHBAH is a tertiary referral hospital and the third-largest hospital in the world. In light of this, the reported deaths could be attributed to the type of hospital setting, among other factors. Other factors that could have contributed to a high mortality rate in the current study could be patient delays in seeking specialist care and the presence of comorbidities.

In the current study, one SAP patient died during the second week after onset (late phase of AP), and the other died two weeks after disease onset. The variation in mortality time could be associated with the MSOF, local complication variations, or both since both patients had comorbidities, MSOF and experienced ICU admission. Only these two SAP patients (8%) were admitted to ICU out of the 24 sampled patients. Furthermore, both patients had MSOF consisting of respiratory, cardiovascular and renal dysfunction.

4.2 The Link Between Immune Cells, Intracellular and Circulating Cytokines and Chemokines in acute pancreatitis (AP) Patients

Injured pancreatic acinar cells trigger the secretion of cytokines and chemokines, promoting inflammation (Szatmary and Gukovsky, 2016; Thomson, Brand and Fonteh, 2018). Secreted cytokines promote leukocyte recruitment to the site of injury, resulting in immune dysregulation and inflammation exacerbation (Nieminen et al., 2014). Characterisation of immune cell populations and subpopulations and their association with intracellular IL-6 in whole blood showed that monocytes were the most expressed leukocytes over time (D3-D15), specifically, classical monocytes ($CD14^{+}CD16^{-}$) when compared to other leukocytes such as lymphocytes. Hence the need to focus on finding ways to better stratify and prognosticate AP using dysregulated immune cells potentially associated with secretion and elevation of IL-6 and other chemokines in AP, to improve clinical interventions and patient outcomes.

Classical monocytes were expressed more in the MSAP and SAP group of patients in the early phase, specifically on D3 and D5, than in the MAP and HC groups. These results were consistent with Nalisa et al. (2021), who reported an increase in monocytes rather than NK cell subsets in the early AP phase. Additionally, in a study by Zhang et al. (2019), classical monocytes were found to be expressed more in the SAP group of patients than in the MSAP and MAP groups. Upregulated expression of monocytes in severe forms of AP is expected since the type of pancreatic acinar cell death in this group of patients is necrotic and leads to the secretion of proinflammatory molecules, which subsequently attract M1 macrophages (Hu et al., 2020). However, in the late phase, specifically D9 and D15, the MAP group had the highest expression of classical monocytes. The reason could be that the MAP patients who were still hospitalised during the second-week post onset of epigastric pain were experiencing complications related to AP or even exacerbation of comorbidities. For instance, increased intrapancreatic pressure during ERCP may damage the pancreas resulting in the upregulation

of immune cells (Pallagi et al., 2020). Additionally, 2/5 MAP patients who were still hospitalised during the second week of AP onset were HIV-positive. In AP, HIV infects and reproduces in macrophages located in liver hepatocytes which indirectly increases the expression of monocytes that promote pancreatic inflammation (New-Aaron et al., 2021).

Although classical monocytes were the most expressed leukocytes, intracellular IL-6 expression was the least in these cells. These findings might suggest that the cells may have secreted most of the intracellularly abundant IL-6 into circulation, hence the observation of elevated IL-6 concentrations in severe patients from the plasma data. This inference is supported by similar findings from Zhang et al. (2019), where classical monocytes were elevated in SAP patients based on immunophenotyping results. Furthermore, in the same study secreted myeloid-associated cytokines in circulation, such as IL-6, was also upregulated in SAP patients in the early phase based on results from the Luminex assay. Thereafter, results from both assays were correlated.

Albeit NK cell subsets were among the least expressed leukocytes across AP patients in the present study, they expressed IL-6 intracellularly across the three AP severity groups. The marked activation of NKs ($CD16^{+}CD56^{+}CD57^{+}$) and NKTs ($CD3^{+}CD16^{+}CD56^{+}CD57^{+}$) was linked with intracellular IL-6 expression, with NK cells expressing more intracellular IL-6 in MAP and MSAP patients and NKTs expressing intracellular IL-6 in MAP and SAP patients. IL-6 from the NK subpopulation ($CD16^{+}CD56^{+}CD57^{+}$) was the highest on D9 of the MAP and MSAP group than HC and the SAP group of patients. In a study by Shifrin et al. (2005), it was reported that NK cells infiltrate the inflamed pancreas from the second day post epigastric pain, peak on day four, and persist for 28 days. Similarly, IL-6 from the NKTs subpopulation was expressed more on D9 of the MAP and SAP group than in HC and MSAP patients. It is worthy to note that sometimes the SIRS and CARS phases of AP may develop simultaneously due to the complexity of various cytokines and molecules regulating the inflammatory process (Kostic et al., 2015). Hence the inconsistent results in terms of the immune cells and intracellular abundance of IL-6 across severity over the two AP phases are not impossible. The expression of intracellular IL-6 in SAP patients on D9 was consistent with what was observed in plasma results, where IL-6 peaked on D9. These findings suggest that, if the SIRS phase and pro-inflammatory cytokines dominate the immune response, patients will develop necrotising AP with a potentially lethal outcome (Kostic et al., 2015).

Increased NKs and NKTs may also be linked with circulating plasma IL-6 based on the NK, and NKTs IL-6 MFI generated from the ICS assay. There was a weak statistically significant ($p=0.019$) positive correlation (0.331) between NKs and IL-6 MFI from NK cells. Furthermore, a weak positive correlation (0.237) that was not statistically significant was observed between NKTs and IL-6 MFI from NKT cells. Intracellular IL-6 MFI was high for both NK and NKTs in SAP patients, which corresponded with increased plasma IL-6 expression in SAP patients. Therefore, it is plausible that NKs and NKTs could be secreting IL-6 into circulation (plasma) rather than retaining IL-6 intracellularly. According to Wei et al. (2019), lymphocytes serve as vital immunoregulatory cells that can secrete cytokines directly or indirectly to regulate the immune response. Although the increase of IL-6 with severity is not novel, an association between IL-6 and lymphocytes in the severe groups of AP patients was established. These observations agree with those reported by Dambrauskas et al. (2010), Choy and Rose-John, (2017) and Nalisa et al. (2021) that the elevation of IL-6 in the severe AP groups is associated with activated cytokine-producing cell subsets such as NKs and monocytes.

Over the years, studies have shown how elevated IL-6 is an excellent predictor of SAP IL-6 (Rao and Kunte, 2017). Plasma results showed increasing IL-6 levels with increasing AP severity (SAP>MSAP>MAP) in the late AP phase. Furthermore, IL-6 peaked on D9 in the SAP group, and an increasing trend compared to HC, MAP and MSAP was maintained until D15. Overall, plasma IL-6 concentrations in MAP patients and the HC group were lower than in the MSAP and SAP patients. However, there was no significant difference across severity and time ($p>0.05$). This could be attributed to the small sample size. MSAP patients (2/4) had complications in the early phase, which could explain the unusual, elevated IL-6 expression in the studied population. Furthermore, as they progressed to the late phase, all the MSAP patients recovered which could be attributed to clinical interventions they received in the early phase or a reversal of organ failure. C-reactive protein was also upregulated in SAP patients over time, with a significant difference ($p=0.019$) reported between CRP and the SAP group of patients. These results were consistent with those of Thomson and colleagues (2018), where IL-6 concentrations and CRP levels were higher in the SAP group in the late AP phase compared to the MAP and MSAP patient groups. In the same study, IL-6 peaked on D9, and CRP was significantly higher in the late phase (Thomson, Brand and Fonteh, 2018). IL-6 induces the synthesis of acute phase proteins such as CRP and procalcitonin in the liver (Kylänpää, Rakonczy and O'reilly, 2012), which explains why CRP levels would increase with

elevated IL-6 expression. According to Munir et al. (2020), once the dynamic balance between SIRS and CARS is disrupted, the condition of SAP patients is likely to deteriorate, eventually leading to mortality. In our study, 2/3 of SAP patients died in the late phase; the two patients who died had persistent OF, and the one who recovered had transient OF.

Secreted CXCL4 by activated platelets stimulates pancreatic neutrophil infiltration and subsequently leads to tissue damage; this process is mediated by CXCL2, which is generated from an inflamed pancreas (Li et al., 2021; Wetterholm et al., 2016). This agrees with the results obtained in the current study, where increased neutrophils ($CD66b^+$) complemented increased intracellular and circulating CXCL4. Increased CXCL4 expression was observed more in plasma than intracellularly in platelets in AP patients when compared to HCs. The increased expression of CXCL4 in this study supports what Wetterholm et al. (2016) reported from a study on animal models. Intracellularly, activated $CD41a^+CD62p^+$ platelets were the ones expressing CXCL4. MAP patients had the least $CD41a^+CD62p^+$ platelets but the highest intracellular CXCL4 over time (D3-D15). In contrast, SAP patients had the most $CD41a^+CD62p^+$ and less intracellular CXCL4 expression than MSAP and MAP patients. In the early phase, the pattern of intracellular CXCL4 expression in MAP patients was consistent with CXCL4 concentrations detected in plasma on D3 and D7, specifically. There could have been cell exhaustion in the late phase of MAP patients. Hence the few platelets detected could only secrete CXCL4 intracellularly and not in circulation. On the other hand, SAP patients had the most activated platelets, and more of the secreted CXCL4 was released into circulation; hence more CXCL4 in plasma than intracellular CXCL4 in the late phase. Another possible reason for elevated plasma CXCL4 in SAP patients in the late phase could be that other cells, such as lymphocytes and pancreatic acinar cells, were secreting CXCL4 into circulation (Bhatia et al., 2005; Garraud et al., 2013).

Overall, no clear trends were observed between plasma and intracellular CXCL4 in MAP, MSAP and SAP patients from D3-D15. Due to the variations observed in CXCL4 association with AP severity within the two AP phases, we propose that CXCL4 independently may not be critical for the stratification of AP severity. However, it may be used in combination with other biomarkers since it plays a role in various disease activities that take place in AP patients (Wetterholm et al., 2016). Despite results showing no significant difference of CXCL4 across severity over time, an association between the expression levels of IL-6 and CXCL4 was noted,

especially in the late phase (D9-D15), where both were upregulated in the plasma of patients in the SAP group than MAP, MSAP and in HCs (Wetterholm et al., 2016). Furthermore, between plasma CXCL4 and intracellular IL-6 in NKTs, a statistically significant (p=0.031) and weak positive correlation (0.291) was reported. Additionally, another significantly different (p=0.012) weak positive correlation (0.338) was found between intracellular CXCL4 and intracellular IL-6 NKTs. It is of note that there are limited studies that explain the relevance of CXCL4 in AP, therefore, a need to explore this marker with other biochemical markers is necessary across both AP phases. Another consequence of platelet activation, besides secretion of chemokines and pro-inflammatory cytokines, is the generation of ROS and nitric oxide (Uhlmann et al., 2008).

4.3 Reactive Oxygen Species Increase with Increasing acute pancreatitis (AP) Severity

In the present study, ROS levels were upregulated as AP severity increased from MAP to SAP over the two AP phases (D3-D15) and (D5-D13) in serum and plasma, respectively. ROS plays various roles in AP, such as intercellular signalling by regulating inflammatory cascades and also by activating inflammatory cells and promoting tissue damage (Yu and Kim, 2014). Since ROS was upregulated in the SAP group of patients (in both plasma and serum) in the studied cohort, most (2/3) severe patients had alcohol-associated AP. According to Sbeit et al. (2017), alcohol enables ROS accumulation which subsequently causes the stimulation of cytochrome P450, enhancing ROS production and leading to cellular damage and, eventually, OF. Additionally, elevated ROS levels in SAP patients promote cell death, which ultimately results in OF or other AP-associated complications (Criddle, 2016).

Apart from intercellular signalling, ROS affects intracellular events by activating nuclear factor kappa B (NF-κB), signal transducer, the activator of transcription 3 (STAT3) and activator protein-1 (AP-1) that regulate the expression of inflammatory cytokines, which subsequently amplifies the inflammatory cascade in AP (Meher et al., 2015). In accordance with findings described by Shi et al. (2005) and Meher et al. (2015), the current study's IL-6 ICS and ELISA results showed activated and upregulated leukocytes and IL-6 in AP patients than HCs from the early to late AP phase. In summary, ROS is a critical factor responsible for the development of pancreatitis-induced remote organ dysfunction via intercellular and interorgan signalling. Therefore it may be useful in stratifying AP severity and as a therapeutic target (Yu and Kim, 2014).

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study provides preliminary findings that explored the interwoven network of immune cells (leukocytes and platelets) associated with cytokine, chemokine and ROS secretion in the AP disease course spanning the early to the late phase of SAP. Despite the unique study design, geographical setting, and small sample size, we believe these preliminary findings have provided insights into a better understanding of the underlying immunopathophysiology of AP in a South African patient cohort. These findings are important as a step to better prognosis and patient stratification. They have the potential to inform therapeutic interventions to focus on certain immune cells, associated molecules and clinical parameters. Demographic and clinical data of the studied patients played a crucial role in identifying, characterising and attributing immune cells and molecules to the different AP severity groups.

A link between leukocytes (NKs, NKTs and monocytes) associated with circulating and intracellular secretion of IL-6 in MSAP and SAP patients was explored. Furthermore, the expression of ROS and the link between platelets and secretion of CXCL4 was also studied/explored. These findings showed that ROS and CXCL4 might be crucial in the development and prognosis of AP. Overall, IL-6, CXCL4 and ROS trends were observed in the late rather than the early AP phase. Furthermore, these molecules were more upregulated in SAP patients in the late AP phase (specifically from D9) than in MSAP, MAP and HCs. Therefore, the feasibility of these markers over time (early to late AP phase) still needs to be supported with further studies to validate the observed trends and interlinks which can be considered in cell lines and animal models throght knockout studies.

In line with findings from other studies, our results provide further support that combining immune markers (cells associated with IL-6 and CXCL4 secretion), IL-6, CXCL4 and ROS with clinical parameters such as CRP may be useful in stratifying AP severity. It may also predict the outcome of patients, especially severe patients, who may not present early to the hospital, subsequently delaying early treatment interventions. This was evident based on the results that correlated various experimental data, where possible links between parameters were observed. For instance, plasma CXCL4 had a positive correlation with intracellular IL-6 from NKT lymphocytes. However, accurate stratification and prognostic potential, clinical and

healthcare resource implications of these markers still require further assessment in a larger patient cohort for validation.

In summary, NKs and NKTs were found to be responsible for intracellular IL-6 secretion in MSAP and SAP patients, respectively, over time (D3-D15). Furthermore, activated immune cells (leukocytes and platelets) were associated with releasing cytokines (IL-6), chemokines (CXCL4) and ROS into circulation, promoting inflammation and associated AP complications. Therefore, the current study suggests that combination therapy targeting different cells, molecules, and steps of the inflammatory cascade, might be the appropriate treatment in AP patients. Future studies demonstrating the link between immune response within the SIRS and CARS phases of AP may prove useful in further elucidation of the AP pathophysiology and assist in identifying more or novel targets for therapeutic interventions.

In conclusion, the link between inflammatory mediators such as IL-6 and CXCL4 and cells associated with their secretion and ROS expression has the potential to be useful in better AP stratification and prognostication to improve the care of AP patients, supporting the original hypothesis.

5.2 Limitations and Recommendations

Although interesting findings have been reported, the current study had limitations that require acknowledgement. Like most clinical studies, adequate sample size is crucial for producing accurate, reliable, and reproducible data. The small sample size was the major limitation in the current study. The study cohort was limited to AP patients recruited from one hospital. Hence a multicentred study may have provided more robust and accurate results in terms of sample size and immune response data from participants. Additionally, the COVID-19 pandemic drastically affected the sampling process and delivery of project-specific reagents due to supply chain issues. Numerous sampling suspensions were encountered due to COVID-19 regulations in South Africa from December 2020 to January 2022. However, it is important to note that an effort to overcome this hurdle was made by relating the study findings to those in the literature on similar work and the overall similarities in the findings are indicative of the validity of this work.

Other limitations included late presentation of patients to the hospital, which could be due to the socio-economic status where patients tend to delay seeking treatment. Since this was a time-sensitive study, early patient presentation to the hospital could have benefited the study cohort for a better understanding of the longitudinal aspect of the disease progression. Consent withdrawal was another limitation due to the longitudinal nature of the study, wherein patients would decline consent days after recruitment for reasons such as discomfort during phlebotomy and sometimes early discharge before the sampling period was over. They would refuse to continue participating, although an incentive to cover travel was offered. It is therefore recommended that more samples are collected to validate these preliminary trend findings in a larger cohort of patients.

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APPENDICES

APPENDIX A: Ethical clearance certificate

we

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

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

TO: Ms M Maimela; Drs J Devar and J Omoshoro
School of Clinical Medicine
Department of Surgery
Medical School
University

E-mail: rurumaimelag@gmail.com

CC: Supervisor: Dr P Fru; Professor M Smith; Ms M Nalisa
<Pascaline.Fru@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: 2020/10/07

REF: *R14/49*

PROTOCOL NO: **M200757** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Cell associated interleukin-6 expression in acute pancreatitis: potential for monitoring 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.

MSWorks2003\Iain\2007\Clearscan.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms M Maimela; Drs J Devar and J Omoshoro

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200757

NAME: (Principal Investigator)	Ms M Maimela; Drs J Devar and J Omoshoro
DEPARTMENT:	School of Clinical Medicine Department of Surgery Medical School University
PROJECT TITLE:	Cell associated interleukin-6 expression in acute pancreatitis: potential for monitoring disease severity
DATE CONSIDERED:	2020/07/31
DECISION:	Approved unconditionally
CONDITIONS:	Approval applies to Charlotte Maxeke Johannesburg Academic Hospital site only; further sites may be approved on submission of evidence of management approval to the HREC (Medical)
SUPERVISOR:	Dr P Fru; Professor M Smith; Ms M Nalisa
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/10/07

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

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature _____ Date _____

APPENDIX B: Patients information sheet

Information sheet for the patients' group

Cell associated IL-6 expression in acute pancreatitis: potential for monitoring 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 cells associated with producing a substance that increases in conditions like AP, known as IL-6, which will assist in early stratification of AP severity and possible complications.

You are invited to take part in this study. This research study is looking for **at least 30 participants (10 from each class of severity) that are newly diagnosed with acute pancreatitis and have no co-existing inflammatory conditions,** to donate **about 16 ml** of blood. The blood will be drawn from a vein in your arm using a syringe at the hepatopancreaticobiliary unit at Chris Hani Baragwanath Academic Hospital 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 also called plasma and cells will be separated and used to test for cells associated with IL-6 expression. Some of these samples such as the plasma cannot be used immediately and will be frozen at -20 or -80 degrees Celsius until needed (within 2 months to 5 years). Tests that will be done will include associating IL-6 expression with different cells in patients with MSAP and SAP. The association of IL-6 expression with organ failure will also be analysed. The results from these samples will be compared to those of participants who lack a history of acute pancreatitis.

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 nurse has been recruited to perform the procedure and to minimize the risks. Experienced doctors are also available to assist in her absence. The procedure will only take about 10 minutes and blood will be obtained from you from day one of onset of AP to 720 hours, within an interval of two days. Participating in this study might not benefit you now but we hope that the results from these studies will benefit patients in the 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 then disposed of in bio-hazardous/ medical waste boxes.

For further information/ reporting study related adverse effects/complaints contact:

Researchers:

Miss Murunwa Grace Maimela (Research student) Email: 2400613@students.wits.ac.za

Prof. Pascaline Fru (Supervisor) Tel: 0117172476, pascaline.fru@wits.ac.za

REC Chairperson – Prof Clement Penny: Clement.penny@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 C: Patients consent form

Patient Group Consent Form: Use of Clinical Sample and Information for Research

Dear Potential Participant,

You are currently admitted to the Chris Hani Baragwanath Academic Hospital (CHBAH) after your diagnosis of acute pancreatitis. The CHBAH 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 and 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, 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.

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. Murunwa Grace Maimela at 071 238 9050, Prof. Pascaline Fru at (011)717-2476

A. Consent Given

I_____hereby give consent for my records to be used as per the above-mentioned conditions for the purposes of research:

PATIENT SIGNATURE: _____DATE: _____

B. Consent Not Given

I _____do not give consent for my records to be used: PATIENT SIGNATURE: _____DATE: _____

APPENDIX D: Information sheet for healthy controls

Information sheet for the control group

Dear potential participant,

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 cells associated with producing a substance that increases in conditions like AP, known as IL-6, which will assist in early stratification of AP severity and possible complications.

You are invited to take part in this research study. This study is looking for **at least 10 healthy participants who have no history of being diagnosed with acute pancreatitis or any co-existing inflammatory conditions,** to donate **about 16 ml** of blood. The blood will be drawn from a vein in your arm using a syringe in the department of surgery laboratories at the University of the Witwatersrand. From the blood, the liquid portion also called plasma and cells will be separated and used to test for cells associated with IL-6 expression. Some of these samples such as the plasma cannot be used immediately and will be frozen at -20 or -80 degrees Celsius until needed (within 2 months to 5 years). Tests that will be done will include associating IL-6 expression with different cells in patients with MSAP and SAP. The association of IL-6 expression with organ failure will also be analysed. The results from these samples will be compared to those of participants who have been diagnosed with acute pancreatitis.

Blood samples will be collected once and you may be subjected to some pain or discomfort caused by the needle, but a qualified and experienced nurse has been recruited to perform the procedure and to minimize the risks. The procedure will only take about 10 minutes. Participating in this study might not benefit you now but we hope that the results from these studies will benefit patients in the future. Participating in this study will not result in any "out of pocket' expenses. Furthermore, your participation in this study is entirely voluntary.

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 then disposed of in bio-hazardous/ medical waste boxes.

For further information/reporting study related adverse effects/complaints contact:

Researchers: Miss Murunwa Grace Maimela (Research student) Email: 2400613@students.wits.ac.za; Prof. Pascaline Fru (Supervisor) Tel: 0117172476, pascaline.fru@wits.ac.za

REC Chairperson – Prof Clement Penny: Clement.penny@wits.ac.za

REC Administrators – Ms. Zanele Ndlovu/ Mr. Rhulani Mkansi/ Mr. Lebo Moeng

Tel 011717 2700/2656/1234/1252, Email: HREC-Medical.ResearchOffice@wits.ac.za

APPENDIX E: Information sheet for healthy controls

Control Group Consent Form: Use of Clinical Sample and Information for Research

Dear potential participant,

Receiving this form implies that you have been requested to partake in an acute pancreatitis study and you have responded. Prior to signing this form, you would have been familiarized with the details of the study. You are expected to donate approximately 2½ teaspoons of blood 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 implies that you grant the researcher permission to analyse your blood to ensure 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 valid up to 5 years after sample collection.

Your participation in this research is entirely voluntary. If you choose not to participate in this study, you will not be compromised 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. Murunwa Maimela 071 238 9050, Dr. Pascaline Fru 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 F: Datasheet for acute pancreatitis patient's demographic and clinical data

Cell associated IL-6 expression in acute pancreatitis: potential for monitoring disease severity SAMPLE DATASHEET:

Sample No: CHAP_____

Age……

Gender…….

Employment status…… Occupation……..

Smoker… ..... (Y/N)

Drinker… .....(Y/N)

Severity of AP…….

Aetiology…….

DOA…….

DOD…….

Days of hospitalization……

In hospital death….....(Y/N)

RVD..... (Y/N)

ARVs (if applicable) ……

Amylase (23 to 85 U/L, also 140U/L) …… Lipase

(0 to 160 U/L) ……

Full blood count	**At ADMISSION**	**DAY 3** No of tubes:	**DAY 5** No of tubes:	**DAY 7** No of tubes:	**DAY 9** No of tubes:	**DAY 13** No of tubes:	**DAY 15** No of tubes:
WBC ($x10^9$/L)							
Haemoglobin g/Dl							
Haematocrit (L/L)							
Platelets ($x10^9$/L)							
Other							
CRP (mg/L)							
Liver function tests							
Albumin							
Total Bilirubin (mg/dL (µmol/L))							
Blood Gas test							
Fi02							
Pa02							
Ph							
pO_2							
pCO_2							
HCO_3 (mmol/L)							
U+E							
Na (mmol/L)							
K (mmol/L)							
Cl (mmol/L)							
Creatinine(µmol/L)							

Urea ((mmol/L)							
Glucose (mmol/L)							
Other							
Acute Renal Failure (dysfunction)							
Organ dysfunction (if yes, state order)							
Comorbidities							
Local Complications							
Admission to ICU	*(Y/N, No of Days)*						
Other surgical procedures							
Additional comments							

APPENDIX G: Interpretation Spearman's correlation (rho)

Table A: Spearman's correlation interpretation

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

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 $\alpha = 0.01$ level (2-tailed) and $\alpha = 0.05$ level (2-tailed). Adapted from Mukaka, (2012)

APPENDIX H: Nine colour panel designed for the IL-6 ICS 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)	Optimized volume(µl)	Catalogue No; Clone
Red 640										
	730/45 BP	Alexa Fluor 700	720		CD19			5	1.5	557922, Clone HIB19
	660/20 BP	APC	660		CD16			5	1.25	559865, Clone 3G8
Blue 488										
A	780/60 BP	PE-Cy7	780			CD56		5	2.0	560916; Clone HIB19
B	695/40	PE	695				CD14	5	1.5	561116; Clone MφP9
F	530/30BP	BB515/FITC	515		CD57			5	1.25	555619, Clone NK -1
	695/40	PerCP-CY5.5	690				CD66b	5	1.5	562316; Clone G10F5
Violet 405										
	655/8	BV650	650		HLA-DR		HLA-DR	5	2.5	564231; Clone G46-6
	450/50 BP	BV421	421		IL-6			5	2.5	562555, Clone MQ2-13A5
UV 355										
	530/30	BUV496	496		CD3			5	1.0	564810, Clone UCHT1

APPENDIX I: Four colour panel designed for the CXCL4 ICS according to configuration of BD LSR Fortessa II

Laser Name	Filter	Parameter	Emission	Platelets	Vol per test (µl)	Optimized volume(µl)	Catalogue No; Clone
Red 640							
	730/45 BP	Alexa Fluor 647	720	CXCL4	5	1.5	557922, Clone 170138
Blue 488							
F	530/30B P	BB515/FIT C	515	PAC-1	5	1.25	43519, Clone PAC-1
Violet 405							
	450/50 BP	BV421	421	CD62p	5	2.5	543215, Clone AK-4
UV 355							
	530/30	BUV496	496	CD41a	5	1.25	55432, Clone HIB8

APPENDIX J: Plagiarism (Turnitin) Report

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