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Lymphocyte ratio, oxidative stress, cell death and metabolic profiling in acute pancreatitis: implications for disease monitoring.

Jeanet Mazibuko

(831447)

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine.



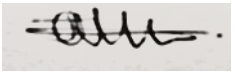
DEPARTMENT OF SURGERY, FACULTY OF HEALTH SCIENCES,

UNIVERSITY OF WITWATERSRAND,

Johannesburg, South Africa 2022

DECLARATION

I, Jeanet Mazibuko, declare that this dissertation is my own work. It is being submitted for the Degree of Master of Science in Medicine by research at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Candidate's Signature):  Date: 23 June 2023

DEDICATION

This dissertation is dedicated to all the Black women who come from disadvantaged communities like me and to everyone who believes in me and continuously supports me; this is for you!!

PUBLICATIONS AND PRESENTATIONS

Publication(s) in preparation:

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Oral Presentation(s)

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ABSTRACT

Acute pancreatitis (AP) is the autodigestion and inflammation of the pancreas that clinically presents with nausea, vomiting, abdominal pain, and increased amylase and/or lipase levels. Mild acute pancreatitis (MAP) is associated with no organ dysfunction and quick recovery. Moderately severe acute pancreatitis (MSAP) has transient organ dysfunction, while severe acute pancreatitis (SAP) is characterized by a significant systematic inflammatory response associated with varying degrees of organ dysfunction. Current systems use many variables to diagnose AP, which are unsuitable for identifying high-risk patients, prognosis, and management. This study aimed to identify metabolic indicators for monitoring AP severity by measuring the CD4:CD8 ratio, apoptosis, hydroperoxides, and metabolites, key pathological responses to the activated immune system. Fifty patients of different severities of AP as well as ten healthy controls (HCs) were recruited from the Chris Hani Baragwanath Academic Hospital. Whole blood samples were collected from the patients within 72 hours (Day 1) of experiencing epigastric pain and every 48 hours for an additional 3 days (Days 3, 5 and 7). The peripheral blood mononuclear cells (PBMCs), plasma and serum were prepared according to standard operating procedures. Flow cytometry was used to quantify the CD4:CD8 ratios while apoptotic gene expression, hydroperoxides and metabolites were analyzed by polymerase chain reaction (PCR), DEPPD assay and nuclear magnetic resonance (NMR) spectroscopy, respectively. As expected, the HCs and the MAP groups had the highest percentage of CD4:CD8 compared to the other AP severity groups. *Caspase 7* expression was high in MAP compared to the HCs, although not statistically significant, while its expression in MSAP patients was statistically significant ($p = 0.0029$) compared to the HCs. Over time, the expression of *caspase 7* expression in MSAP patients increased on day 5 ($p = 0.00994$) and slightly on day 7 ($p = 0.0249$). The expression of *caspase 7* in SAP patients decreased over time from day 5 to day 7. The MSAP group displayed the highest levels of hydroperoxide on day 5 with statistical significance to the HC than the other groups in both serum ($p = 0.0012$) and plasma ($p = 0.0008$), between the SAP and the HC in serum on day 5 ($p = 0.0009$) as well as in plasma ($p = <0.0001$ between MAP and the HC. An unsupervised analysis of metabolites separated the AP patients into two clusters consisting of MAP+MSAP and another of MSAP+SAP patients. The main metabolites differentiating the groups were phenylalanine 0.54 ($p< 0.001$); mannose 0.57 ($p< 0.001$); lactate 0.67 $p< 0.001$), acetoacetate 0.63 ($p<0.001$), 3-hydroxybutyrate 0.46; ($p< 0.003$) lipid alpha-CH2 0.45($p=0.006$) with AP severity. Ascorbate 0.46 ($p< 0.003$); glutamine -0.55 ($p<0.001$); methanol -0.46 ($p< 0.003$); ethanol -0.64 ($p< 0.001$); lipid=CH-CH2-CH=-0.55($p<0.001$), lipid beta-CH2 -0.52 ($p<0.001$), lipid-CH3 -0.44($p=0.005$) and protein-NH -0.75 ($p<0.001$) decreased as severity increased. Taken together, the measured parameters i.e., CD4:CD8 ratio, apoptosis, ROS, and metabolites, suggest that as AP severity increases, integrating immune and metabolomics data may help in understanding the pathophysiological conditions of AP for early stratification, management, and prognosis of patients. MSAP represents the earliest shift toward progression without the comorbidities associated with SAP.

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LIST OF ABBREVIATIONS

APACHE II	Acute physiology and chronic evaluation
ARDS	Acute respiratory distress syndrome
APCs	antigen-presenting cells
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
BUV	BD Horizon Brilliant™ Ultraviolet 266
BD	Becton Dickinson
BMI	Body mass index
BV	Brilliant Violet™
cDNA	Complementary Deoxyribonucleic Acid
CD	Cluster of differentiation
CHBAH	Chris Hani Baragwaneth Academic Hospital
CDCl3:CD3OD: D2O	Chloroform-d:methanol-d:water-d
CRP	C - reactive protein
CT	Computed tomography
cDNA	Complementary deoxyribonucleic acid
DEPPD	N, N-Diethyl-p-phenylenediamine
DIPE	Diisopropyl ether: ethyl acetate
EDTA	Ethylenediamine tetraacetic acid
ERCP	Endoscopic retrograde cholangiopancreatography
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDR	False discovery rate
GDP	Gross domestic product
HAART	Highly activated antiretroviral therapy
HDL	High-density lipoprotein
HDL-P	HDL-Particle
HIV	Human immunodeficiency virus
HPB	Hepatopancreaticobiliary
HTG	Hypertriglyceridemia
ICU	Intensive care unit
IDL	Intermediate-density lipoprotein
IL	Interleukin
LDL	Low-density lipoprotein
LOS	Length of hospital stay
LpX	Lipoprotein X
MAP	Mild acute pancreatitis
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MSAP	Moderate severe acute pancreatitis
MODS	Multiple organ dysfunction syndrome
n.d	No date

NF-κβ	Nuclear factor kappa beta
NOESY	Nuclear 55 Overhauser Effect Spectroscopy
NRTIs	Nucleoside reverse transcriptase inhibitors
NTC	No template control
OF	Organ failure
PBMCs	Peripheral blood mononuclear cells
PCT	Plasma procalcitonin
PE	Phycoerythrin
Pis	Protease Inhibitors
RPMI	Roswell Park Memorial Institute
RAC	Revised Atlanta classification
ROS	Reactive oxygen species
Rho	Correlation coefficient
RPL 13A	Ribosomal Protein L13a
rtPCR	Real-time polymerase chain reaction
SIRS	Systemic Inflammatory Response Syndrome
TAP	trypsinogen activation peptide
TG	Triglycerides
UK	United Kingdom
USA	United States of America
VLDL	Very-Low Density Lipoprotein
WBC	White blood count
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

There has been a rise in hospital admissions of the African population in patients diagnosed with acute pancreatitis (AP). In South Africa, a third-world country with limited resources, this increase in admissions has put a strain on the health system due to the poor prognosis of AP, as well as the time spent to triage the patients by the clinicians. This results in a delay in the diagnosis and treatment of patients. Worldwide, there is an increase in hospital admissions of patients with AP associated with alcohol abuse and gallstones (Fagenholz *et al*., 2007; Yadav and Lowenfels, 2013; James and Crockett, 2018). Acute pancreatitis is the autodigestion and inflammation of the pancreas that clinically presents with nausea, vomiting, abdominal pain, and increased amylase and/ or lipase (Dragovic, 2013). The Revised Atlanta classification (RAC) of AP from 2012 is an international multidisciplinary classification of the severity of AP. It's a consensus that includes an agreed-upon classification of AP severity, with standardized terminology for pancreatitis and its complications. The RAC recognizes AP's early and late phases which can also be interstitial edematous and necrotizing (Banks *et al*., 2013). The severity of AP is classified into three categories: mild, moderately severe, and severe (Banks *et al*., 2013).Most patients are diagnosed with interstitial edematous AP, which has a favorable prognosis as it is mild and self-limiting (Beger and Rau, 2007). Mild acute pancreatitis (MAP) is associated with no organ dysfunction and quick recovery related to apoptotic acinar cell death (Pandol *et al*., 2007). Moderately severe acute pancreatitis (MSAP) is almost similar to severe acute pancreatitis (SAP), however, it has organ dysfunction that reverses before 48 hours, exacerbation of co-morbidities or local complications (Banks *et al.,* 2013). SAP is characterized by a significant systematic inflammatory response associated with varying degrees of organ dysfunction, including cardiovascular failure, respiratory insufficiency, or renal failure (Banks *et al.,* 2013). The necrotizing form of AP develops in 15% of AP patients where complications such as sepsis, multi-organ failure, and death develop due to infected necrosis (Zerem, 2014). AP has diverse presentations; the pathophysiology is still unclear, and the clinical extent differs widely from mild features to Systematic Inflammatory Response

Syndrome (SIRS). The heterogeneity of the disease makes prognosis difficult because there is no simple way to predict the course of the disease.

Several severity scoring systems are developed for clinicians to triage AP patients and assist with prognosis (Sharma *et al*., 2015). However, the disadvantage is that these tools are time-consuming; they use many parameters that make their use difficult and are ill-suited for assessing patients during admission. Recently, blood components such as plasma/serum, platelets, white blood cells, and red blood cells have been studied to predict disease severity and possibly be used as metabolic indicators of pancreatitis (Cho *et al*., 2018). Biomolecules such as C-reactive protein (CRP), plasma procalcitonin (PCT), and white blood count (WBC) are widely used in combination and are reliable as diagnostic and prognostic biometabolic indicators of infection and severity (Magrini *et al*., 2014) . However, CRP and WBC are not specific and sensitive to bacterial infection whereas PCT is raised in other conditions that lead to the release of proinflammatory cytokines (Magrini *et al*., 2014).

This study aims to potentially identify immunometabolic perturbations in acute pancreatitis patients i.e., lymphocyte ratio, cell death, oxidative stress, and metabolic changes. The RAC system was used to recruit the study participants at the Chris Hani Baragwanath Academic Hospital (CHBAH). The clinicians working in the Hepatopancreaticobiliary (HPB) Unit of the CHBAH diagnosed the AP patients as either MAP, MSAP, or SAP. Blood samples were collected over four days within 72 hours of pain starting from days 1, 3, 5, and 7 from the AP patients after obtaining informed consent. The clinical data were obtained from the patients' hospital files, and these included the demographic data, weight, aetiology, AP severity, results of laboratory investigations, and comorbidities. Healthy volunteers of the same age, sex, and weight were recruited as the controls for the study. The peripheral blood mononuclear cells (PBMCs), serum, and plasma were obtained from the blood samples of the patients and stored in the -70°C freezer until needed. The cell subpopulations were measured by immunophenotyping using flow cytometry from the blood samples of seven patients using three antibodies to measure the CD4/CD8 ratio. The polymerase chain reaction (PCR) technique was used to determine caspase gene expression in the PBMCs of 35 patients diagnosed with AP. *Caspase 2, 3, 7,* and *8* genes were assessed to determine the apoptosis status of the cells. Ribosomal Protein L13a (*RPL 13A),* a reference gene, was used to normalize relative fold expression. The AP-associated reactive oxygen species (ROS) production

was assessed in the different severities using N, N-Diethyl-p-phenylenediamine (DEPPD), and iron sulphate to quantify the concentration of ROS in the sera of patients using a spectrophotometer. Analysis of biofluids using metabolomics is currently being used to better understand diseases on the molecular level. For this study, Nuclear magnetic resonance (NMR) spectroscopy was used to quantify and assess the metabolites present in the serum samples.

The findings of this study should inform on the potential and correlation between T lymphocytes, apoptotic genes, ROS and metabolites to be used as prognostic metabolic indicators that may be more accurate in stratifying the risk profiles of patients at the early phase of AP and potentially improve management strategies.

1.2 Literature review

1.2.1 The Pancreas: Development, Function, and Regulation

The pancreas is part of the foregut and located behind the stomach and is about 12–15 cm long (Slack, 1995). The exocrine pancreas secrets fluid that contains digestive enzymes into the small intestine via the ampulla of vater. This fluid contains bicarbonate, which neutralizes the hydrochloric acid from the stomach, and digestive enzymes, which break down the food entering the small intestine from the stomach (Tanase *et al*., 2010). The pancreas is observed at four weeks of development in humans as the dorsal bud emerges from the duodenum's proximal end (Reichert and Rustgi, 2011). The head of the pancreas connects the duodenum through the pancreatic duct on the right of the abdomen, and the tail as shown in **Figure 1.1**.

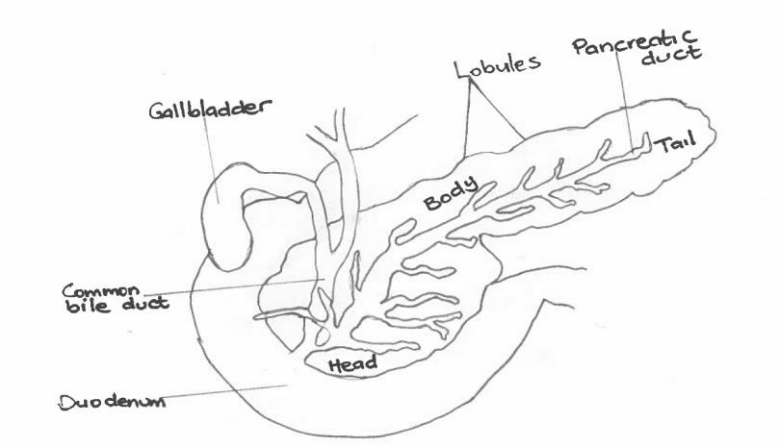


Image by Jeanet Mazibuko

Figure 1.1 Diagram of a pancreas. The pancreas shows three parts: the head, body, and tail, with the pancreatic ducts across.

The development of the pancreas is strongly regulated, with the exocrine and endocrine parts developing from the same progenitor cells (Mastracci and Sussel, 2012). Progenitor cells are cells that differentiate into a specific type of cell. The islet cells of Langerhans make up the endocrine part of the pancreas (Da Silva Xavier, 2018). This section consists of alpha (α), beta (β), delta (δ), and epsilon (ε) polypeptide cells arranged into islets. They secrete insulin and glucagon into the bloodstream (Brereton *et al*., 2015).

The pancreas produces enzymes that are crucial in metabolism, converting ingested food into energy. It also plays a key role in the regulation of digestion i.e. metabolism and homeostasis by releasing various digestive enzymes and pancreatic hormones (Lema-Pérez, 2021). Insulin lowers blood sugar levels by causing the liver to convert glucose to glycogen, and it assists the cells to take up glucose. Glucagon elevates blood sugar levels by promoting the breakdown of glycogen and increasing gluconeogenesis. The alpha cells of the pancreas produce glucagon when blood glucose levels decrease. Maintaining blood sugar levels is critical to the proper functioning of major organs like the kidney, liver and brain (Ritter, 2017). The pancreatic hormones, insulin, and glucagon are present in the human fetal circulation as early as the fourth or fifth month of fetal growth (Murphy *et al*., 2006).

The exocrine component contains glands that produce enzymes such as proteases, lipases, and amylases. These enzymes break down proteins, lipids, and carbohydrates during digestion. The

digestive enzyme alpha-amylase is the main exocrine enzyme secreted by the salivary glands and the pancreas. Studies of the first two years of prenatal fetal and post-natal production of amylase have shown that most of the enzymes produced initially are salivary amylase (Georgountzou and Papadopoulos, 2017). The secretory response to producing amylase is poor in infants and newborns, meaning it is either absent or minimal at birth and only acquired postnatally. This suggests that the exocrine pancreas only becomes "functional" after birth (Georgountzou and Papadopoulos, 2017).

1.2.2 Diseases of the pancreas

Pancreatitis, pancreatic cancer, and diabetes are common diseases of the pancreas. Pancreatitis can be acute (AP) or chronic (CP). AP develops instantaneously, it is a short-term illness with a complete recovery provided appropriate medical care is administered (Zerem, 2014). During an AP attack, the pancreas is damaged by its digestive juices, resulting in tissue swelling and death. AP is characterized by an injury to the acinar cells, which is localized and systematic, interceded by inflammatory chemokines and cytokines (Clemens *et al*., 2014). CP is a continuing inflammatory and fibrosing process of the pancreas that results in morphological changes and permanent exocrine and endocrine dysfunction. The damage is irreversible and continues to the different glands in the body and may lead to diabetes and significant exocrine insufficiency (Witt *et al*., 2007). Chronic pancreatitis patients are at a higher risk of developing pancreatic cancer at a later stage (Etemad and Whitcomb, 2001). Pancreatic cancer starts in the pancreas' tissues and proliferates quickly to surrounding organs. There are no apparent symptoms at the disease's initial stages, making it difficult to diagnose early. Pancreatic cancer has the poorest prognosis of all cancers, with a 5-year survival rate which is below 3 % (Greenlee *et al*., 2000). Diabetes mellitus (diabetes) is a metabolic disease that causes elevated blood sugar levels. Deficiency/resistance in insulin secretion or insulin activity leads to the destruction of the β-cells of the pancreas resulting in resistance to insulin action (Mastracci and Sussel, 2012). Over time, significant damage to the kidneys, eyes, heart, blood vessels, and nerves occurs (Tan, 2020).

1.2.3 Prevalence of AP

The global incidence of AP has increased significantly in recent years, with an annual increase of 2.7% reported (Roberts *et al*., 2013). It is the most typical gastrointestinal disorder that requires

hospitalization (Granger and Remick, 2005). In the US, about 5000 new AP cases are recorded annually, with a mortality rate of 2% (Gapp and Chandra, 2021). In Africa, published data are scarce. In South Africa, alcohol-induced AP accounts for about 78% of hospital AP admissions (Anderson *et al*., 2008).

1.2.4 Aetiology of AP

In Western countries, gallstones, and alcohol account for 80% of AP cases and 20% are idiopathic (Williams *et al.*, 2007; Roberts *et al*., 2017). In a study done between 2001 and 2006 at Addington Hospital in Durban, South Africa, 82% of AP cases were associated with alcohol and 14% were attributed to gallstones (Anderson *et al*., 2008). The study also revealed that AP was a common comorbidity in people living with HIV (PLWH) with an incidence of 5.7% (Anderson *et al*., 2008). The aetiology of AP varies with economic, geographic, environmental, individual habits, and seasonal factors. These factors may affect the occurrence, severity, and clinical outcomes of AP. In South Africa, most cases are presented after a long weekend, during the festive season, and Easter holidays after binge drinking. Therefore, understanding the aetiology will assist in achieving favorable outcomes to prevent recurring AP and implement effective management strategies.

1.2.4.1 Alcohol-induced AP

Excessive alcohol consumption is a risk factor for both AP and CP. When AP does not resolve, it may lead to transient organ failure or progress to cause systemic inflammation and multi-organ failure. Repeated pancreatitis attacks may lead to diabetes, fibrosis, pancreatic calcification, or pancreatic insufficiency (Klochkov *et al*., 2021). The recommended daily dose of alcohol for women is not more than one drink (90 mL) and two drinks (120 mL) for men (Boston and Ma, 2012). When alcoholic drinks more than the recommended daily dose are consumed, the alcohol is broken down into substances that are toxic to the pancreas. The metabolism of alcohol over time in the human body may increase the risk of pancreatitis. This is influenced by pancreatic function, and gastrointestinal and genetic predisposition in individuals who abuse alcohol for many years (Chowdhury and Gupta, 2006). The acinar cells break down ethanol via the oxidative and non-oxidative pathways, which are toxic to the cells (Apte *et al*., 2016). Studies suggest that the events

that begin in the pancreas' acinar cells play a role in the instigation of AP (Gorelick and Thrower, 2009). The acinar cells are the majority in the pancreas and produce the enzymes required to digest food. They are packed into vesicles called zymogen in an inactivated state (Slack, 1995). Under normal conditions, the pancreatic enzymes get activated in the small intestine, but they get released and activated before they reach the small intestine during an AP episode. AP is initiated when ethanol and its metabolites release inflammatory mediators, sensitize the pancreas, and lower its threshold for injury (Clemens *et al*., 2014). The acinar cell signaling spectrum changes during AP and includes the activation of the pancreatic enzymes and inflammatory mediators, changes in cell permeability, and the pathways involved in cell death are stimulated (Gorelick and Thrower, 2009).

1.2.4.2 Biliary-induced AP

The passage of a stone from the common bile duct through the ampulla of Vater into the small intestine is thought to be the pathophysiology of gallstone pancreatitis (Freund *et al*., 1976, Chatila *et al*., 2019). Smaller stones pass through the cystic duct more easily (Wang *et al*., 2009). Cholestasis is the stoppage of blood secretion and flow. The symptoms include an increase in alkaline phosphatase, bilirubin, cholesterol, lipoprotein X (LpX), and bile acids (Assy *et al*., 1999). Patients diagnosed with gallstone-induced pancreatitis usually have a mild clinical course of the disease, however, when not treated, the risk of recurrence in gallstone pancreatitis ranges from 32 to 61% (Chatila *et al*.,2019). Women have a higher probability of getting biliary pancreatitis than males since gallstones occur with increased frequency in women, and the incidence increases with age (Yadav & Lowenfels, 2013). Complications of AP include local complications and systemic complications. Local complications include acute necrotic collections, peripancreatic fluid collections, pseudocyst and walled-off necrosis. These complications are defined according to the time (Pandol *et al*., 2007). There have been many investigations regarding the role of the main etiological risk factors that may affect the clinical course and outcome of AP (Cho *et al*., 2015; Kim *et al*., 2017). In a study by Cho *et al.* (2015) where researchers were comparing the clinical course and outcome between the two main aetiologies of AP, they found that more severe forms of the disease are found in alcoholic-associated AP, while some studies have found no significant differences between the two main aetiologies of pancreatitis (Anderson *et al*. 2008; Cho *et al*., 2015). In contrast, some report that biliary-associated AP is more severe, and the mortality is

higher than alcohol-induced pancreatitis. Other risk factors of AP include an unhealthy lifestyle which involves smoking and consuming unhealthy foods, endoscopic retrograde cholangiopancreatography (ERCP), hypertriglyceridemia and the use of certain highly active antiretroviral therapies (HAART). Sometimes the cause of AP is unknown.

1.2.5 The pathogenesis of acute pancreatitis

The pancreas secretes proteases, lipases, and amylases. The role of these enzymes is to break down proteins, lipids, and carbohydrates during digestion and fight against pathogens in the body (Gurung *et al*., 2013). The damage to the pancreas occurs due to the early activation of pancreatic enzymes before they reach the small intestine (Adrian, 2007). The immune system gets activated, which causes SIRS and involves granulocytes, macrophages, cytokines, and intracellular signaling pathways. The immune activation is the main contributing factor to organ dysfunction and early death within the first week of admission (Akbarshahi *et al*., 2012). The severity of the inflammatory response in AP determines which patients will suffer organ failure. SIRS is exacerbated by a complex cytokine cascade which leads to a weakened immune system (Osterbur *et al*., 2014). The protease cascade does not influence the severity of AP, it is the T-lymphocytes that are responsible for cell-mediated immune responses (Wang *et al*., 2017). The CD4 and CD8 lymphocyte number and ratio are reduced extensively, suggesting that T lymphocytes, primarily CD4 T-helper (Th) cell depletion contributes to severe pancreatitis (Yang *et al*., 2015). Early alterations to the T lymphocyte subsets influence the prognosis of AP patients i.e. decreased lymphocyte counts have been observed in SAP patients although no changes have been detected in MAP (Shen *et al*., 2015). Advanced technology such as flow cytometry and PCR has made it possible to measure the components in blood to better understand the immune responses and to determine the extent of cell death by indicating apoptosis and necrosis (Brauchle *et al*., 2014).

1.2.6 The diagnosis of AP

The RAC diagnosis acute pancreatitis when two of the criteria are present, typical history of epigastric pain radiating the back, laboratory tests confirming amylase and lipase raised to at least three times the upper limit of normal or cross-sectional imaging confirming AP (Banks *et al*., 2013). The main disadvantage of the CT scan is ionizing radiation and contrast agents that contain iodine (Lusic and Grinstaff, 2013). In patients with renal disease or radiographic contrast allergy,

this is problematic as the iodine may worsen renal function, and the latter can cause cardiac arrest (Andreucci *et al.*, 2017). Therefore, finding a more effective, non-invasive tool for easy diagnosis and early intervention is crucial. There have been many attempts to develop a laboratory tool that can predict disease severity and progression of AP with ease – that is cheap, rapid, and reliable (Werner *et al*., 2003). Several biochemical metabolic indicators have been studied for early diagnosis and severity over the past few decades. Currently, lipase and amylase are the cornerstones in the laboratory diagnosis for AP, although they are only useful for diagnosis, and not prognosis (Meher *et al*., 2015).

Some scoring systems and metabolic indicators of inflammation have been developed to assess and predict the course of the disease. These predictive and prognostic biometabolic indicators are used to help optimize ideal treatments and to prevent disease progression. The 2012 RAC of AP suggests that three organ systems should be examined to define organ failure: respiratory, renal and cardiovascular (Banks *et al.*, 2013). The modified Marshall scoring system predicts organ failure (Zhou *et al*., 2019). The Multiple Organ Dysfunction Score (MODS), Ranson's criteria, and Acute Physiology and Chronic Health Evaluation II (APACHE II) systems are mainly utilized to predict the course of AP (Zhou *et al.*, 2019). Ranson's score uses 11 parameters, which are recorded soon after the patient presents and again 48 hours after admission (Rao and Kunte, 2017). The APACHE II score is derived from parameter variables that are repeated during the patient's hospital stay (Rao and Kunte, 2017). All these scoring systems require at least 48 hours of hospitalization for completion.

Amylase levels in the blood are one of the diagnostic metabolic indicators used in conjunction with other diagnostic tools to support the diagnosis of pancreatitis (Matull *et al.*, 2006). Amylase is produced in the salivary glands and the pancreas. It is also present in small quantities in some tissues (des Gachons and Breslin, 2016). Amylase levels in patients with pancreatitis vary depending on the severity of the disease. During an AP episode, serum levels rise quickly within twelve hours and remain elevated for up to 72 hours, and are eventually excreted by the kidneys (Munoz and Katerndahl, 2000). The specificity and sensitivity of amylase as a tool for diagnosis depend on its threshold value. The sensitivity is around 55-84%, while its specificity can be up to 95% at a cut-off level of 1000 IU (Matull *et al*., 2006). Increased serum levels may be present in various inflammatory conditions, such as in patients who have salivary disorders and those with

kidney disease. In some cases, amylase activity is delayed when the patient presents at the hospital or in people with exocrine pancreatic insufficiency due to chronic alcoholism (Matull *et al*., 2006). Lipase is a better marker than amylase since it is four times more sensitive and unlikely to be affected by exocrine pancreatic insufficiency (Keller and Layer, 2005). Lipase has a 60% specificity and 80% sensitivity (Meher *et al*., 2015). Its concentration increases within 3-6 hours of the onset of the disease and peaks in 24 hours. The increase levels remain for 7-14 days before they return to normal levels (Meher *et al*., 2015). This makes lipase the better marker, especially in patients whose presentation is delayed. The disadvantage of using lipase is that its increase in serum can be seen in many diseases such as appendicitis, cholecystitis, intestinal ischemia, and renal failure (Meher *et al*., 2015). At 600 IU, the specificity is above 95%, but the sensitivity is 55-100% (Esmaili *et al*., 2017). Lipase lacks specificity to the pancreas, although it has better sensitivity than amylase.

In pancreatitis induced by ERCP, the concentration of trypsinogen increases within an hour of the injury (Matull *et al*., 2006). Urinary trypsinogen-2 is detected by a "dipstick method" devised to detect AP (Matull *et al*., 2006). The autodigestion of pancreatic tissue is the cause of premature trypsinogen (trypsinogen-1 and trypsinogen-2) activation to trypsin in a pancreatic disease (Dixit *et al*., 2016). The subsequent local and systematic inflammation is the earliest event during the initiation of AP, whereby the acinar cells secrete trypsinogen into the pancreatic fluid (Saluja *et al*., 2019). This causes the increased vascular permeability of significant quantities of trypsinogen to enter the systemic circulation (Meher *et al*., 2015). The trypsinogen activation peptide (TAP) is released and can be detected in the urine and blood of AP patients. The TAP concentration in the urine and blood rises within a few hours and decreases to standard levels within 3 to 5 days (Dixit *et al*., 2016). The dipstick method has lower specificity and sensitivity compared to amylase and lipase and despite it being a great tool in excluding the diagnosis of acute pancreatitis, it is less frequently used in routine clinical practice (Cevik *et al*., 2010).

Haematocrit (HCT) measures the volume percentage of red blood cells (RBC) in the blood. It forms part of the patient's complete blood count which is done when a patient is admitted to a medical facility due to illness (Zhou *et al*., 2019). RBC transfers oxygen from the lungs to tissues and therefore becomes a point of reference for delivering oxygen to the blood system. When HCT levels are too high or too low it can be an indication of the presence of a disease in the body

(Penttilä *et al*., 2017). The HCT test is used as an early prognostic marker for the severity of AP (Penttilä *et al*., 2017). It is a simple cost effective tool and is being used in primary health care and emergency setting for early risk stratification and early transfer to intensive care units if needed (Nieminen *et al*., 2014). However, HCT is not specific as increased hematocrit may be due to an increase in the number of red blood cells in the body or dehydration (Kundrapu and Noguez, 2018). Decreased values may be due to kidney failure, anemia, overhydration, or chronic inflammatory conditions (Kundrapu and Noguez, 2018).

Plasma procalcitonin (PCT) is an amino acid peptide of calcitonin, a hormone that is produced in humans by the parafollicular cells (neuroendocrine cells) (Maruna *et al*., 2000). It is always used to diagnose sepsis and bacterial infection as it is highly specific (Granger and Remick, 2005; Dias *et al*., 2015). It is used as an early, noninvasive, and good prognostic indicator in AP patients. In studies where procalcitonin has been compared to CRP as a prognostic indicator as well as its usefulness to predict antibiotic requirement, PCT is always accurate in identifying those patients (Chan *et al*.,2003). PCT also predicts the likelihood of organ failure and the severity of the disease (Modrau *et al*., 2005; Dias *et al*., 2015). It is more efficient as a prognostic indicator than Ranson's score and CRP. The disadvantage of PCT is the release of proinflammatory cytokines may be due to other factors such as trauma, organ hypoperfusion, and cardiogenic shock, which all raise PCT levels (Dias *et al*., 2015).

1.2.7 Cell death and pathways involved in AP.

Apoptosis and necrosis are crucial in the development of AP because cell death affects the clinical presentation of the disease. While both processes result in cell death, the events that take place are quite different (Clemens *et al*., 2014).

1.2.7.1 Apoptosis

Apoptosis is essential during growth and senescence and to maintain equilibrium for the preservation of cell populations in tissues. During apoptosis the death is programmed, it involves the pre-determined elimination of cells, and no injury to the cells is involved. The lymphocytes are produced as a defense mechanism against infection as well as when cells are attacked (Furman and Davis, 2015; Elmore, 2007). During apoptosis there is minimal inflammation because the cells undergoing apoptosis do not release their cellular components, as a result, the integrity of the

organelle is maintained throughout this process and the plasma membrane is intact. The apoptotic cells get phagocytosed by surrounding cells, there are no anti-inflammatory cytokines released and therefore the severity of pancreatitis is reduced (Bhatia, 2004). *Caspases* are cysteine proteases that cause apoptosis and function as a cascade of molecular events (Takeyama, 2005). *Caspases* are activated from their inactive forms in response to stimuli and cleave a range of substrates such as plasma membrane proteins, nuclear proteins, and mitochondrial proteins (Yang *et al*., 2015). There are initiator *caspases* (*caspase 2, 8, 9* and *10*) and the executioner *caspases* (*caspase 3, 6* and *7*) (Bhatia, 2004; Elmore, 2007; Meher *et al*., 2015). There are two pathways involved during apoptosis, the intrinsic mitochondrial pathway and the extrinsic pathway known as the *caspase* 8 pathway (Elmore, 2007).

1.2.7.2 Necrosis

Necrosis represents the death of tissue or cells; the cell membrane is ruptured during the process. The cytoplasmic components are released into the surrounding tissue and signals are sent for the recruitment of inflammatory cells (Elmore, 2007). This type of cell death causes severe AP (Takeyama, 2005). *In vivo* studies have shown that pancreatitis severity is increased when there is necrotic cell death (Zhou *et al*., 2019).

1.2.8 Inflammatory metabolic indicators and AP.

About 20 – 30 % of AP patients develop a severe form of the disease which is characterized by local or systematic inflammation and is the cause of organ failure and/or death (Leppäniemi *et al*., 2019). Blood serum and plasma are the primary sources of circulating proinflammatory mediators such as cytokines which can be used for diagnostics or therapeutics. Cytokines are secreted by the immune cells in the body and are released into the bloodstream as early as 30 minutes after the initiation of the disease. They are the perfect candidates to be used as metabolic indicators for disease progression and manipulated for therapeutic purposes (Schütte and Malfertheiner, 2008).

The CRP is a marker of severity that is synthesized by the hepatocytes and is induced by the release of Interleukin 6 (IL-6) in the liver (Meher *et al*., 2015). CRP has an 85% sensitivity in the first three days after the onset of symptoms at concentrations greater than 130 mg/L (Sproston and Ashworth, 2018). The use of CRP and Ranson's score can distinguish SAP from MAP with the specificity, sensitivity, and precision of 80.9%, 82.9%, and 81.1%, respectively (Khanna *et al*.,

2013). CRP is easy to measure and inexpensive and has become the sole biomarker to assess severity in AP (Meher *et al*., 2015). A concentration of 150 mg/L or greater is used to predict severe AP (Meher *et al*., 2015). The disadvantage is that this marker is not specific and is usually consistently elevated in inflammatory diseases such as pneumonia and cholangitis therefore, these ailments must be ruled out before the measurement of CRP *(*Khanna *et al.*, 2013).

The neutrophil-to-lymphocyte ratio (NLR) is a biomarker that conjugates the neutrophils and the lymphocytes, therefore, it merges the innate immune and the adaptive immune responses (Suppiah *et al*., 2013). Neutrophils recruit, activate and interact with other immune cells and secrete pro-inflammatory and immunomodulatory cytokines and chemokines (Selders *et al*., 2017). Neutrophils are thus highly reflective of the immune response as they fight infections (Malech *et al*., 2014). This proves that the neutrophils and lymphocytes ratio is a better marker than the white blood count (WBC) because it reflects the balance between the immune response to systemic inflammation (Malech *et al*., 2014) The neutrophils are also responsible for the release of neutrophil granules, the generation of oxidative stress and the formation of neutrophil extracellular traps (Manda-Handzlik and Demkow, 2015; Rosales, 2018).

Nuclear factor-kappa beta (NF-κB) activation is responsible for immune regulation and inflammation in the development of pancreatitis (Jakkampudi *et al*., 2016). NF-κB activation is behind the initial inflammatory cascade during AP and the subsequent death in SAP patients (Zhou *et al*., 2019). Consequently, if NF-κB and the associated inflammation are reduced, the reduced inflammation can be beneficial for pancreatitis prevention and treatment. NF-κB is currently being evaluated as a therapeutic target for AP (Jakkampudi *et al*., 2016). Some inducers of NF-κB activity are tumor necrosis factor-alpha (TNFα), interleukin 1-beta (IL-1β), and the reactive oxygen species (Dambrauskas *et al*., 2010; Jakkampudi *et al*., 2016). The NF-κB initiates the transcription processes for inflammatory factor genes (Liu *et al*., 2017). The inflammatory targets of NF-κB include immune receptors, chemokines, adhesion molecules, and cytokines (Liu *et al*., 2017).

Interleukins are produced by the immune cells to counter TNF-α (Liu *et al*., 2017). They are part of the immune response and the inflammatory process. They enable cell growth, circulation, and differentiation of inflammatory T cells (Kundrapu and Noguez, 2018). Studies have confirmed that IL-6, IL-8, and IL-10 are elevated in patients with severe pancreatitis (Fisic *et al*., 2013;

Jakkampudi *et al*., 2016; Nalisa *et al*., 2021; Rao and Kunte, 2017). IL-1β is found in the plasma and serum of AP patients, with higher levels observed in those who are seriously ill (Ueda *et al*., 2006). IL-6 is produced in the pancreas during AP. It is a great predictor of organ failure, thus a helpful severity marker for AP (Khanna *et al*., 2013). IL-6 is elevated in patients who present with severe AP and peaks at 72 hours (Meher *et al*., 2015). IL-6 induces the production of CRP and other proteins that increase or decrease in response to inflammation (Pereira *et al*., 2014). A study was done on the inhibition of IL-6 activity where a cerulein model of AP was used, and IL-6 knock-out mice were found to have substantial inflammation, suggesting that high levels of IL-6 can be used as an anti-inflammatory mediator *in vivo* (Cuzzocrea *et al*., 2002).

TNF is a cytokine that induces necrosis in tumors. Several studies have documented TNF-isolated *in-vitro*-cultured acinar cells and cerulein models of AP (Jakkampudi *et al*., 2016). TNF has also been detected in the serum of animal models when AP is induced (Granger and Remick, 2005; Clemens *et al*., 2014). Therefore, TNF suits the conventional pattern of acinar cell damage, which progresses to systematic inflammation. However, this theory worked on experimental animal models. In humans, the timing was found to be critical as TNF is rapidly cleared from blood circulation (Granger and Remick, 2005).

1.2.9 Oxidative stress in AP

Oxidative stress plays a role in the pathogenesis of many diseases. Clinical studies have shown high oxidative stress levels in AP patients compared to healthy individuals (Simon *et al*., 2000; Robles *et al*., 2013). The reactive oxygen species (ROS) are produced as products of cellular metabolism and include hydrogen peroxide, hydroxyl radical, and superoxide anion (Hayashi *et al*., 2007). These reactive free radicals change the structure and functioning of lipids, nucleic acids, and proteins.

Various factors contribute to the development of oxidative stress. These include infection, exposure to toxins, inflammation, stress, alcohol, and smoking. The pancreatic acinar cells are predisposed to oxidative stress (Armstrong *et al*., 2013), and this has been substantiated in earlier studies by Kasahara *et al* (1997) who revealed that ROS induces apoptosis in living cells. In their study, the authors compared the rates of apoptosis in neutrophils isolated from healthy controls to neutrophils of patients diagnosed with chronic granulomatous disease. They observed that

apoptosis of normal neutrophils was markedly inhibited by the reduction of intracellular levels of hydrogen peroxide (Kasahara *et al*., 1997). These findings suggest that the cell death of neutrophils may be stimulated by endogenous oxidative products. Another way ROS can be produced and released is during an inflammatory response by the circulating neutrophils. The neutrophils are involved in the events during the pancreatic injury and the systematic complications of AP (Meher *et al.*, 2015).

The ROS are generated during normal respiration due to mitochondrial electron transfer processes, which produces adenosine triphosphate (ATP). Some electrons leak and add to the oxidative stress signal (Zorov *et al*., 2014). During the development of AP, there is an overload of cytosolic calcium, which damages mitochondria affecting ATP production and resulting in cell death (Mukherjee *et al*., 2016). The ROS can also induce apoptosis by opening the pores of the mitochondria using proteins of the Bcl-2 gene (Simon *et al*., 2000). Mitochondria oxidizes and releases cytochrome c (Simon *et al*., 2000). The Bcl-2 gene is transcribed to RNA and translated into a regulator protein responsible for cell death by inducing or inhibiting apoptosis (Leibowitz and Yu, 2010).

Bile acids also induce ROS production in the acinar cells of humans and animal models (Criddle, 2016). These acids modify Ca^{2+} signaling occurrences in the acinar cell during the development of AP. The cascade of events can also lead to lipids and proteins being oxidized due to ROS release and the pancreatic membrane being destroyed (Robles *et al*., 2013). There have been efforts by scientists and clinicians to develop drugs that prevent mitochondrial dysfunction caused by Ca^{2+} (Criddle, 2016; Mukherjee et al., 2016). Criddle, (2016) and Mukherjee *et al*., (2016) attempted to maintain homeostasis of the pancreatic function that prevents the premature activation of enzymes and prevents necrosis, which is a determinant of disease progression and increases the mortality rate (Criddle, 2016; Mukherjee *et al*., 2016). These researchers investigated the potential treatment strategies that utilize antioxidant therapy by inhibiting ROS production. Criddle, (2016) found that ROS elevation and generation occurred within the mitochondrion and inhibited N-acetylcysteine, an antioxidant. While the potential treatment strategies by inhibiting ROS production using antioxidants are promising *in vitro*, the value of ROS suppression in real life appears to be less convincing. A cocktail of methionine, vitamins C and E, selenium, and β-carotene improved AP progression (Criddle 2016; Mukherjee *et al*., 2016). The ROS generated within the acinar cells,

during the inflammatory response when immune cells are activated showed the actions of antioxidants are ineffective, and they worsen the disease process (Criddle, 2016). The complex physiology and biochemistry can explain the adverse outcomes of these findings in the human body, and during AP, ROS are generated in multiple areas in the body; therefore, a blanket inhibition technique using general antioxidants may influence other processes in the cells.

1.2.10 Metabolites as metabolic indicators of AP.

A biomarker is a measured characteristic that is used as an indicator of normal or changes in biological processes (Strimbu and Tavel, 2010). The advancement of analytical techniques has made it possible to quantify and identify a wide range of metabolites as biomarkers. Metabolomics studies metabolites present in a sample; metabolites are the downstream product of genes, substances made or used when the body breaks down food, drugs or chemicals, or its tissues. Metabolomics can provide unique insight by analyzing low molecular weight compounds in biological systems (Owusu-Sarfo *et al*., 2014). Mass Spectrometry (MS) and NMR spectroscopy are well-established methods for this purpose. The advantages of NMR includes its high analytical reproducibility, easy sample preparation, and sample recovery is excellent for further analyses (Lusczek *et al*., 2013). Chromatography techniques can also be used, including gas chromatography-mass spectrometry (GC-MS) which is suitable for volatile samples and provides high resolving power (Nicholson *et al*., 2002). Liquid chromatography (LC)-MS offers the broadest coverage of compounds such as alcohols, polyamines, and lipids (Nicholson *et al*., 2002). Ion chromatography (IC)-MS is suitable for charged molecules that are difficult to analyze by LC-MS (Grebe and Singh, 2011).

Metabolic profiling has been successfully applied to investigate diseases using minimal biofluid samples such as serum and plasma obtained from the blood by centrifugation methods. Costello (2018) investigated the potential of utilizing metabolomics techniques such as GC-MS and LC-MS for pancreatic cancer diagnosis. Plasma and serum samples were used for analysis and produced a validated biomarker signature that faultlessly distinguished pancreatic cancer from chronic pancreatitis. Although the biomarker signature was not inferior to CA19-9, an antigen released by pancreatic cancer cells, it was sufficient for distinguishing cancer from non-pancreatic controls. A total of 477 metabolites were identified the majority being lipids and fatty acids.

Xiao *et al*. (2017) were able to characterize AP samples against the healthy controls in serum using metabolomics. In this study, AP patients were found to have higher levels of fatty acid, hexadecanoic acid than the healthy controls. The mannose, galactose, and glucose levels were also elevated. These findings confirmed that fatty acid metabolism in AP pathogenesis should receive attention as altered fatty acid metabolism can cause hyperglycemia, a disease that affects people with diabetes. In the severe AP cases, the metabolites found in abundance were citric acid and 3-hydroxybutyric acid. The results suggest that these metabolites may be possible biomarkers for stratifying mild and severe patients (Xiao *et al*., 2017). AP diagnosis is mainly by the detection of amylase/lipase levels. Multivariate analysis differentiated the AP patients from healthy volunteers. Concentrations were higher for metabolites such as d-mannose, d-galactose, and d-glucose. Changes were also noted for 3-hydroxybutyric, glycerol, serotonin, phosphoric acid, citric acid, and hexadecanoic acids and they were all adopted as possible biomarkers for AP detection (Xiao *et al*., 2017). Another study by Lou *et al*., (2022) identified four biomarkers for AP stratification and prognosis which were eicosatrienoic acid (C20:3), thiamine triphosphate, 2-Acetylfuran, and cis-citral. These biomarkers were from plasma extracellular vesicles whose function is to deliver nucleic acids, proteins, and metabolites to recipient cells.

In a recent study in South Africa by Candy *et al*. (2017), the authors used serum samples of chronic pancreatitis and pancreatic cancer patients to determine differences in metabolites. The branched-chain amino acids such as valine and leucine decreased in pancreatic cancer whereas they remained unchanged in chronic pancreatitis. They also found altered lipid concentrations and ketone bodies in pancreatic cancer (Candy *et al*., 2017). Hypermetabolic conditions in acute pancreatitis due to the early release of pancreatic enzymes lead to complications such as organ and tissue damage. These conditions result in inflammation due to the disruption in the homeostatic metabolism of lipids, carbohydrates, and amino acids in the body. Additionally, the resulting inflammatory response due to the damage in the pancreas' cells triggers a hypercatabolic state, resulting in increased energy requirements for protein, lipid, and nucleotide synthesis, and catabolism of major nutrient substrates (Lakananurak and Gramlich, 2020). Conventional metabolic studies have identified disturbed lipid class changes such as unsaturated fatty acids elevations in AP patients and the associated episodes tend to be more severe (Kota *et al*., 2013; Peng *et al*., 2021). The main parameters of the lipid profile which are low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein (VLDL-C) triglycerides (TG), are altered during biliary acute pancreatitis

(Hong *et al*., 2018). Taken together, the existing immune, biochemical, and metabolic metabolic indicators of progressive AP are associated with biosignature represented by low CD4:CD8 ratio, high NLR, necrosis, high ROS levels, and perturbed lipid metabolism. Table 1.1 below shows parameters that are involved in the pathogenesis and affect the severity of AP. These studies used conventional metabolic assays and only focused on MAP, SAP, and the healthy controls, they did not investigate MSAP patients.

Table 1.1 Parameters that are involved in the pathogenesis and affect the severity of AP.

Parameter	MAP	SAP	Reference
CD4:CD8 ratio	No changes of T lymphocytes in patients with mild acute pancreatitis	Cytotoxic T cells are decreased in SAP. A reduced number of cytotoxic T cells is associated with overexpression of Fas/FasL, which induces apoptosis of lymphocytes in AP. Significant decrease of both CD4+ and CD8+ T cells in SAP and associated with SIRS	(Wang *et al*., 2021) (Ding *et al*., 2020; Pietruczuk et al., 2006) (Shen *et al*., 2015) (Pinhu *et al*., 2014) (Yang *et al*., 2015)
NLR	NLR was lower in the mild AP groups than in the severe AP groups	Elevated baseline NLR correlates with severe acute pancreatitis and organ failure. The neutrophil-to-lymphocyte ratio is associated with severe AP. NLR was more sensitive and accurate in judging the severity of HTG-AP compared with WBC.	(Jeon and Park, 2017) (Kokulu *et al*., 2018) (Huang *et al*., 2019)
Apoptosis	MAP is associated with extensive apoptotic acinar cell death. Caspases mediate apoptosis also protects from necrosis	SAP involves extensive acinar cell necrosis but very little acinar cell apoptosis. Pancreatitis correlates directly with necrosis and inversely with apoptosis. Mitochondria dysfunction-mediated oxidative injury releases proteases to degrade cytosolic proteins that cause pancreatic acinar cell death	(Bhatia, 2004) (Kang *et al*., 2014) (Mareninova *et al*., 2006)
Oxidative stress		The oxidation of lipids and proteins and disruption of the pancreatic membrane Oxidative stress affects the severity of the systemic inflammatory response. Increased concentrations of lipid peroxides in the plasma and tissue of AP patients	(Criddle, 2016) (Armstrong *et al*., 2013) (Robles *et al*., 2013) (Schulz *et al*., 1999)
Metabolites		Differences in cis-11,14,17-eicosatrienoic acid (C20:3), thiamine triphosphate, 2-acetyl furan, and cis-citral of MAP and SAP samples. Metabolites of intestinal microflora as therapeutic approaches for the treatment of SAP.	(Lou *et al*., 2022) (Ye *et al*., 2021)

1.3 Study rationale

AP has diverse presentations, the pathophysiology is unknown, and the clinical heterogeneity of the disease makes prognosis difficult. Various severity scoring systems are utilized to assist clinicians in triaging AP patients and predict their prognosis (Sharma *et al*., 2015). However, these systems are laborious; they make use of many parameters which makes them difficult to use in assessing patients during admission. There have been many attempts to develop a laboratory tool that can be used to predict disease severity and progression in AP with ease that is cheap, rapid, and reliable (Werner *et al*., 2003). In response to the pancreatic injury, pro-, and anti-inflammatory mediators are released by the immune cells into the bloodstream as early as 30 minutes after the initiation of the disease. Thus, they are the ideal candidates to be used as metabolic indicators for diagnosis, and disease progression and to be manipulated for therapeutic purposes.

Previous studies have been done to test lipase and amylase; lipase was found to lack specificity to the pancreas but has better sensitivity, whereas, with amylase, the disadvantage is that its activity may also be raised in other intra-abdominal pathologies or renal insufficiency (Gomez *et al*., 2012). Hematological components such as neutrophil to lymphocyte ratio (NLR) were investigated to predict disease severity and for use as immune biomarkers of AP disease (Cho *et al*., 2018). NLR is a great biomarker because it reflects the balance between the immune response to inflammation. Other biomarkers which are currently being used for diagnosis and prognosis of AP lack specificity. The biomarkers which lack specificity to the pancreas include CRP, which can predict severity, HCT is usually consistently either elevated or lower in inflammatory conditions and PCT, which is a great prognostic marker (Asim Riaz *et al*., 2022; Meher *et al*., 2015). IL-6 can diagnose severe AP and TNF recruits inflammatory cells and promotes apoptosis; however, these biomarkers get cleared quickly in the blood (Asim Riaz *et al*., 2022; Meher *et al*., 2015).

The link between the immune response, apoptosis, and the reactive oxygen species makes it essential to use metabolomics to hopefully identify linkages to the observed immune perturbations in AP. This study sought to establish the link between T lymphocyte ratio, apoptosis, and ROS and the metabolites secreted in the different AP severities i.e., MAP, MSAP, and SAP and over time. Findings should inform on the potential of using these metabolic parameters as prognostic metabolic indicators that may be more accurate in stratifying the risk profiles of patients at the early phase of AP and potentially improve treatment strategies.

1.4 Aim

The main aim of this study was to establish a relationship between the immune response, apoptosis, and oxidative stress in the biofluid of pancreatitis patients with the metabolites and their potential for monitoring disease in different severities of acute pancreatitis.

1.5 Study objectives

The objectives of the study are:

- To measure the ratio of CD4+/CD8+ T lymphocytes in patient samples compared with healthy controls.
- To determine the expression of apoptotic genes in PBMCs from patients with different severities of AP using real-time PCR (polymerase chain reaction).
- To measure ROS in the serum and plasma of AP patients.
- To use NMR spectroscopy to obtain metabolic profiles of mild, moderate, and severe AP patients.
- To correlate clinical and research parameters.

In **Chapter 2**, the overall patient demographics will be discussed. This will be followed by **Chapter 3** where we cover the immune parameters, specifically lymphocyte ratios, changes in apoptosis genes, and ROS. In **Chapter 4**, the findings from the metabolic profiling of the samples using NMR will be provided. In **Chapter 5**, we provide a summary of the findings and correlation from the clinical and laboratory-based results to find links between the immune responses, apoptosis, oxidative stress and prominent metabolites and to provide insights for translational application of the findings. In **Chapter 6,** we revisit the objectives and provide overall recommendations, limitations and conclusions.

CHAPTER 2: DEMOGRAPHIC AND CLINICAL DATA

2.1 Introduction

This chapter looks at the demographics such as sex, age, aetiology, and the biochemical parameters used in the diagnosis of acute pancreatitis patients. The major risk factors associated with AP are excessive alcohol consumption and gallstones. AP aetiology incidences vary significantly among populations and are region-specific. Approximately 35% of acute pancreatitis cases are associated with alcohol abuse in developing countries (Clemens *et al*., 2014). In South Africa, there has been an increase in patients admitted with gallstone disease within the African population (Khan *et al*., 2020). Most cases of AP are mild and resolved without serious complications (Gapp and Chandra, 2021). The severe form of AP is associated with high morbidity and mortality (Zerem, 2014).

Pancreatitis associated with gallstones is a costly pathology. Cholecystectomy is an expensive surgical procedure for the treatment of symptomatic gallstones and other gallbladder conditions. Gallstones (cholelithiasis) are caused by abnormally high levels of either cholesterol or bilirubin in bile (Tanaj a *et al*., 2021, Kim *et al*., 2017). Pancreatic duct obstruction promotes pancreatitis by increasing duct pressure in the pancreas and releasing pancreatic enzymes (Wang *et al*., 2009). The adoption of the western diet by Black South Africans has played an enormous role in the increase of biliary AP (Khan *et al*., 2020). This diet includes eating high-cholesterol, high-fat, low-fibre, and inactive lifestyles, leading to people being overweight and obese. The body mass index (BMI) has pointed out the risk of obesity in patients with AP and has been included as a criterion for predicting severity (Dobszai *et al*., 2019). A BMI of 25 kg/ m^2 or higher represents 'overweight' while 'obesity' is defined as a BMI equal to 30 kg/m^2 or higher. The prevalence of gallstones has increased significantly with age and the female gender also has a significant contribution to biliary-associated AP (Kim *et al*., 2017). In younger people, the gallstones are smaller and so more likely to attempt passage out of the gall bladder (Heaton *et al*., 1991). Oestrogen increases gallstone formation by enhancing hepatic synthesis and secretion of cholesterol and reducing bile salt synthesis. This may explain, in part, why gallstone prevalence is higher in women than in men (Sun *et al*., 2009). AP associated with alcohol is treated by analgesics, intravenous fluid therapy, and electrolyte replacement (Klochkov *et al*., 2021). Additionally, a nutritionist and repeated counseling appointments are recommended by the health

care workers to get patients to decrease alcohol use. Intensive care for organ support is also administered for those patients diagnosed with MSAP or SAP. Pancreatitis associated with alcohol is highest in males between 35 and 54 years old (Klochkov *et al*., 2021). In some cases, the recurring attacks of alcoholic AP progress to chronic pancreatitis, which is 20 times more likely to lead to the development of pancreatic cancer, a disease with a grim prognosis (Klochkov *et al*., 2021).

Understanding the mechanisms by which ethanol predisposes acinar cells to damage promises targets for therapeutic intervention (Gorelick and Thrower, 2009). Several factors are believed to be triggers of alcoholic pancreatitis. Alcohol and its by-products sensitize the pancreas triggering the initiation of AP (Clemens *et al*., 2014). Additional risk factors for AP associated with alcohol are: smoking cigarettes, genetic predisposition and high lipid diet (Yadav and Lowenfels, 2013). The other risk factors of AP are endoscopic retrograde cholangiopancreatography (ERCP), anti-retroviral treatment, type II diabetes and sometimes, a reason for pancreatitis is never found.

The symptoms of AP are pain that radiates to the back, vomiting, and nausea. However, doctors run biochemical tests to confirm the clinical diagnosis of the disease. Amylase and lipase are the most commonly used biometabolic indicators for the diagnosis of AP (Matull *et al*., 2006). Amylase is produced in the pancreas as well as in the salivary glands, it is also present in small quantities in some tissues, and it breaks down carbohydrates during digestion (Meher et al., 2015). Lipase is produced in the salivary glands, stomach, and pancreas; it breaks down fats for easy absorption in the small intestine (Meher et al., 2015). CRP is a marker of severity that is synthesized by the hepatocytes (Stirling *et al*., 2017). HCT measures the volume percentage of red blood cells (RBC), the RBC transfer oxygen from the lungs to tissues (Penttilä *et al*., 2017). Haemoglobin is iron-rich protein found in the RBC which carries oxygen throughout the body (Pérez *et al*., 2015a). Platelets are colorless fragments that form clots, they prevent or stop bleeding and are made in the bone marrow or they assist in the prognostic evaluation during AP (Kefeli *et al*., 2014). The combination of these biometabolic indicators is essential in the diagnosis and prognosis of AP.

2.2 Objective

To assess the aetiology, demographic characteristics, and clinical parameters and outcomes of AP patients recruited for this study. The findings will be used to make conclusions on the other laboratory-based investigations by correlating them to clinical outcomes.

2.3 Materials and methods

2.3.1 Recruitment of patients

Ethics clearance for this study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (Ethics No. M190407) which was part of the bigger study (Ethics No. M180133), **Appendix I** and **II** respectively. The Revised Atlanta Classification (RAC) system was used to recruit participants at the Chris Hani Baragwanath Academic Hospital (CHBAH). Participants included in the study were: patients whose age was greater or equal to 18 years old, who self-identified as of African descent, and patients who had started to experience AP-related symptoms within 72 hours of admission. 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 for the following reasons: those patients who had been diagnosed with a chronic medical condition associated with inflammation, including auto-immune diseases, patients who were undergoing recent (within one month of current admission) therapy with immunomodulatory medications and patients with comorbidities requiring the use of antibiotics within a month before admission.

Clinical parameters which are currently being used for the AP diagnosis were documented from the patient files according to the clinician's notes in the datasheet in **Appendix VII**. The clinical data were recorded from the day of patient admission and each day of sample collection. Only data collected on day 3 was assessed since this was the day with many more data points and will be used in the subsequent chapters for correlation studies i.e., for oxidative stress analysis and the analysis of the metabolites. Demographic data such as age, gender, and weight of our healthy controls were also collected in **Appendix VIII**.

2.3.2 Sample collection

Blood samples were collected from fifty-five patients. The PBMCs, serum, and plasma were prepared through centrifugation using standard protocols. The collected samples were classified according to the RAC i.e., MAP n = 33, MSAP n=14, SAP n = 8, and the 10 healthy controls.

2.4 Data Analysis

Data analysis and relevant figures were generated in Microsoft Excel version 7 (Washington, USA). The age, body mass index (BMI), and length of hospital stay (LOS) were presented as median and interquartile ranges. The Wald test was used to calculate the statistical significance with p-values < 0.05 considered as statistically significant. To account for multiple testing, a false discovery rate (FDR) of <10% was applied for the LFTs parameters.

2.5 Results

2.5.1 Patient Demographics and characteristics

Patients were recruited according to the RAC from August 2018 until March 2021. A total of sixty-five patients diagnosed with AP who met the prescribed inclusion criteria were considered for the study. Fifty-five AP who fit the recruitment criteria were enrolled in the study. Ten patients were excluded based on: refusal to consent (n=3) and the recruitment period was beyond the first week of disease onset (n=7). When looking at the sampled population, the gender distribution was 56% (31/55) females and 44% (24/55) males **Figure 2.1**. The severity distribution was MAP 33 (60%), MSAP 14 (25%), and SAP 8 (15%) **Figure 2.2**. The most common aetiology of AP was biliary 26 (47.2%), alcohol 25 (45.5%), antiretrovirals (ARVs) 3 (5.5%), and ERCP 1 (1.8%) **Figure 2.3**.

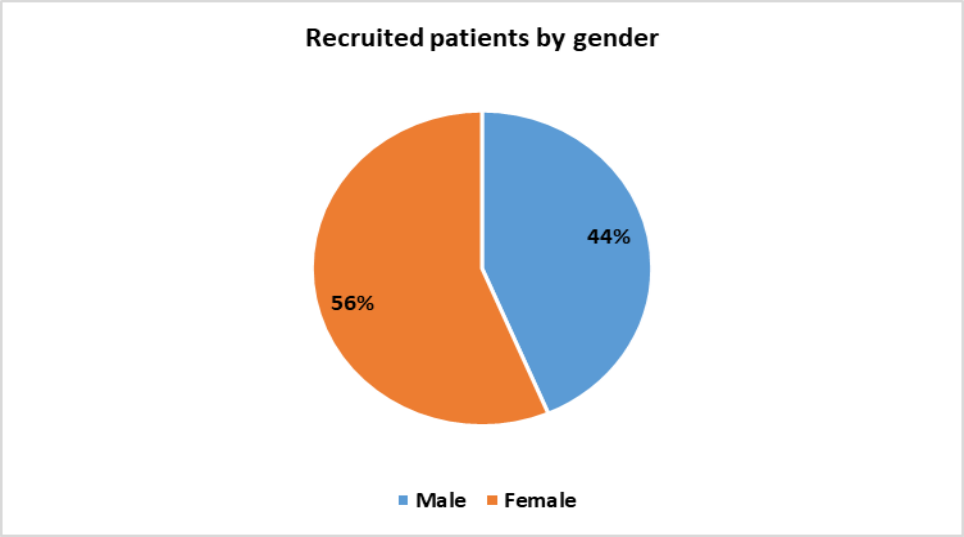


Figure 2.1: The percentage (%) of AP patients by gender. The distribution of gender in our study consisted of 44% (n=24) males and 56% (n=31) females.

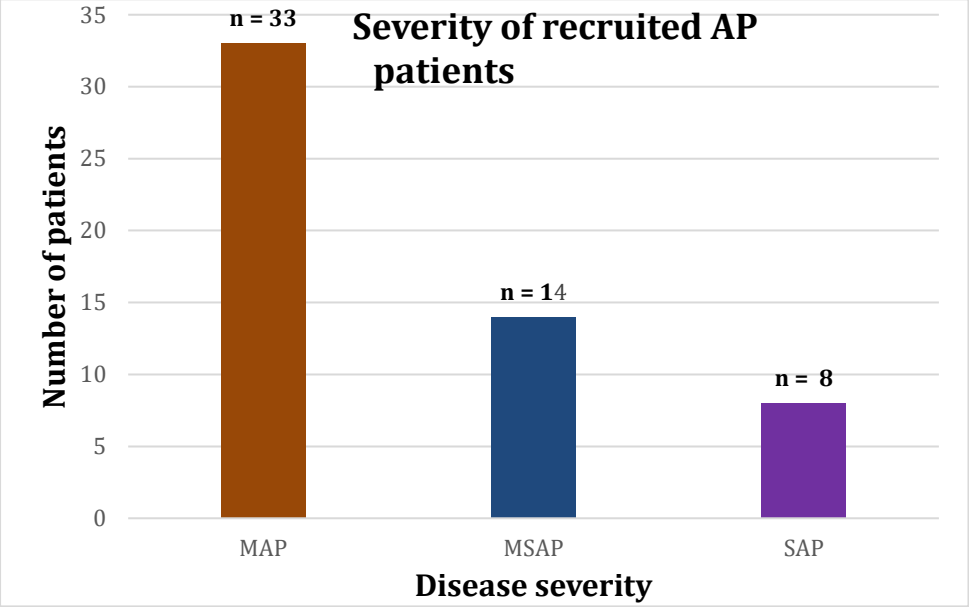


Figure 2.2: The number of recruited AP patients by severity. The severity of our AP patients' cohort consisted of MAP=60% (n= 33) MSAP=25% (n= 14) and SAP=15% (n=8). Notes: MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

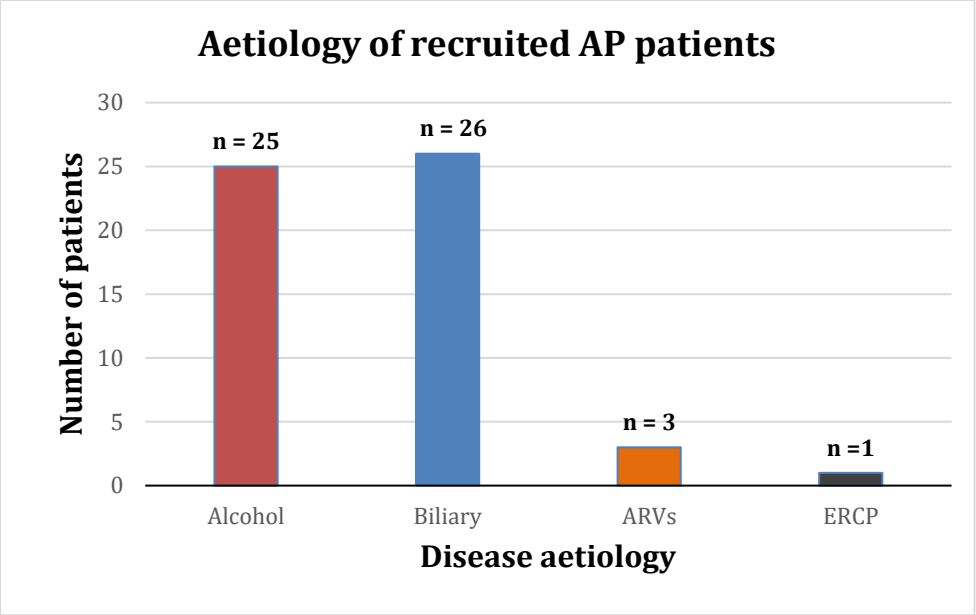


Figure 2. 3: Aetiology and risk factors of recruited AP patients. Biliary-associated pancreatitis had 47.2% (n=26) patients while alcohol aetiology had 45.5% (n=25). The ARVs and ERCP had 5.5% (n=3) and 1.8% (n=1) patients respectively. **Notes**: ARV – antiretroviral drugs, ERCP: Endoscopic retrograde cholangiopancreatography.

Out of the fifty-five recruited AP patients with a median age of 38, the interquartile range (IQR) was 26 to 76 years, and 41 participants had the data required for calculating the BMI, Table 2.1. AP patients who were categorized as obese made up 48.80 % (n = 20) of the population and from these 80 % had biliary-associated AP diagnosis. Those who were overweight were 17% (n = 7) while 24.4 % (n = 10) were healthy and 9.76% (n = 4) were underweight. The LOS of our study participants was six with an IQR of 2- 24 days for the MAP, eleven with an IQR of 5- 20, for the MSAP, and thirty-one with an IQR of 13- 40 days for SAP. The SAP and the MSAP groups of patients had the most number of patients who were admitted into the ICU and those who died in the hospital, four and five patients respectively. MAP and MSAP patients had the most number of patients who underwent surgical procedures such as cholecystectomy and ERCP at eight and two respectively. The commonest comorbidity was found to be human immunodeficiency virus (HIV) infection at 21.8 % (n= 12) followed by type II diabetes at 7.3% (n = 4). The PLWH were on antiretroviral therapy (ART). Two patients were both HIV positive and had type II diabetes. Six patients died in the hospital while being admitted to ICU due to organ failure, one patient had MSAP was male and had alcohol-associated AP and the other five had SAP. Two female patients

were on ART treatment for HIV and 1 of them had Type II diabetes. Two patients had alcohol-associated SAP and were female and only one male SAP had a biliary-associated AP.

Table 2.1 Demographic and clinical characteristics of the patients recruited into the acute pancreatitis study.

Parameter	Total cohort (N=55)
Demographics	
Age in years [median (IQR)]	38 (26-76)
BMI kg/m^2 [median, (IQR)]	26.6(9.76 – 48.8), (n=41)
MAP [median, (IQR)]	24.8 (16.6-54.3)
MSAP [median, (IQR)]	26.2 (17.3-32.5)
SAP [median, (IQR)]	34.0 (26.6-41.4)
Hospital outcomes∞	
Median length of Hospital Stay (LOS) MAP [median; IQR]	6 (2-24)
Median LOS MSAP [median; IQR]	11 (5-20
Median LOS SAP [median; IQR]	31 (13-40)
Total Number of ICU admissions [n, %]	8(20)
MAP group (n)	1(12.5)
MSAP group (n)	3(37.5)
SAP group (n)	4(50)
Death in hospital [n, %], Total	6(10.9)
MAP group (n)	0
MSAP group (n)	1(16.6)
SAP group (n, %)	5(83.3)
Surgical procedures	
Total Laparoscopic cholecystectomy [n, %]	7(18)
MAP group (n)	6
MSAP group (n)	1
SAP group (n)	0
ERCP [n, %],	4(10)
MAP group (n)	2
MSAP group (n)	1
SAP group (n)	1
Comorbidities	
HIV positive [n, %]	12(21.8)
Diabetes [n, %]	4 (7.3)

Notes: LOS - length of stay, ICU - Intensive Care Unit, BMI - Body Mass Index, IQR - interquartile range, ARVs - antiretroviral drugs, ERCP - Endoscopic retrograde cholangiopancreatography, HIV - Human immunodeficiency virus, MAP - mild acute pancreatitis, MSAP - moderately severe acute pancreatitis, SAP - severe acute pancreatitis.

2.5.2. Routine Biochemical tests

The data collected on day 3 of the onset of epigastric pain from patients (day 3 had the most clinical data collected for all our patients) is shown in **Table 2.2** below. No statistical significance was observed between the severity of AP and routine biochemical tests. The median values of liver function parameters (LFTs), which are total bilirubin, conjugated bilirubin, alanine transaminase (ALT), aspartate transaminase (AST) and Alkaline phosphatase (ALP) were elevated above the physiological range for SAP group of patients.

Table 2.2: Routine haematological parameters used for the diagnosis of AP.

Features	Physiological range	Mild, median	Moderate, median	Severe, median	*p*-value	FDR
Amylase (U/L)	28- 110	1009	652	794	0.820	0.865
Lipase (U/L)	13-60	649	760	1170	0.640	0.846
WBC (10x^9/L)	3.92-10.40	10.8	12.75	8.2	0.538	0.846
Haemoglobin (g/dL)	13.4- 17.5	12.8	14.35	13.95	0.328	0.846
Haematocrit, (L/L)	0.390 - 0.510	0.35	0.4	0.4	0.142	0.846
Platelets (10x^9/L)	171-388	230	227.5	224.5	0.668	0.846
Na (mmol/L)	136- 145	136	136	137	0.618	0.846
K (mmol/L)	3.5- 5.1	4.2	4.3	4.5	0.523	0.846
Cl (mmol/L)	98-107	96	100	85	0.578	0.846
Urea (mmol/L)	2.1-7.1	4	8.75	9.6	0.364	0.846
C Reactive protein (mg/L)	<10	83	115	232	0.793	0.865
Liver function test						
Total protein, (g/L)	60-78	71.5	69	62	0.391	0.846
Albumin, (g/L)	35-52	37	35.5	34.5	0.391	0.846
Total bilirubin, (µmol/L)	5-21	20	17.5	48.5	0.400	0.846
Conjugated bilirubin (µmol/L)	0-3	12	5.5	31.5	0.468	0.846
Alanine transaminase, (U/L)	10-40	34	33.5	102.5	0.509	0.846
Aspartate transaminase (U/L)	15-40	35	36	164	0.102	0.846
Alkaline phosphatase (U/L)	53-128	91	111	144	0.901	0.901
Gamma-glutamyl transferase (U/L)	< 68	375	119	397	0.749	0.865

Notes: MAP - mild acute pancreatitis, MSAP - moderately severe acute pancreatitis, SAP - severe acute pancreatitis, FDR- false discovery rate.

From **Figure 2.4A,** it is evident that AP patients with values of CRP higher than 150 mg/L or a value of Albumin lower than 35 g/L had a higher incidence of in-hospital death or organ

dysfunction. These were patients: CHAP013, CHAP014, CHAP017, CHAP046, and CHAP052. A comparison of the biochemical tests among different aetiologies of AP showed that both amylase ($p = 0.000875$), ALP ($p = 0{,}000153$), and ALT, ($p = 0.0134$) were statistically significant, **Appendix IX**. Although both amylase and ALP showed a statistically significant and higher concentration in biliary-associated AP, according to **Figure 2.4B,** only their combination can clearly identify biliary-associated AP from alcohol-associated AP. The median values of LFTs, which are total bilirubin, conjugated bilirubin, ALT, AST, and ALP were elevated above the physiological range for the SAP group of patients **Table 2.2**.

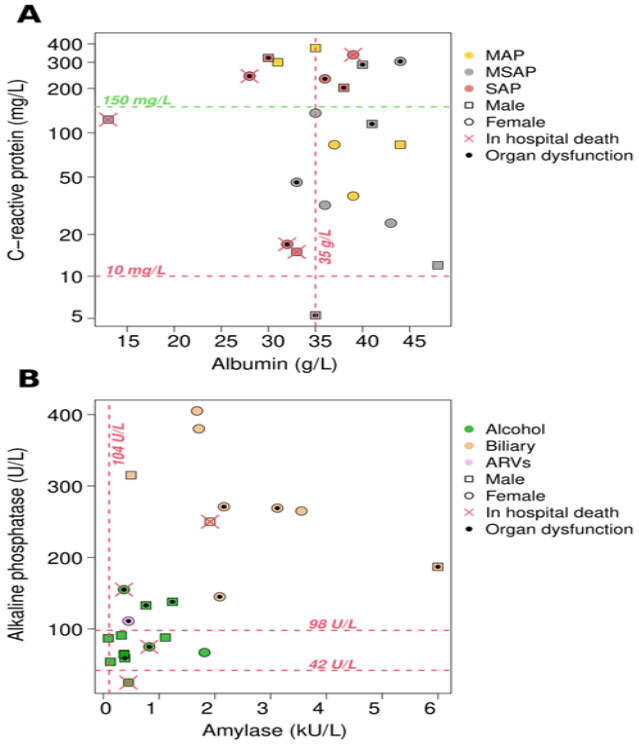


Figure 2.4 Plots showing (**A**) the correlation between albumin and CRP, CRP levels>150mg/L and albumin<35g/L are associated with an increase in the incidence of hospital death while (**B**) ALP values above 200 U/L correlate with biliary AP.

The biochemical tests: ALT showed statistical significance among the 3 different aetiologies of AP with (p-value=0.013; FDR=0.085) and AST did not show any statistical significance (p-value=0.074; FDR=0.209) as shown in **Table 2.3**.

Table 2.3. Comparison of biochemical tests for the different aetiologies of acute pancreatitis

Feature	Biliary, median [IQR] (N=12)	Alcohol, median [IQR] (N=16)	ARVs, median [IQR] (N=2)	p-value	FDR
Amylase	1914 [1696.5 2642]	450 [370.5 794]	450 [450 450]	0.001	0.008
Lipase	1129.5 [529.5 1678.25]	749 [489.75 1031]	NA	0.339	0.496
WBC	13.1 [10.9 14.7]	8.05 [6.875 10.875]	12.1 [8.95 15.25]	0.032	0.154
Haemoglobin	13.4 [11.2 14.8]	13.95 [12.675 15.25]	12.55 [11.525 13.575]	0.733	0.871
Haematocrit	0.4 [0.325 0.475]	0.4 [0.35 0.4]	0.4 [0.35 0.45]	0.914	0.961
Platelets	235 [212 303.5]	209.5 [133.75 252.75]	264 [256 272]	0.183	0.387
Na	140 [135 140]	134.5 [129 137]	137 [137 137]	0.240	0.432
K	4.4 [4 27.375]	4.4 [3.95 5.175]	3.2 [3.2 3.2]	0.325	0.496
Cl	54 [29.2 83.5]	100 [96 101]	97 [97 97]	0.570	0.774
Urea	7.5 [5.8 8.8]	8.5 [2.9 12.45]	8.7 [8.7 8.7]	0.961	0.961
C.Reactive.protein	46 [32 136]	162.5 [70.25 297.25]	337 [337 337]	0.145	0.344
Total. protein	66.5 [60.75 71.25]	66 [57.5 72]	62 [62 62]	0.908	0.961
Albumin	36 [35 38.5]	35 [31 41]	29.5 [24.75 34.25]	0.715	0.871
Total.bilirubin	42.5 [15.5 91.75]	15 [8 21]	31 [22 40]	0.250	0.432
DBil.	21.5 [11.25 57.5]	5 [4 6]	26 [16.5 35.5]	0.077	0.209
ALT.	160 [82.5 232]	23 [22 37]	27 [22 32]	0.013	0.085
AST.	91.5 [44.25 231.75]	33 [27.25 46.25]	50 [42 58]	0.074	0.209
ALP.	267 [231.25 304]	75 [64.25 90.25]	93.5 [84.75 102.25]	<0.001	0.003
GGT.	468 [328.5 1028.25]	175.5 [65.25 317.75]	192 [117.5 266.5]	0.053	0.200

2.6 Discussion

Hospitals have seen an increase in emergency admissions due to AP in recent years. Globally, the incidence of AP has risen by a factor of 10 (Iannuzzi *et al*., 2022). In South Africa, no incidence studies have been reported except for the two prevalence studies which were done in the 90s (Funnell *et al*., 1993; John *et al*., 1997). In both studies, alcohol was found to be the main cause of AP. A study by Anderson *et al*., (2008), also revealed that alcohol was the main aetiology of AP. However, in recent years, biliary-associated AP has become the main aetiology in Europe and the United States of America (Roberts *et al*., 2017; Sharma *et al*., 2021; Yadav and Lowenfels, 2013). Studies were done on the African population in South Africa, by Thomson *et al*., (2018), and biliary-associated AP accounted for 50% while alcohol was 38%. In a recent study by Nalisa *et al*., (2021b), both the main aetiologies of AP had an equal number of patients 44% for both biliary and alcohol-associated AP.

In our current investigation, biliary AP was the main aetiology of AP at 47.2 % compared to the 45.5% alcohol with the majority of AP cases being female. Biliary-induced AP has surpassed alcohol-induced AP due to the changes in the lifestyle of Black South Africans resulting from urbanization, access to fast foods, and inactive lifestyles. Recently, Khan *et al*., (2020) examined cholecystectomy rates for gallstone disease in South Africa. An increase in costly surgical

procedures such as ERCP and laparoscopic cholecystectomy, which are performed to remove stones or any blockages in the pancreas, suggests an increase in biliary disease.

Age is a risk factor for AP (Roberts *et al*., 2017), the median IQR for the patients in our AP study was 38 years old. The age of AP patients was associated with biliary etiology, i.e., older female patients are more likely to have biliary-associated AP in European countries (Gullo *et al*., 2002; Sharma *et al*., 2021). Thus, the aetiology of AP is affected by gender, age as well as geographical region. The risk of biliary AP increases with age and alcohol-associated AP affects younger individuals (Yadav and Lowenfels, 2013). As of 2021, the population in South Africa is approximately 60 million, with a majority of the population falling under 0- 44 years old (South Africa: Population, by Age Group 2021 n.d.). This means that South Africa consists of a younger population that is prone to the social lifestyle of eating fast foods, drinking alcohol, unhealthy lifestyle without any physical activities. The World Health Organization (WHO) defines overweight and obesity as excessive fat accumulation (Goetjes *et al*., 2021). Women are more obese and overweight compared to men in South Africa and the obesity risk among women increases with age (Goetjes *et al*., 2021).

When looking at the BMI of the patients recruited into this study, the median was 27, while for MSAP and SAP patients it was 26 and 34 respectively. Consumption of simple sugars and saturated fat has been mostly associated with a higher risk of obesity and several clinical investigations have shown that obesity is a risk factor for AP (Emerenziani *et al*., 2019; Khatua *et al*., 2017; Moran *et al*., 2018). Obesity can also negatively impact clinical outcomes mostly by reducing response to a specific treatment, which results in longer hospital stays, admission to ICU, and even death (Pandol *et al*., 2007).

MAP resolves within the first week of hospital admission and has been associated with apoptotic acinar cell death which is a form of programmed cell death that does not lead to the destruction of the cell. The median IQR for the length of hospital stay for our study group was six days, simply because 60% of our study population was MAP, and symptoms in the mild group resolved within a week. It is not a surprise then that SAP and MSAP patients experience longer hospital hospitalization, admission to ICU, and hospital death because they experience the relentless form of the disease (Zerem, 2014). LOS stay was longer in MSAP and SAP patients with a median IQR of eleven and SAP median IQR of thirty-one respectively. Of the patients who were admitted to

ICU, 50% were SAP patients and in-hospital death were six, one MSAP, and five SAP. The severe form of AP is associated with high morbidity and mortality (Yadav and Lowenfels, 2013).

Previous studies have shown that the prevalence of comorbidities may affect clinical outcomes during the course of AP (Moran *et al*., 2018). This is also true for our study population where these factors correlate with the severity of acute pancreatitis. Within Sub-Saharan Africa, South Africa has the highest prevalence of HIV infections compared to respiratory infections, malaria, and diarrhoeal diseases *(Lim et al*., 2016). And thus, despite the increases over the past two decades in the Department of Health's share of gross domestic product (GDP) by the government, most of the allocated budget goes to ART and TB treatment, leaving little to no money allocated for other diseases (Blecher *et al*., 2016). Almost half of the population in South Africa is infected with HIV and is on ART treatment (Anderson and Thomson, 2017). Hence, it is not a surprise that 12 (21.8%) AP patients recruited into our study cohort were already on ART treatment, which is a risk factor for AP. Additionally, the prevalence of HIV infection is associated with an increase in gallstone-related pancreatitis in women (Anderson and Thomson, 2017).

The severe form of AP (SAP), which is necrotizing and life-threatening, develops in 20 – 30 % of AP cases and organ failure (OF) persists for more than 48 hours (Houghton *et al*., 2018). In our study group, 7.3 % of cases had type II diabetes, and some studies have suggested that patients with type II diabetes are likely to develop pancreatitis (Urushihara *et al*., 2012; Richardson and Park, 2021). There are some data to suggest that diabetes predisposes patients to structural changes in the pancreas and an increased risk of type II diabetes mellitus has been reported recently in several countries (Girman *et al*., 2010; Urushihara *et al*., 2012).

Serum amylase and lipase tests are used only to diagnose AP and do not predict severity. The sensitivity of amylase is reduced by late presentation whereas serum lipase activity remains elevated for up to two weeks (Matull *et al*., 2006; Rompianesi *et al*., 2017). The downside of using lipase is that this enzyme is not specific to the pancreas, it is raised in other intra-abdominal diseases (Matull *et al*., 2006). An increase in ALP indicates an obstruction in the ducts which makes ALP a useful marker for biliary AP (Güngör, Çağlayan, *et al*., 2011). Knowing the aetiology of pancreatitis, the associated risk factors, and the role of biochemical tests helps to administer the appropriate treatment strategies for biliary pancreatitis patients.

2.7 Conclusion

AP is a non-communicable disease that may lead to death in MSAP and SAP individuals. Our study cohort had an almost equal number of biliary and alcohol-associated AP. This is mainly due to the type of lifestyle patterns of the study population. Biliary AP is associated with obesity and being overweight, female gender, and older age. Alcohol-associated AP is more common in young males. The complications associated with AP are experienced by the MSAP and SAP because of the inflammatory response following the pancreatic injury which may lead to organ failure and death. These patients require specialized treatment, hence admission to ICU and longer hospitalization to recover. The presence of comorbidities such as HIV infection and being on ART treatment as well as type II diabetes were also risk factors in our study group. The routine haematological metabolic indicators did not show statistical significance, however, the LFTs were out of the physiological range in SAP patients. Additionally, amylase and ALP in combination were able to distinguish between the two main aetiologies of AP. Whilst ALT was able to distinguish between the three aetiologies of our recruited AP patients i.e., alcohol, biliary and ARVs.

CHAPTER 3: IMMUNE RESPONSE CHANGES WITH ACUTE PANCREATITIS SEVERITY

3.1 Introduction

The infiltrating immune cells are involved during the pathogenesis of AP and they also influence disease severity (Peng *et al*., 2021). The complications which occur in AP such as organ failure which may lead to death in 20 -30 % of AP cases are due to the excessive inflammation in response to the pancreatic injury (Garg and Singh, 2019). Thus, immunomodulatory therapy can potentially be used to regulate the imbalance of the inflammatory response and improve the prognosis of AP patients. Other factors such as the activation of caspases, which contributes to cell death and oxidative stress play a role in the pathogenesis and inflammation in AP (Kang *et al*., 2014; Robles *et al*., 2013). Oxidative stress can exacerbate inflammation when there is an imbalance in the production and elimination of reactive oxygen species (Albano *et al*., 2022; Hussain *et al*., 2016).

3.2 The immune cells and AP

The immune system is made up of a variety of immune cells which perform specific tasks aimed at recognizing and attacking invaders. The innate immune cells are the first to be activated to defend the host within minutes of injury (Aristizábal and González, 2013). The innate immune system consists of white blood cells called leukocytes which identify and eliminate pathogens (Peng *et al*., 2021). These cells include phagocytes i.e., neutrophils, macrophages, and dendritic cells), basophils, mast cells, and eosinophils (Marshall *et al*., 2018). The adaptive immune system involves antibody and cell-mediated immune responses; it is systemic and specialized and includes lymphocytes (Marshall *et al*., 2018). Lymphocytes are white blood cells and are divided into two types, the B-cells and the T-cells (Cano and Lopera, 2013). The lymphocytes produce antibodies that attack bacteria and destroy toxins or cancerous cells. Lymphocytes are made in the bone marrow and/or move to the thymus where they mature into T cells (Cano and Lopera, 2013; Marshall *et al*., 2018). These types of cells only get activated when they encounter a specific antigen (Ag) and then bind to the surface of antigen-presenting cells (APCs) (Marshall *et al*., 2018). The APCs have on their surface major histocompatibility complex (MHC) class proteins that present the Ag to the T cells. The T cells have T cell receptors on their surface and depending on which MHC class, the Ag is presented to either CD4 or CD8 cells (Wieczorek *et al*., 2017).

The CD4 cells are T-helper cells that can differentiate into two major subsets: T helper (Th) 1 and Th2 (Luckheeram *et al*., 2012). Th1 cells activate macrophages and CD8+T cells by secreting IL-2 and IFN-γ to support cell-mediated immune responses (Bhat *et al*., 2017). Th2 cells promote immunoglobulin (Ig) class switching to IgE. This process is mediated by IL-4 which activates the immune system and induces their degranulation and the secretion of chemokines, cytokines, and proteases which recruit more inflammatory cells (Cano and Lopera, 2013). CD3 is a generic T-cell receptor that identifies T- helper cells such as CD4+ and CD8+ T cells and is involved in cell-mediated immunity (Yang *et al*., 2015). Diseases target and destroy CD4 cells resulting in a lower CD4 cell count than the CD8 cells and thus CD4:CD8 ratio tends to decrease in sick individuals. In a study conducted by Yang, the authors characterized the role of inflammatory mediators and discovered that the reduction of peripheral blood CD4+ T lymphocytes is associated with persistent organ failure in AP (Yang *et al*., 2015). Pro-inflammatory cytokines such as, IL-1β, IL-6, IL-8 and TNF-α have been shown to be present in animal studies where AP was initiated by trypsin (Gea-Sorlí and Closa, 2010; Machado *et al*., 2012). Machado *et al*., (2012) demonstrated that peritoneal lavage leads to an increase in the anti-inflammatory cytokines IL-10, and a decrease in IL-6 and TNF-α. Neutrophils contain host defense peptides such as alkaline phosphatase, Myeloperoxidase, acid phosphatase, lysozymes, and lysozymes that act against pathogens (Peng *et al*., 2021).

3.2.1 Cell death in acute pancreatitis

Apoptosis is programmed cell death; it plays a role in the pathogenesis of AP as it regulates immune homeostasis. Apoptosis occurs as a defence mechanism in response to cell damage or normally during development and aging (Elmore, 2007). There is no fragmentation or release of cell constituents as the cells and tissues are not damaged. Thus, apoptosis protects from the systematic inflammatory response that most SAP patients experience. *Caspases* are cysteine-dependent, aspartate-specific proteases that cleave their substrates at aspartate residues (Seaman *et al*., 2016). *Caspases* are responsible for the hydrolysis of peptide bonds in a reaction that results in substrate inactivation during the intrinsic and extrinsic pathways during apoptosis and inflammation (McIlwain *et al*., 2013). Necrosis is caused by a lack of blood and oxygen to the tissue/organ, a form of cell injury that results in the premature death of cells in living tissue by autolysis (Miller and Zachary, 2017). Necrosis results in loss of membrane integrity and cellular

fragmentation and content release. Necrosis thus augments the inflammatory response as measured in AP patients.

The *caspases* involved in apoptosis are classified by their mechanism of action and are either initiator *caspases* such as *caspase*-8 and-9 or executioner *caspases* such as *caspase*-3,-6, and -7 (McIlwain *et al*., 2013). Initiator *caspases* activate executioner *caspases* that eventually coordinate their activities to break down key structural proteins and activate other enzymes (Unsain and Barker, 2015). The initiator *caspases* normally exist in an inactive form and are activated by the signaling events that induce caspase dimerization and activation (Parrish *et al*., 2013; McIlwain, Berger *et al*., 2013). Following the cleavage mediated by an initiator *caspase*, effector *caspases* act directly on specific cellular substrates to dismantle the cell. This cleavage between the large and small subunits allows a conformational change that brings the two active sites of the executioner *caspase* dimer together and creates a functional mature protease (Riedl and Shi, 2004; Brown-Suedel and Bouchier-Hayes, 2020). Proteases are enzymes that break down proteins that are involved in or cause inflammation. Once the mature protease gets activated, it becomes possible for a single executioner *caspase* to cleave and activate the other executioner *caspases* (McIlwain *et al*., 2013). The intrinsic and the extrinsic pathway of apoptosis are the programmed-cell-death pathways, and they are intertwined. The intrinsic pathway is activated when an injury occurs within the cell whereas, the extrinsic pathway of apoptosis begins outside a cell, i.e. involves receptors and is influenced by the extracellular environment (Hedrick *et al*., 2010). In both pathways of apoptosis, signaling results in the activation of *caspases* that act in a proteolytic cascade to break and eliminate the dying cell (Peter, 2011). Therefore, the activation of *caspases* activates the immune response, and their activation or inactivation underlies major efforts to design better therapies for inflammatory diseases.

3.2.2 Oxidative stress in acute pancreatitis

The reactive oxygen species are by-products of oxygen metabolism; they are highly reactive chemicals. They include superoxides, peroxides, and hydroxyl radicals. The pathological consequences such as damaged mitochondria accelerate the production of ROS, and the oxygen radicals act as a molecular trigger of various inflammatory processes. ROS can cause irreversible damage to DNA as they oxidize and modify some cellular components and prevent them from

performing their original functions (Pérez *et al*., 2015). The generation of ROS has been implicated as a possible initiator and agent responsible for disease progression in AP (Mittal *et al*., 2014).

ROS induction in the acinar cells promotes the less severe form of cellular injury, apoptosis, as it does not structurally damage the cells (Rosales, 2018). In contrast, inhibition of ROS production results in reduced ATP which leads to necrosis because it activates the inflammatory response that leads to local and/or systemic inflammation (Mittal *et al*., 2014). The oxidative stress which is activated during the inflammatory response to acinar injury may be responsible for the further propagation of local and systemic inflammation (Leung and Chan, 2008). The depletion of antioxidants such as glutathione has been shown to be involved in the early phase of AP and also to influence the extent of disease severity (Maciejczyk *et al*., 2019; Pérez *et al*., 2015). The ROS generated from the activated neutrophils during AP is a result of mitochondrial dysfunction (Dunn *et al*., 2015; Mittal *et al*., 2014). They are implicated in cellular activity which attacks cell membranes and triggers the production of neutrophils which causes a variety of inflammatory responses and complications (Rosales, 2018). Antioxidant therapy has been investigated in previous studies and its therapeutic efficacy has already been demonstrated to reduce oxidative stress in experimental AP (Esrefoglu, 2012; Hackert and Werner, 2011).

3.2 Objectives

This chapter aimed to measure the CD4:CD8 subpopulations, assess the type of cell death and measure the oxidative stress in the samples of AP patients. This would confirm whether our cohort from South Africa resembles published data.

3.3 Materials and methods

3.3.1 Blood sample collection and processing for the assays

The participants for the study were patients presenting to the Hepatopancreaticobiliary (HPB) unit of the CHBAH. These patients were diagnosed by the clinicians as either MAP, MSAP, or SAP. A participant information sheet, **Appendix III**, detailing the research, was provided to the patients and explained in the presence of a health care practitioner. The patients signed a consent form, **Appendix IV**, before phlebotomy. Blood samples were collected using two 10 mL blood collection tubes i.e., the BD vacutainer® EDTA blood collection tubes and another 10 mL of blood and the BD vacutainer® Clot activator blood collection tube (BD Biosciences, New Jersey, USA). The blood samples were drawn from the AP patients over four days starting from days 1 (within 72 h post epigastric pain), 3, 5, and 7. The clinical data of the patients were obtained from the patient's hospital files and recorded on the RedCap® (Tennessee, USA) database. The patients' recorded information included demographic data, weight, aetiology, AP severity, clinical test results, and comorbidities. After being age and sex-matched with the AP patients, healthy volunteers were recruited into the study. These participants were colleagues and students at the University of the Witwatersrand, Faculty of Health Sciences campus. They were also given a participant information sheet, **Appendix V**, detailing the research and had to sign the consent form, **Appendix VI**.

Within 6 hours of phlebotomy, 100 µL of whole blood from the EDTA blood collection tubes (BD Biosciences, New Jersey, USA) was aliquoted into a sterile 2 mL corning cryogenic vial (Corning Incorporated, New York, USA). A volume of 1000 µL FACS Lysing Solution (BD Biosciences, New Jersey, USA) was added 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 for immunophenotyping assay. The remainder of the whole blood in the blood collection tubes was allowed to stand upright for 30 minutes at room temperature then centrifuged for 30 minutes at 300 *xg*. The plasma was aliquoted in volumes of 200 µL in 1.5 mL and stored at -70°C until needed for the bioassays.

The BD clot activator tubes (BD Biosciences, New Jersey, USA) were allowed to stand upright for 30 minutes at room temperature to allow the blood to clot. The blood collection tubes were

centrifuged for 30 minutes at 300 *xg*. After centrifugation, 200µL of the supernatant or serum component was carefully aspirated into sterile 0.6mL microcentrifuge tubes using a pipette. Aliquots were stored at –70° C until they were required for the ROS assay and metabolomics analysis.

Peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll-Paque™ (GE Healthcare, Illinois, USA) technique as per the manufacturer's instructions. The cells were made up to a concentration of $1x10^5$ to 10^6 cells/ml PBMCs and stored at -70°C until required for RNA extraction in the apoptosis assays. Briefly, after removing the plasma from the BD vacutainer® EDTA blood collection tubes, the remaining blood was diluted in a 1:1 ratio using Roswell Park Memorial Institute (RPMI) medium (Sigma Aldrich, St Louis, MI, USA). The diluted blood was layered onto ficoll at a 2:1 ratio, followed by centrifugation at 300 *xg* for 30 minutes, with the brakes off. The PBMCs were transferred to a new 50 mL falcon tube and washed by centrifugation at 300 *xg* using 30 mL of RPMI (Sigma Aldrich, St Louis, MI, USA). Red blood cells were lysed using ammonium–-chloride–potassium (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.2) for 5 minutes before washing at 300 *xg* with 15 mL of RPMI media. The cells, at a concentration of $1x10^5$ to 10^6 cells/ml were stored at -70ºC in a freezing medium (10% Dimethyl sulphoxide, Sigma. Aldrich, Missouri, USA, and 90% Gibco Fetal Bovine Serum FBS, Thermo Fischer, Massachusetts, USA) until required for the experiments. The flow diagram shown in **Figure 3.1** below describes the type of samples collected, the assays performed for the study.

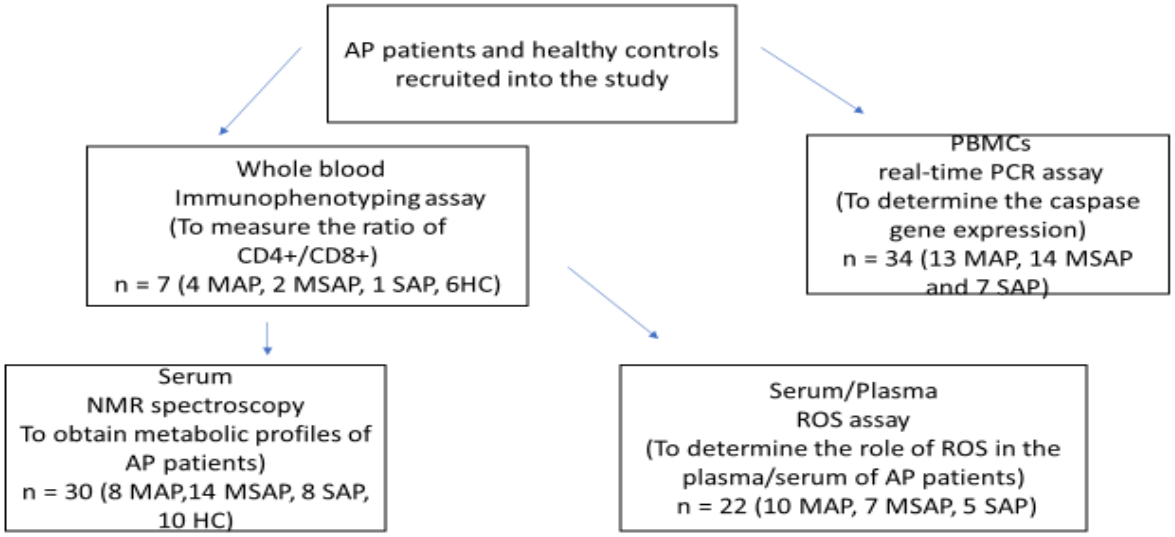


Figure 3.1: Overview of sample size and type of blood samples used. Immunophenotyping, apoptosis gene expression, and the reactive oxygen species assay for the mild, moderately severe, and severe acute pancreatitis patients recruited at CHBAH. The immunophenotyping assay consisted of 4 patients in the MAP group, 2 patients from the MSAP group, and 1 patient from the SAP group sampled on days 3, 5, and 7. The apoptosis assay consisted of 13 MAP, 14 MSAP, and 7 SAP patient samples. To measure the amount of oxidative stress experienced by AP patients, 10 MAP, 7 MSAP, and 5 SAP patient samples were used. **Notes**: AP: acute pancreatitis, MAP- Mild acute pancreatitis, MSAP- Moderately severe acute pancreatitis, SAP- Severe acute pancreatitis, HC- Healthy controls, PBMCs: peripheral blood mononuclear cells.

3.3.1.1 Immunophenotyping lymphocytes for CD4+/CD8+ ratio assessment

Principle: Flow cytometry is a technology that utilizes lasers as light sources to examine single cells in solution as they move in a stream to produce both scattered and fluorescent light signals that are read by detectors such as photodiodes. Immunophenotyping is a flow cytometric approach that simultaneously analyses mixed populations of cells for multiple cellular parameters. The cells are stained with fluorochrome-conjugated antibodies that are targeted against antigens i.e., CD numbers on the cell surface (McKinnon, 2018).

a) Preparation of antibodies, titrations, and immunophenotyping

Due to the patients presenting late to the hospital, limited time, and resources, only seven AP patients' blood samples collected on days 3, 5, and 7 of onset of AP symptoms were analyzed for the T cell metabolic indicators i.e., CD3+ for lymphocyte identification, CD4 (T helper cells) and CD8 (cytotoxic T cells) to enable CD4:CD8 ratio calculation. An additional six healthy participants' blood samples were used as controls. Prior to immunophenotyping, there was the optimization of the flow cytometer and experiment-specific parameters that include instrument quality control to determine baseline parameters, antibody titrations (for optimal staining and resolution of subpopulations), voltage optimization (for negative and positive controls), and compensation controls to subtract spill over from overlapping fluorochromes, shown in **Table 3.1** below:

Table 3.1: Colour panel designed for this study according to the configuration of the BD LSR Fortessa II. The cell type optimized titrated volume, catalog number, and antibody clone are shown.

Laser name	Filter	Parameter	Emission	Lymphocytes	Volume per test(µL)	Optimised volume(µL)	Catalog number/Clone
Power 40	730/45 BP	Alexa Fluor 700	720	CD4	5	1.0	557922/ RPA-T4
Power 50	605/12	BV605	605	CD8	5	0.5	564115/ SK1
Power 50	530/30	BUV496	496	CD3	5	1.0	64810/ UCHT1

Notes: Surface Marker- CD3; Cell type- CD4 and CD8 T cells

Antibody Titration: The titration of the controls was performed under the same conditions as the experimental assay. Following the incubation for 15 minutes, centrifuged at 277 xg for 5 minutes. The supernatant was discarded, and the cells were washed twice with 3 mL of 1x PBS (Sigma-Merck, Missouri, USA) and then suspended in 500µL of 1xPBS. A master mix which consisted of 50 µL of stain buffer (1X PBS, 5% FBS, and 0.1% NaN3) and 1 µL FC blocker (BD Biosciences, New Jersey, USA) was prepared and vortexed briefly. The FC blocker blocks the non-specific binding of antibodies to the Fc receptor-expressing cells and reduces background noise thereby improving the accuracy of results. Fifty microliters of the master mix were added to the cells and resuspended. The sample was then acquired by flow cytometry on a BD LSR II Fortessa (BD Biosciences, New Jersey, USA).

The volumes of the antibodies in **Table 3.1** were prepared in the dark and included a control tube without any antibodies. A fluorescence-labeled antibody at double the concentration recommended by the manufacturer (5 µL per antibody) was added to tube 1 and two-fold serial dilutions were done in staining buffer for a total of 7 concentrations. The 8th tube did not receive the antibody and was used as an unstained tube for setting up the flow cytometer's forward scatter area (FSC-A) and side scatter area (SSC-A) parameters. Fifty microliters of the antibody solutions were then transferred to the washed fixed white blood cells in all tubes, mixed thoroughly, and incubated in the dark, at room temperature for 20 minutes. The stained cells were resuspended by adding 1000 µL of 1X BD Perm/Wash™ buffer (BD Biosciences, New Jersey, USA) and washed at 277 x*g* for 10 minutes. Afterward, stained white blood cells were resuspended in 300 µL staining buffer before the flow cytometric analysis. Approximately 100,000 cells were acquired on BD LSRFortessa™ II flow cytometer (BD Biosciences, New Jersey, USA) for each tube.

To determine the optimum concentrations for antibodies to use in the panel, a plot of antibody volume against the stain index (which is calculated by taking the difference between the MFI of positive and negative populations divided by two times the standard deviation of the negative population) which is shown in Figu**re 3.2**. Serial dilutions were made of each antibody and acquired using LSR II Fortessa. (BD Biosciences, New Jersey, USA). The results of the respective antibody titration findings are shown in **Figure 3.2** below:

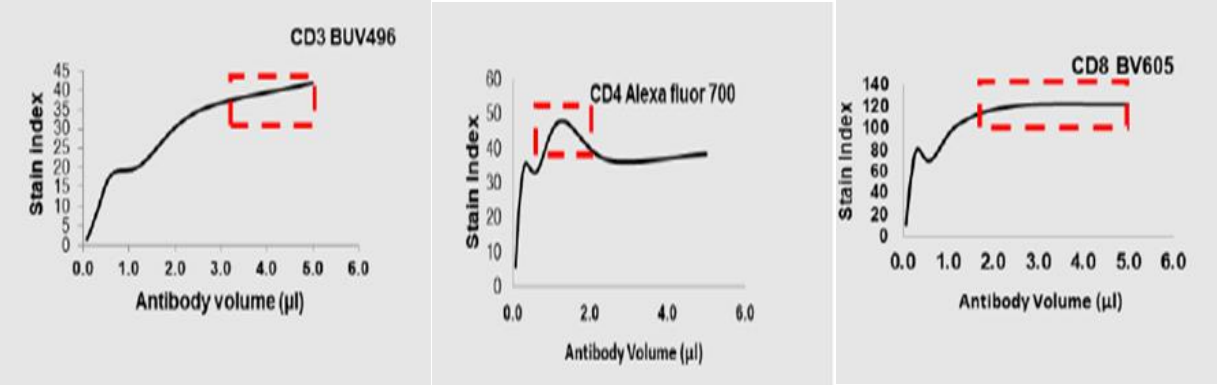


Figure 3.2: Antibody titration and verifying cell populations. Serial dilutions were made of each antibody to determine optimal concentrations for antibodies in the panel. Data was acquired using a BD LSRFortessa™ II flow cytometer. The dotted red line represents the peak and the optimum volume of respective antibodies.

The volume corresponding to the highest stain index was selected for use in the experiment. For volumes less than 0.5 µL and it was assumed that 0.5 µL was sufficient. The dotted red line represents the peak and the optimum volume for respective antibodies. From this, a plot of antibody volume against the SI was used to decide on the optimal volume for the use, which was the volume corresponding to the highest stain index. The dynamic range, which is the total range of fluorescent values for the assay, was established in the process.

b) Optimizing PMT voltages and the preparation of the compensation controls

The PMT voltages were optimized to eliminate electronic noise from cell debris. To obtain consistent results, the voltages of the three fluorochromes were optimized to establish the positive and negative populations of the target parameters. Voltages of all antibody fluorochromes were optimized for the assays to ensure consistent results. Voltage optimization established negative and positive populations of target parameters and their linearity. The following voltages were set and used to acquire data: The forward side scatter, which is the measurement of cell size (FSC) 380v, and side scatter, the measurement of complexity/granularity of cells (SSC) 345v. As well as BUV496 400v, Alexa Fluor 700v, and BV650 530v, approximately 10 000 events were recorded per sample at a threshold setting of 20 000.

c) Compensation Controls

Compensation controls used to subtract fluorescence spillover between overlapping spectra were prepared using CompBeads (Anti-Mouse Ig, κ/Negative Control Compensation Particles Set; BD Biosciences, New Jersey, USA). Cells were acquired on the flow cytometer using FACSDiva™ software version 5 (BD, Biosciences, New Jersey, USA). 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 Set3; (BD Biosciences, New Jersey, USA) were added into 5 mL Corning Falcon tubes (Corning Incorporated, New York, USA) containing 50µL of 1X PBS (Sigma-Merck, Missouri, USA). The optimized 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 *xg* for 5 minutes. The supernatant was decanted, and the pellet was 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.

d) Gating strategy

Cells were gated as singlets and then as lymphocytes using forward scatter height (FSC-H) and FSC area (FSC-A). FSC-A and Side Scatter Area (SSC-A) were used to discriminate the lymphocytes from the granulocytes and monocytes, based on their size and granularity or complexity. The antibodies used were CD3BUV496, CD4Alexa Fluor, and CD8BV605 as shown in **Table 3.1**. The populations were represented as percentages of their parent populations in **Figure 3.3.**

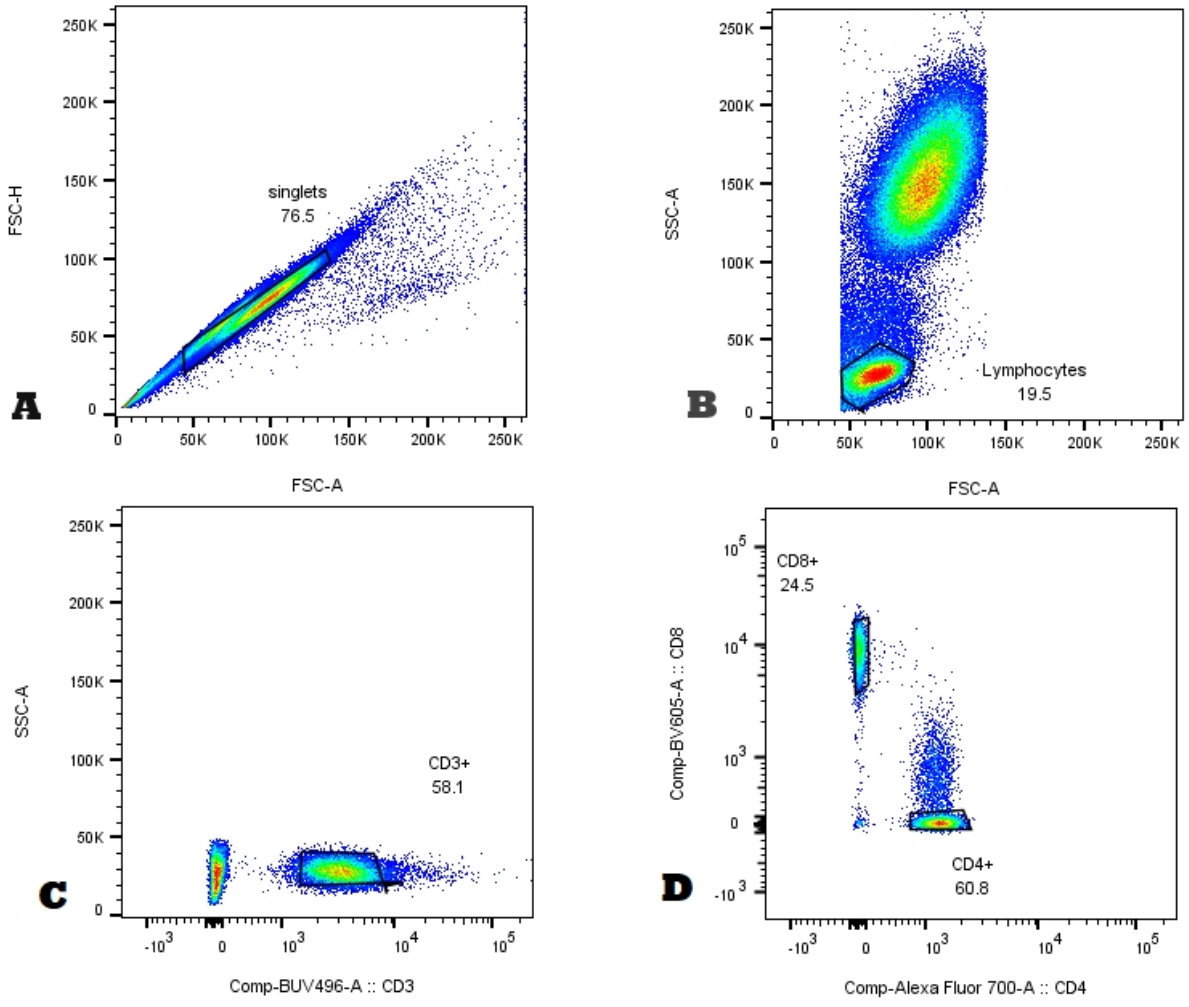


Figure 3.3: Gating strategy for the lymphocytes. Cells were gated into singlets (FSC-A versus FSC-H) shown in A, as well as to differentiate cells based on size, i.e., lymphocytes, smallest and least granular, shown in B. C, Graph showing CD+ cells. D, Lymphocytes were observed by gated T-cells subsets; CD3+CD4+ and CD3+CD8+.

3.3.1.2 Apoptotic gene expression studies

Principle: real-time polymerase chain reaction (PCR)/q-PCR estimates the quantity of starting material in a sample. Since the products are detected as the reaction proceeds, the fluorescent reporter dye is used as an indirect measure of the amount of nucleic acid present during each amplification cycle. The increase in fluorescent signal is directly proportional to the quantity of exponentially accumulating PCR product molecules (amplicons) produced during the repeating phases of the reaction.

Ribonucleic acid (RNA) extractions

The PBMCs that were processed using the protocol described in 3.3.1 above were retrieved from the -70 ºC freezer and allowed to thaw at room temperature. The cells were washed at 500 x*g* for 5 minutes and the supernatant was discarded. One ml of TRI Reagent® (Sigma-Aldrich, Missouri, USA) was added to the pellet and left at room temperature for 5 minutes to lyse the PBMCs, and then pipetted up and down. A volume of 200 µL of chloroform was added to the mixture and shaken vigorously for 15 seconds, then left at room temperature for 10 minutes. The sample was then centrifuged at 12,000 x*g* for 15 minutes at 4 °C. The colorless upper aqueous phase containing RNA was pipetted into a fresh tube followed by the addition of 500 µL of isopropanol. The mixture was centrifuged at 12,000 x*g* for 10 minutes at 4 °C. The RNA precipitate was washed three times with 1 mL of 75% ethanol, at 7,500 x*g* for 5 minutes at 4°C. The RNA was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA). A total of 34 (13 MAP, 14 MSAP, and 7 SAP) RNA samples including 6 healthy controls with an A260/280 ratio ≥ 1. 8, representative of sample purity were aliquoted into three tubes of 10µl each to be used for cDNA synthesis.

a) Complementary Deoxyribonucleic acid (cDNA) synthesis

The RT2 First Strand Kit (QIAGEN, Hilden, Germany) components were thawed at room temperature and centrifuged for 15 seconds at 300 *xg*. The RNA of 50ng/µL concentration was used for the genomic elimination. The genomic DNA elimination mix provided by the manufacturer for each RNA sample was calculated according to **Table 3.2**.

Table 3.2: Genomic DNA elimination mix provided by the manufacturer.

Component	Amount
RNA	50ng/µL
Buffer GE	2µL
RNAse free water	variable (to make 10µL)
Total volume	10µL

The genomic DNA elimination mix was incubated for 5 min at 42°C in a SimpliAmp™ Thermocycler (Thermo Fischer, Massachusetts, USA), then immediately placed on ice for at least 1 minute.

Table 3.3: Reverse transcription master mix preparation according to manufacturer's instructions.

Component	Volume/ reaction (µL)	Volume/reaction (µl) x n* (+1)
5 x Buffer Bc3	4	4n+1
Control P2	1	1n+1
Reverse transcriptase mix	2	2n+1
RNase-free water	3	3n+1
Total volume	10	10n+1

n= number of samples

A volume of 10 µL reverse-transcription master mix: RT2 First Strand Kit (QIAGEN, Hilden, Germany) was added as shown in **Table 3.3** to each tube containing 10 µL genomic DNA elimination mix and mixed gently by pipetting. The samples were then incubated at 42°C for 15 minutes and the reaction stopped by incubating at 95°C for 5 minutes. The total RNA samples in each severity group were pooled together after the concentration was adjusted to 250 ng/µL. cDNA synthesis was performed from 250 ng/µL of total RNA using the Photoscript® II First Strand cDNA Synthesis Kit E6560S (New England BioLabs® Inc.). A volume of 2 µL of d(T)23VN (50 µM) was added to 10 µL of Photoscript II Reaction Mix (2x) followed by adding 2 µL Photoscript II Enzyme Mix (10x) to the mixture. Two microlitres of RNA template were added followed by Nuclease free water to make up 20 µL and was then incubated at 42 ºC for 1hr, denatured at 85 ºC, and then allowed to cool down at 4 ºC.

b) Real-time PCR

The total cDNA was adjusted with nuclease-free water to 100 ng. The PCR hood was prepared by wiping with 70% ethanol, the pipettes wiped down with RNAse away (Sigma Aldrich, St Louis, Missouri, USA) and UV radiation switched on for 20 minutes. The PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was swirled gently to allow mixing. The PCR Master Mix (MM) was prepared according to the volumes in **Table 3.4** below and mixed thoroughly by pipetting up and down in a 96-well plate placed on ice.

Table 3.4: Calculations and volumes of PCR Master Mix (MM)

Component	Volume (10 µL/well)
PowerUp™ SYBR™ Green Master Mix (2X)	5 µL
reverse primer	0.5 µL
forward primer	0.5 µL
cDNA template	2 µL
Nuclease free H20	2 µL
Total	**10 µL**

The primers for the respective *caspases* used for this study are shown in **Table 3.5**.

Table 3.5: Primer sequences used for the real-time PCR.

Name		Sequence
RPL 13A	Forward:	5′- CATAGGAAGCTGGGAGCAAG- 3′
	Reverse:	5′- GCCCTCCAATCAGTCTTCTG- 3′.
Caspase 2	Forward:	5′- GTCACACAAGAAGGGAGGAGA- 3′
	Reverse:	5′- ACCTTCACCCATGGAACGG- 3′.
Caspase 3	Forward:	5′- TTATTCAGGCCTGCCGTGGT- 3′
	Reverse:	5′- AGCATGGCACAAAGCGACTG- 3′.
Caspase 7	Forward:	5′- AGTGGATGCTAAGCCAGACCG- 3′
	Reverse:	5′- TCGAACGCCCATACCTGTCA- 3′.
Caspase 8	Forward:	5′- ATAGGCCTGTGACGAAGGTGC- 3′
	Reverse:	5′- GCGGAATGTAGTCCAGGCTCA- 3′.

A total of 34 (13 MAP, 14 MSAP and 7 SAP) cDNA samples and 6 healthy controls with an A260/280 ratio ≥ 1. 8 were used for real time PCR. The samples were loaded in duplicate and included the different severity groups and healthy controls. **Table 3.5** lists the genes that were analysed were *caspase 2*, *caspase 3*, *caspase 7*, and *caspase 8*. *Caspases 2* and *8* are initiator genes, whereas *caspases 3* and *7* are executioner genes. *RPL13A* was used as a stable reference gene to

perform accurate and reproducible real-time PCR analysis. The 96-well PCR plate was sealed with an optical adhesive cover and then centrifuged briefly to spin down the contents and eliminate any air bubbles. The QuantStudio 1 ™ Thermocycler (Thermo Fischer, Massachusetts, USA) was set for the following conditions:

The Uracil-N-glycosylase gene (UDG) activation was done at 50°C for 2 minutes, Dual-lock DNA polymerase at 95°C for 2 minutes, DNA was denatured at 95°C 15 seconds for 40 cycles, annealing temperature was set at 55°C for 50 seconds and 40 cycles and extension of the primers at 72°C 1 minute 40 cycles.

3.3.1.3 Reactive Oxygen Species (ROS) analysis using N, N- diethyl -para-phenylenediamine.

Principle: The Fe (2+)-dependent decomposition of peroxides oxidizes N, N- diethyl -para-phenylenediamine (DEPPD) sulphate when it reacts with serum or plasma. DEPPD is rapidly oxidized to a stable radical cation which can be measured spectrophotometrically (Hayashi *et al*., 2007). A total of 22 (10 MAP, 7 MSAP, 5 SAP) frozen serum and plasma samples and 10 healthy controls previously stored at -70°C were left to thaw at room temperature. A standard curve was prepared using H_2O_2 at different concentrations i.e., 50, 25, 6.25, 3.13, 1.56, 0.78, and 0.39 µM prepared in duplicates. A 0.1 M sodium acetate buffer at pH 4.8 was prepared and 140 µL of the sodium acetate buffer was added to each well of a NUNC 96-well plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA). In a 500 mL bottle, 5.56 g of iron sulphate was dissolved using the acetate buffer. A volume of 1.0 mL DEPPD was added to 9 mL of the acetate buffer and 400 µL of the mixture was added to 9.6 mL of the iron sulphate solution in a 50 mL tube. A total of 100 µL of the reagent mixture was added to each well of a 96-well plate and 5 µL of serum or plasma sample was added to the mixture. A plate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to kinetically record colour development at 505 nm, at 25 °C for 30 cycles.

3.4 Data analysis

The immunophenotyping data were analyzed using FlowJo LLC version 10 (BD, Biosciences, New Jersey, USA) with previously linked compensation controls from BD BioSciences LSR Fortessa FACSDiva™ software. The $2^{-\Delta\Delta C}T$ was used which is a convenient method to calculate relative fold changes in gene expression of the caspases. The determination of ROS units of each

sample was done off a standard curve. Statistical analysis of the gene expression and ROS data was done using Microsoft Office Excel 2016 and GraphPad Prism 8 (GraphPad Software, San Diego, CA). An unpaired, nonparametric t-test Kruskal-Wallis test was performed followed by Dunn's post hoc test for significant differences ($p < 0.05$) in AP patient samples of different severities and compared to the healthy control samples.

3.5 Results

3.5.1 Immunophenotyping of lymphocytes to determine CD4+/CD8+ ratio.

The immune cell metabolic indicators CD3, CD4, and CD8 were assessed in the lymphocyte cell populations of six healthy controls and seven patients (4 MAP, 2 MSAP, and 1 SAP) using CD3BUV496, CD4Alexa Fluor, and CD8BV605 as shown in **Table 3.1**. The whole blood samples were collected on days 3, 5, and 7 for the different severities of AP, in total 13 blood samples of AP patients were assayed. The cells were gated using the gating strategy as previously described in **Figure 3. 3 A – D**. The healthy control group had the highest percentage of lymphocytes and CD3+CD4+ T-cells compared to the AP severity groups. Four healthy control samples had CD4:CD8 ratios above two and the other two were below one. As expected, MAP patients had a higher CD4:CD8 ratio of above 1 or closer to one when compared to the other severity groups of AP i.e., MSAP and SAP. MSAP and SAP had lower ratios o below 1 except for one gated MSAP case whose ratio was 2.48 (highlighted in **Table 3.6**). Over time, for the three AP patients i.e., AP 1, 2, and 4 who had samples collected over the entire study days, the CD4:CD8 ratio for the MAP patient increased with time. For the MSAP the CD4:CD8 ratio increased slightly on day five and decreased on day seven. The CD4:CD8 ratio of the SAP patient increased slightly and remained somewhat constant on day seven.

Table 3.6: Percentage lymphocyte cell populations in the blood of patients with different severities of AP (4 MAP, 2 MSAP, and 1 SAP) and 6HC using the BD LSRFortessa™ II flow cytometer.

Sample number	Severity	Lymphocytes	CD3 (%)	CD4 (%)	CD8 (%)	CD4:CD8 (%)
AP1 day 3	MAP	8.57	33.80	47.30	41.20	1.15
AP1 day 5	MAP	5.22	0.66	10.87	11.45	0.95
AP1 day 7	MAP	19.70	59.00	60.80	24.30	2.50
AP3 day 5	MAP	20.60	45.80	43.90	22.90	1.92
AP6 day 5	MAP	4.88	0.20	10.00	10.00	1.00
AP7 day 5	MAP	5.78	0.56	11.20	12.40	0.90
AP2 day 3	MSAP	14.60	1.28	15.50	39.70	0.39
AP2 day 5	MSAP	12.40	1.10	16.20	33.90	0.48
AP2 day 7	MSAP	41.90	7.69	17.20	65.80	0.26
AP5 day 5	MSAP	19.50	58.10	60.80	24.50	2.48
AP4 day 3	SAP	22.80	0.72	11.50	69.20	0.17
AP4 day 5	SAP	26.20	1.70	16.00	62.80	0.25
AP4 day 7	SAP	29.60	6.93	16.70	72.20	0.23
HC 1		26.60	6.68	17.10	27.40	0.62
HC 2		14.10	15.00	23.70	32.30	0.73
HC 3		27.09	54.02	68.10	28.65	2.38
HC 4		14.70	15.60	67.00	26.00	2.58
HC 5		22.50	15.00	62.32	25.25	2.47
HC 6		18.30	61.80	63.90	25.00	2.56

Notes: MAP- Mild acute pancreatitis, MSAP- Moderately severe acute pancreatitis, SAP- Severe acute pancreatitis, CD- Cluster of differentiation

3.5.2 Apoptosis gene expression level changes

To determine gene expression in the PBMCs of recruited AP patients as a marker of severity, initiator *caspases 2* and *8* and executioner *caspases 3* and *7* were assessed using real-time PCR. The gene expression results from 16 MAP, 12 MSAP, 6 SAP, and 6 healthy control participants are shown in **Figures 3.4** and **3.5**. In general, the expression of the caspases was more notable in MAP and MSAP patients and appears to never change in the healthy controls.

3.5.2.1 Expression of initiator caspases: *Caspases 2* and *8*

Gene expression profiles of *Caspase 2* and *Caspase 8* are shown in **Figure 3.4 A-H**. Overall, MSAP (5.88±3.11) patients had the highest level of *caspase 2* expression compared to MAP (3.23±1.42), SAP (0.98±0.24), and HC (1.99±0.91), however, this was not statistically significant. Interestingly, MAP patients showed decreasing levels of *caspase 2*, over time from days 3 – 7.

Whereas, MSAP patients experienced an increased level of *caspase 2* gene expression, especially on day 5 but this decreased on day 7. There was little to no *caspase 8* expression in all the severities of AP including the healthy controls, and consequently, no statistically significant differences were observed when the groups were compared. On days 3 and 5, MSAP patients showed a slight increase in *caspase 8* expression, compared to MAP, SAP, and HC. However, at day 7, *caspase 8* levels in MSAP patients decreased and showed a similar pattern in other groups.

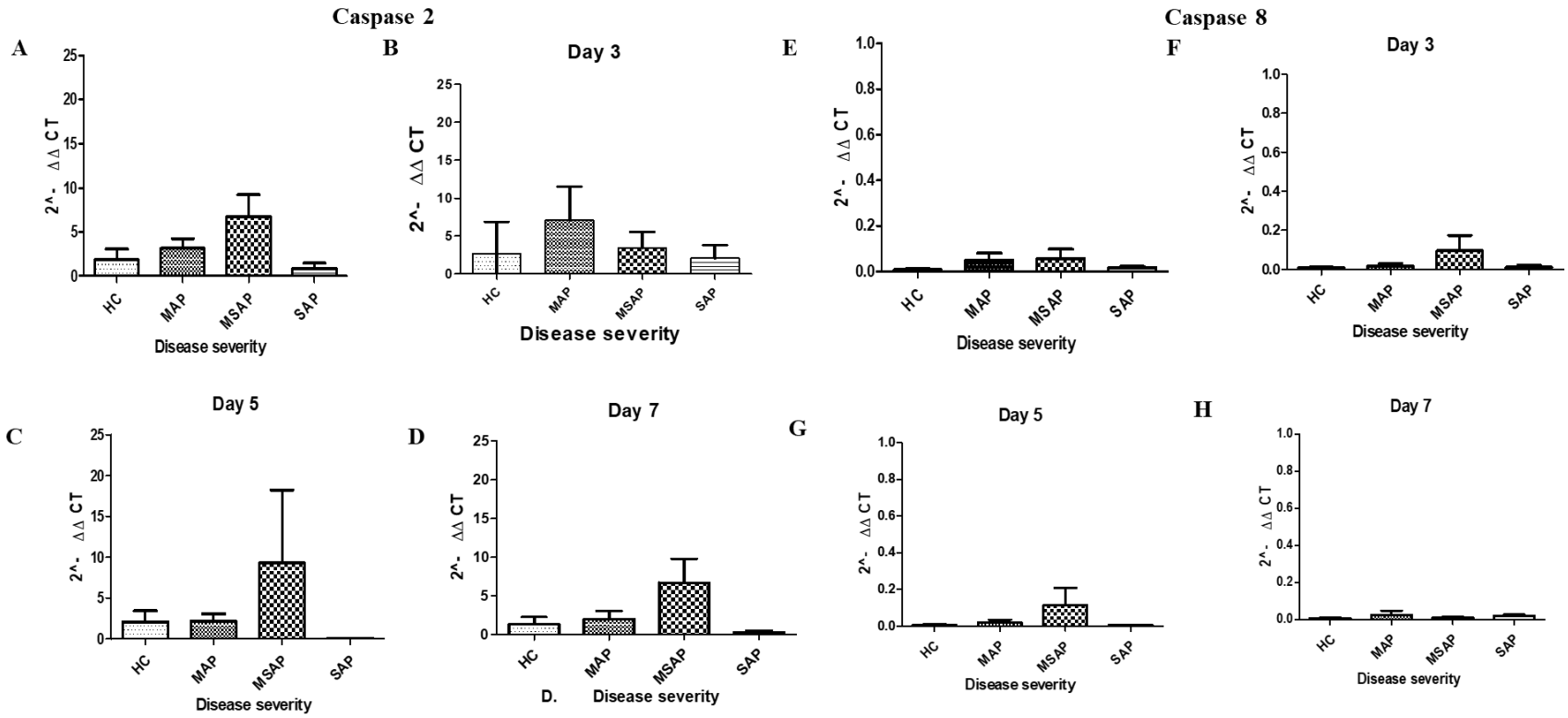


Figure 3.4 *Caspase 2* and *caspase 8* gene expression in the PBMCs of AP patients (MAP =16, MSAP = 12, SAP = 6, and healthy controls = 6). The data presented shows that there were no statistically significant differences between all the severity groups and the HCs. The SAP patients experienced the least amount of caspase activity. **A** Shows the cumulative average for days 1, 3,5, and 7 for the severity groups of AP, and MSAP patients show increased levels of *caspase 2* compared to the other groups**. B** there were higher levels of *caspase 2* expression in the MAP patients compared to the other groups. **C and D** there were higher levels of *caspase* 8 in MSAP patients on day 5 but decreased on day 7. In MAP patients the levels of expression in the cells decreased over time from day 3 to

day 5 and remained unchanged on day 7. The fold changes were MAP 3±1, MSAP6±3, SAP1±0.2, and HC 2±1. E Shows the cumulative average for days 1, 3,5, and 7 for the severity groups of AP and there were low levels of *caspase 8* expression in the severity groups of AP, especially in SAP. **F- H** Over time, *caspase 2* expression in MSAP was higher compared to the but decreased on day 7. The expression values are represented as mean ± SEM. **Notes**: AP: Acute Pancreatitis; HCs: healthy controls; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.5.2.2 Expression of executioner caspases: Caspases 3 and 7

Gene expression profiles of *Caspase 3* and *Caspase 7* are shown in **Figure 3.5 A-H**. Overall, *caspase 3* expression was higher in the MSAP (4.89±0.92) followed by MAP (4.76±0.99) compared to the SAP (1.83±0.10) and the HC (2.49 ±0.52), there was no statistical significance in all the groups. Over time, *caspase 3* expression was high in MAP patients on day 3 compared to the HCs and SAP but the level of expression in the MAP decreased as the days progressed to day 7. However, the MSAP patients experienced on average, a higher level of *caspase 3* expression compared to the MAP and the HCs on day 5 and decreased on day 7.

In SAP patients, the level of *caspase 3* expression seemed to increase over time from day 3 to 7. *Caspase 7* expression was high in MAP compared to the HCs although not statistically significant, while its expression in MSAP patients was statistically significant ($p = 0.0029$) compared to the HCs. Over time, the expression of *caspase 7* was high in MAP on day 3, decreased on day 5, and started to increase on day 7. While *caspase 7* expression in MSAP patients increased on day 5 ($p = 0.00994$), and slightly on day 7 ($p = 0.0249$) compared to the HCs. The expression of *caspase 7* in SAP patients decreased over time from day 5 to day 7.

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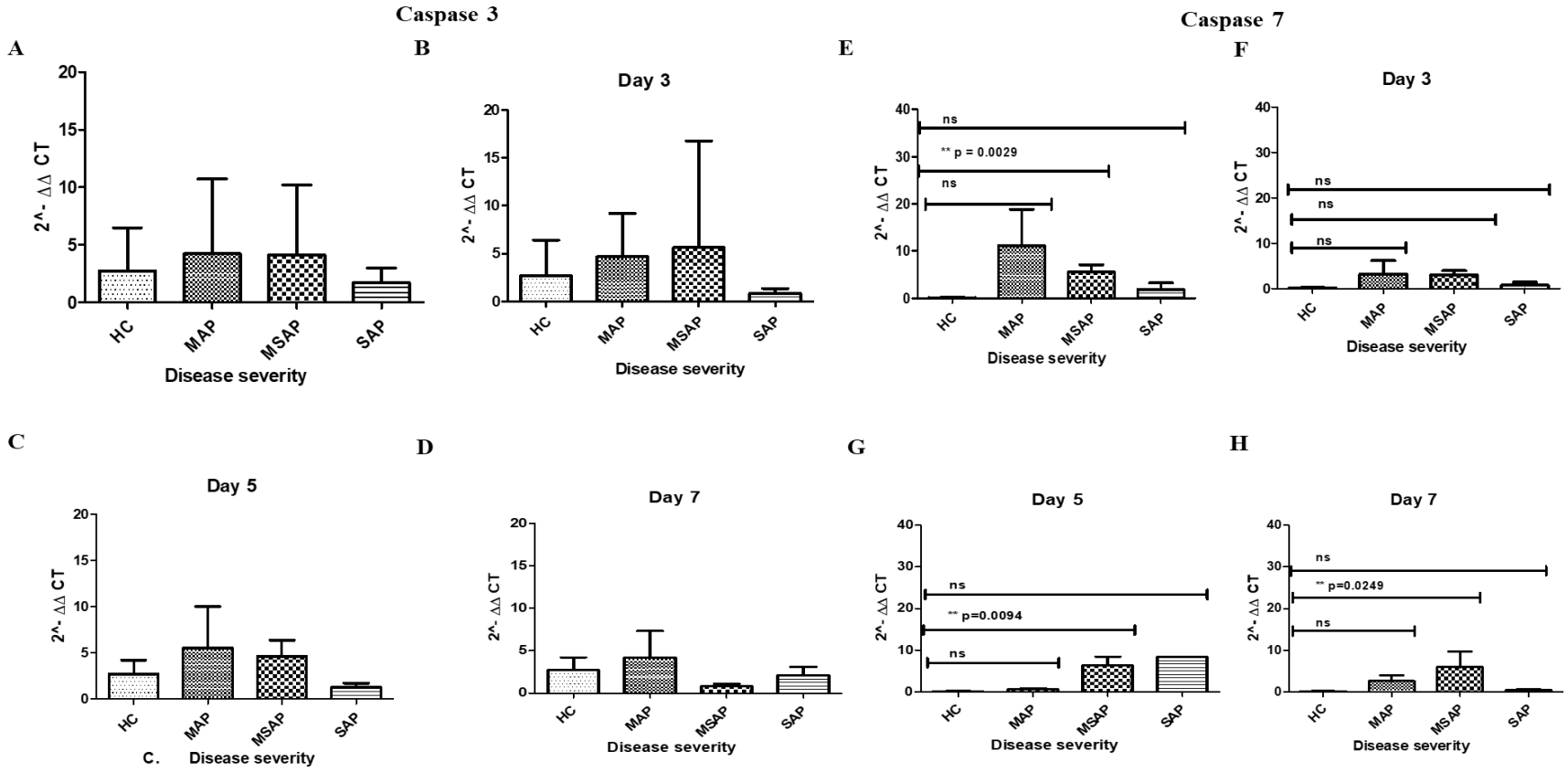


Figure 3.5 *Caspase 3* and *caspase 7* expression in the PBMCs of AP patients (MAP =16, MSAP = 12, SAP = 6, and healthy controls = 6). **A** Shows the cumulative average for days 1, 3,5, and 7 for the severity groups, and *caspase 3* expression was higher in the HCs, MAP, and MSAP compared to the SAP. The data presented shows that there were no statistically significant differences between all the severity groups and the HCs. The fold changes MAP (4.76±0.99), MSAP (4.89±0.92), SAP (1.83±0.10), and HC (2.49 ±0.52). The SAP patients experienced the least amount of caspase activity. Over time, **Figure B – D** there was not much difference in the levels of *caspase 3* expression in MAP patients over time. It is clear that *caspase 3* expression was high in MSAP patients on day 3 but decreased

as the days progressed to day 7. In SAP patients, the level of *caspase 3* expression seemed to increase over time from day 3 - 7. No statistical significance was observed in all the severities of AP compared to the HCs. **E**. The data presented shows the cumulative average for days 1, 3,5, and 7 for the severity groups, and *Caspase 7* expression was higher in the MAP compared to the MSAP, SAP, and healthy controls. There was statistical significance between the MSAP and the healthy controls ($p = 0.0029$). **F - G**, *caspase 7* expression in MAP decreased from day 3-5 but increased on day 7. *Caspase 7* expression which is statistically significant in PBMCs of MSAP to the healthy controls increased on day 5 and slightly on day 7 ($p = 0.00994$, 0.0249 respectively. Expression values are represented as mean ± SEM. **Notes**: AP: Acute Pancreatitis; HCs: healthy controls; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis.

3.5.3 Oxidative stress changes with AP disease severity

The levels of hydroperoxides in AP patients of different severities were measured in their serum and plasma samples, respectively. Twenty-two AP patients (10 MAP, 7 MSAP, 5 SAP and 10HC) whose blood samples were collected over days 3, 5, and 7 and 10 healthy controls were used in the assay. Overall MSAP and SAP patients experienced high levels of hydroperoxides expression in serum and plasma samples compared to the MAP patients and the healthy controls **Figure 3.6 A** and **B**. Data are shown as SEM. The MSAP and SAP groups compared to the healthy controls showed statistically significant differences in the level of ROS measured ($p < 0.0001$) in both the serum and the plasma samples. The concentrations of hydroperoxides were more clear/evident in plasma.

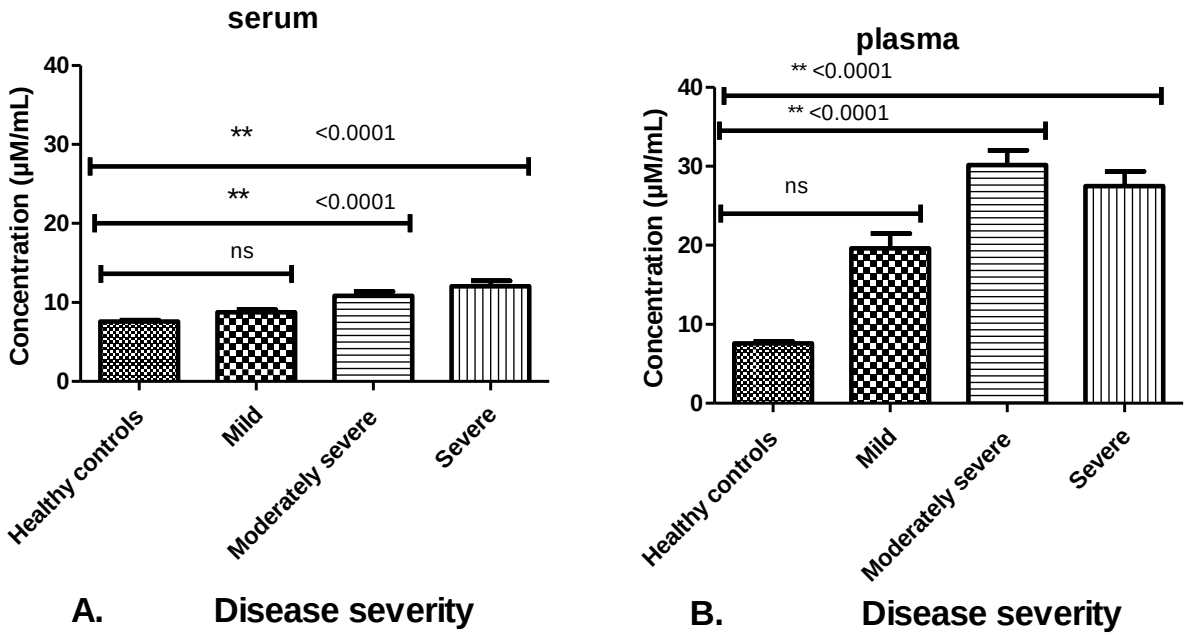


Figure 3.6 A The overall expression levels of ROS from serum and plasma **B** from AP patients (10 MAP, 7 MSAP, and 5 SAP). Bar graphs show a statistically significant difference between the hydroperoxides concentration of the MSAP and the HCs and between the hydroperoxides concentration of the SAP and HCs ($p<0.0001$). Overall MSAP and SAP patients had higher levels of ROS in the serum and plasma samples compared to the MAP patients and the healthy controls. ROS levels were especially higher in the plasma than in serum samples. Data are shown as SEM. **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 = 10 MAP, 7 MSAP, 5 SAP, and 10 HCs.

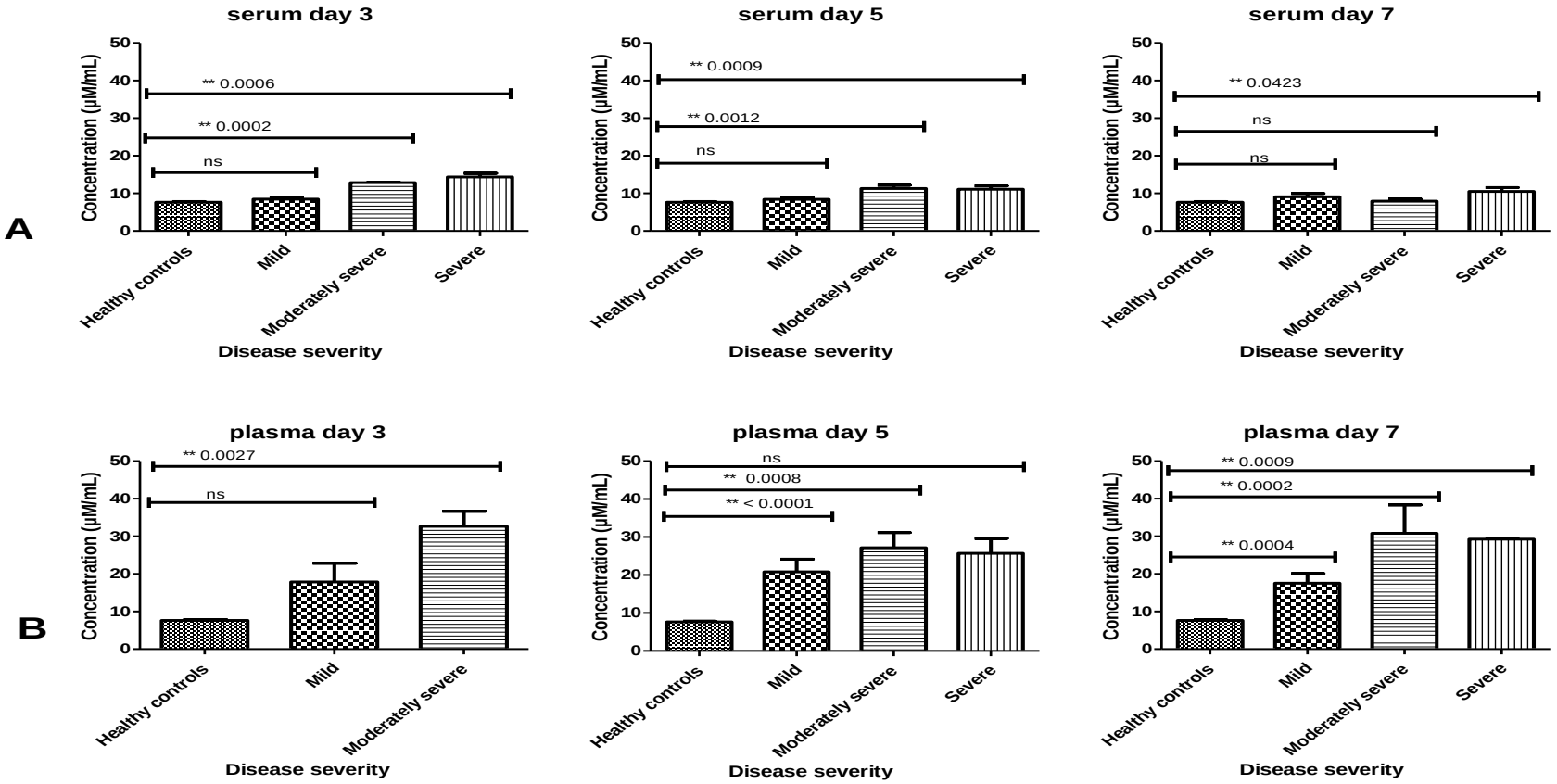


Figure 3.7 ROS concentration in serum and plasma measured by quantifying hydroperoxide levels using the DEPPD assay among the three severity groups and HCs over time in AP patients. Bar graph showing the levels of hydroperoxides expressed in the different AP severities compared to the HCs. The amount of hydroperoxides experienced by the AP patients did not decrease over time. The MSAP group displayed the highest levels of hydroperoxide in patients on day 5 with statistical significance to the HC than the other groups in both serum (0.0012) and plasma (p = 0.0008), and between SAP and the HC in serum on day 5 (p = 0.0009) as well as in plasma (p = <0.0001 between MAP and the HC. The hydroperoxide levels in the plasma of MSAP were more distinct than in serum, especially on days 3 and 7. There were no SAP plasma samples available for day 3. Data are shown as SEM. **Notes**: AP: Acute pancreatitis; HCs: healthy controls; MAP: mild acute pancreatitis; MSAP: moderately severe acute pancreatitis; SAP: severe acute pancreatitis. n = 10 MAP, 7 MSAP, 5 SAP, and 10 HCs.

There was no decrease in the hydroperoxides levels experienced by the AP patients over the 7 days when blood samples were collected from the AP patients In the serum and plasma samples, **Figure 3.7 A** and **B**, on day 3 MSAP and SAP displayed higher levels of hydroperoxides relative to the controls in serum samples ($p = 0.0002$ and 0.0006) respectively, and in plasma ($p = 0.0027$) between the MSAP and the HCs. On day 5 in plasma samples, MAP patients also presented with a statistically significant increase in the hydroperoxides relative to the HC ($p < 0.0001$). In serum, SAP patients had an increase in hydroperoxides relative to the HC ($p = 0.0009$). on day 5 MSAP patients had higher levels of hydroperoxides compared to the severities of AP in serum ($p = 0.0012$) and plasma ($p = 0.0008$). plasma samples generally had high levels of hydroperoxides, especially on day 7 where MAP, MSAP, and SAP hydroperoxides levels were significantly higher when compared to the HC ($p = 0.0004$, $p = 0.0002$, and $p = 0.0009$) respectively. In the serum on day 7, the hydroperoxide levels in the SAP group were borderline significant ($p = 0.042$). the concentration of the hydroperoxides in the plasma samples was thus always higher than in serum samples. There were no samples available for day 3 SAP patients.

3.6 Discussion

In this chapter, the CD4:CD8 ratios, cell death, and oxidative stress were measured in the samples collected from AP patients. AP is characterized by local or systematic inflammation and is the cause of organ failure and/or death (Leppäniemi *et al*., 2019). Blood serum and plasma are the primary sources of circulating proinflammatory mediators. Apoptosis is programmed cell death, during apoptosis, there is minimal inflammation because the cells undergoing apoptosis do not release their cellular components, as a result, the integrity of the organelle is maintained throughout this process and the plasma membrane is intact (Bhatia, 2004). Various factors contribute to the development of oxidative stress. These include infection, exposure to toxins, inflammation, stress, alcohol, and smoking. The pancreatic acinar cells are predisposed to oxidative stress (Armstrong *et al*., 2013), and this has been substantiated in earlier studies by Kasahara *et al*., (1997) who found that ROS induces apoptosis in living cells.

3.6.1 Immunophenotyping lymphocytes for CD4+/CD8+ ratio assessment

The CD4/CD8 ratio reflects the state of the immune system. In healthy individuals, the CD4/CD8 ratio is greater than 1 and ranges between 0.9- 1.9 (Jiang et al., 2004). Understanding the immune

system in relation to AP pathogenesis and management of diseases is important where the immune system gets activated in response to injury. Therefore, monitoring of lymphocyte subsets past onset and at the admission of patients will help with the prognosis of AP patients. The immune cell metabolic indicators CD3, CD4, and CD8 were assessed in determining the lymphocyte cell populations of our study participants.

The MSAP and SAP groups had low CD4 percentages and high CD8 percentages, hence a lower CD4:CD8 ratio than the normal range. The depletion of the CD4 and an increase in CD8 lymphocytes are responsible for early and late mortality in severe acute pancreatitis (Pietruczuk *et al*., 2006). It is known that a lower ratio of CD4:CD8 is associated with a higher risk of morbidity and mortality, whereas a higher ratio is associated with an increased immune function (McBride and Striker, 2017). The SIRS is considered the leading cause of death in the early phase of acute pancreatitis (Bhatia *et al*., 2000). Therefore, the dysregulation of the immune cells correlates to the severity of acute pancreatitis. One MSAP patient had a high CD4:CD8 ratio, which was not expected. This patient had a BMI of 28.33 m^2/kg which falls in the overweight category. Positive correlations have been reported between BMI and total white blood cell count and T-cell numbers in peripheral blood (Jesri *et al*., 2007; Yoshimura *et al*., 2015) and could be the contributing factor to these findings.

Four of our healthy controls had high percentages of CD4 and lower CD8 hence a higher CD4:CD8. Two healthy volunteers had a CD4:CD8 ratio below 1. As we did not obtain any clinical information from our healthy controls other than the absence of pancreatic disease and demographic data we cannot make any conclusions about these two healthy controls. These healthy participants also fall on the overweight and obese spectrum. Research in non-obese diabetic mice has shown that the development of diabetes requires both the CD4+ and CD8+ T cells (van der Weerd *et al*., 2012). T lymphocytes are cells that can permeate the pancreas and recruit other cell types such as macrophages leading to a release of high concentrations of damaging IL-7 and CCL5 (van der Weerd *et al*., 2012). Other factors which may affect the CD4:CD8 ratio such as older age, obesity, diabetes, being HIV positive, and taking treatment for HIV have been considered however they are just speculations in the absence of clinical data. All our healthy controls were under the age of 65 as they were recruited on campus. Altogether, several lines of evidence suggest a direct

link between obesity and a deregulated T-cell accumulation within adipose tissue, in contrast, being on HIV treatment also leads to a higher CD4:CD8 ratio (McBride and Striker, 2017).

3.6.2 Apoptotic gene expression studies

It is widely accepted that inflammation and apoptosis are key pathological responses of AP and determine disease severity. Mitochondria-mediated cell death is essential for the initiation of AP. The activation of caspases is known to induce apoptosis and thus protect the cells from an inflammatory response. The initiator *caspases 2* and *8* and executioner *caspases 3* and *7* were assessed to determine the apoptosis status of the cells of AP patients. The gene expression of initiator *caspase 2* was noted in mild and some moderately severe pancreatitis patients as well as in the healthy controls. MSAP patients generally experienced the highest level of this initiator gene expression compared to the other severity groups and the healthy controls, but this decreased over time from day 3-7. Compared to MAP and MSAP, the SAP patients experienced the least amount of *caspase 2* expression. *Caspases 2* and *Caspases 8* are initiator caspases and are responsible for the activation of executioner caspases during acute pancreatitis (McIlwain *et al*., 2013).

The *caspases* are proteases responsible for the biochemical and morphological features taking place during apoptosis and their activation is the hallmark of apoptosis. There was little to no *caspase 8* expression for the different severities of AP and the healthy controls. *Caspase 8* plays an important role in apoptosis and its activity is often downregulated in cancer (Fianco *et al*.,). *Caspase 8* exists as inactive procaspase monomers that are activated by dimerization, not cleavage. When a gene is downregulated, the rate of a physiological response or the expression of a gene is reduced in the cells. Analysis of microarray gene expression has shown that high levels of *caspase 8* expression are associated with the worst prognosis in glioblastoma (Fianco *et al*., 2017). Thus, the absence of *caspase 8* expression, suggests that apoptosis was initiated by *caspase 2*, which activated the executioner *caspases.* The results also suggest that apoptosis served as protection of the MAP and MSAP patients against SAP. Apoptosis limits the inflammatory cascade; the apoptosis level of acinar cells is inversely related to AP severity. Therefore, the initiation of apoptosis in pancreatic acinar cells has a protective effect whereas suppression of apoptosis by caspase inhibitors increases the severity of AP.

With respect to the executioner caspases, *Caspase 3* expression was higher in the HCs, MAP, and MSAP but the level of caspase *2 expression* either increased or decreased as the days progressed. In SAP patients, the level of *caspase 3* expressions seemed to increase over time. The increases and decreases in *caspase 3* activity were not statistically significant. *Caspase 7* gene expression was higher in MSAP patients compared to the other severities of AP and statistically significant compared to HC. The levels of *caspase 7* expression in SAP also increased over time. Executioner *caspases* get directly activated by the initiator *caspases*. Once the executioner *caspases* get activated, they cleave specific sets of substrates which leads to apoptosis. Apoptosis involves genetically determined cell elimination and it does not break the cell membrane and its constituents (Elmore, 2007). In acute pancreatitis, apoptosis is an important feature and determines disease severity (Bhatia, 2004b; Kang *et al*., 2014). *Caspases* mediate apoptosis and protect from the aggressiveness of necrosis in AP. *Caspase 7* expression in cells was the only of the caspases tested which showed statistically significant differences between the groups.

3.6.3 Reactive Oxygen Species (ROS) analysis using N, N- diethyl -para-phenylenediamine.

Oxidative stress plays a role in the development of inflammation involved in the initiation and progression of AP. The activity of ROS determined by the measurement of hydroperoxides experienced by AP patients of the different severity groups was measured in both serum and plasma samples. Overall SAP and MSAP patients experienced higher levels of hydroperoxides expression in serum and plasma samples compared to the MAP patients and the healthy controls. There was no decrease in the amount of hydroperoxides experienced by the AP patients over time. MSAP patients experienced higher amounts of hydroperoxides compared to the other severities of AP, especially on day 5.

Oxidative stress occurs when there is an imbalance in the production of reactive oxygen species and the antioxidant defence systems (Pizzino *et al*., 2017). The excessive production of reactive oxygen species is closely related to the initiation of AP by disruption of cell membranes, DNA, and proteins (Criddle, 2016), and thus mediate tissue damage. The reactive oxygen species activity in the AP patients was measured by hydroperoxide concentration and seem to increase with the disease severity. Oxidative stress and poor antioxidant status are associated with complications and mortality in severe acute pancreatitis patients (Baser *et al*., 2016; Pérez *et al*., 2015b). Furthermore, disease progression is due to oxidative stress and subsequent inflammatory processes

through the recruitment and release of proinflammatory mediators, which leads to a systemic inflammatory response (Mittal *et al*., 2014; Pérez *et al*., 2015). ROS are also generated during normal respiration and metabolic enzymatic activities.

However, high levels of oxidative stress predict a severe course and antioxidants have been associated with a reduction in the length of hospital stay in AP patients (Gao *et al*., 2021; Jeurnink *et al*., 2015). Biological systems have cellular antioxidant defense mechanisms such as glutathione peroxidase, catalase, and superoxide dismutase to regulate the production of ROS and protect from hydroperoxides (Pizzino *et al*., 2017). Antioxidant therapy has been investigated in previous studies and its therapeutic efficacy has already been demonstrated to reduce oxidative stress in experimental acute pancreatitis (Esrefoglu, 2012; Swentek *et al*., 2021).

3.7 Conclusions

The alterations in the CD4/CD8 ratios in AP patients are due to inflammation of the pancreas caused the immune system activation. Monitoring of lymphocyte subsets during the hospitalization of patients will help in determining the state of the immune system in patients and with the prognosis of AP patients. The activation of *caspases* activates the innate immune response, and their dysregulation underlies major efforts to design better therapies for inflammatory diseases such as AP. Understanding the molecular mechanisms of pancreatic cell death would provide valuable insights into clinical pancreatic disorders and may offer potential pharmacological interventions. An increase in ROS can lead to cellular dysfunction and even the death of healthy cells. The oxidative stress that is activated during the inflammatory response to acinar injury may be responsible for local and systemic inflammation. Alterations in CD4/CD8 ratio, the activation of caspases and their interactions with ROS might help in predicting prognosis in AP and are significant in developing novel therapeutic strategies.

CHAPTER 4: SERUM METABOLOMIC AND LIPOPROTEIN PROFILING OF ACUTE PANCREATITIS PATIENTS AND CORRELATION OF ASSAYS

4.1 Introduction

Acute pancreatitis has diverse presentations and the clinical variations of the disease make its prognosis laborious and challenging. The detection of abnormalities at a molecular level will provide a greater understanding of the pathophysiological events which take place during AP. Currently, the prognosis and diagnosis of AP rely on clinical assessments and grading systems which use many variables which make their use unsuitable for the evaluation of patients at the time of admission. These scoring systems have been designed to assist clinicians to triage AP patients and to predict their prognosis (Sharma *et al*., 2015). There have been many attempts to develop a laboratory tool that can be used to predict disease severity and progression in AP with ease, that is cheap, rapid, and reliable (Werner *et al*., 2003).

In recent years advanced technology has made it possible to use non-invasive techniques such as metabolomics whereby investigations are conducted through the analysis of urine (Wang *et al*., 2012), blood serum/plasma (Kiseleva *et al*., 2021), and saliva (Gardner *et al*., 2020). Through metabolomics, the metabolic profiles and patterns of diseased and healthy subjects can be determined. Nuclear Magnetic Resonance (NMR) Spectroscopy / ^{1}H NMR can identify clinical biometabolic indicators to provide early diagnosis and an understanding of the mechanisms involved in pathologies. NMR can quantitate a wide range of low molecular weight substances present in a biological sample using high-throughput analysis with minimal invasion (Cacciatore *et al*., 2021). A high-frequency magnetic field is applied to samples which causes the atoms to move from a lower energy to a higher energy state. A different frequency is used to create the transition as each element is different and therefore requires a different magnetic pulse. Different frequencies are released as the atoms return to the lower energy and an external magnetic field is applied to a sample and atoms align in the direction of the field. The data released is converted to a spectrum. The data is analyzed using various statistical tools to calculate statistical significance and correlate data.

Various metabolites are produced from the metabolism of foods or when chemicals are either enzymatically converted or modified for specific biological functions within the body. Also, the tissue repair process due to tissue damage or the activation of the immune cells during inflammation has metabolic alterations (Kominsky *et al*., 2010). These deviations from normal ranges may show on the metabolic profile of a patient on suspicion of disease. The metabolomic analysis allows for the quantification of multiple metabolites simultaneously, which could lead to the discovery of metabolic indicators of AP progression. NMR has been used to investigate the urinary and plasma metabolic phenotype of AP patients to determine the diagnostic and prognostic potential of metabolites as biometabolic indicators (Villaseñor *et al*., 2014). Patients' samples were identified by high levels of lipids, urinary ketone bodies, glucose, plasma choline, and decreased levels of urinary hippurate, creatine and plasma-branched chain amino acids. AP could be diagnosed with a high degree of sensitivity and specificity using panel of discriminatory biometabolic indicators consisting of guanine, hippurate and creatine (urine), and valine, alanine and lipoproteins (plasma). Additionally, metabolic phenotyping could differentiate between cholelithiasis and colonic inflammation in those without AP. AP is an inflammatory disease; therefore, the analysis may reveal systemic metabolic outcomes and provide novel targets for intervention. The metabolites released into the bloodstream due to the pathophysiological changes that take place during AP can be analyzed using NMR for the discovery of new metabolic indicators. Metabolites found in patients with an AP diagnosis can be studied in detail at a molecular level. Findings should inform on the potential of using these metabolic parameters as prognostic metabolic indicators that may be more accurate in stratifying the risk profiles of patients at the early phase of AP and potentially improve management strategies.

4.2 Objective

To use ^{1}H NMR spectroscopy to measure and identify altered metabolites in mild, moderately severe, and severe AP patients.

4.3 Materials and methods

4.3.1 Serum sample preparation

Thirty frozen serum samples (MAP = 8, MSAP = 14, SAP =10, HC = 10) previously processed and stored at -70°C were left to thaw at room temperature. A 300 µL 0.75M phosphate buffer at

pH of 7.4 consisting of 65 mg Sodium azide (NaN_3), 5.81 mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in deuterated water (D_2O). Once thawed, 300 µL of the serum and 300 µL of the phosphate buffer were added into an Eppendorf tube using a pipette. The mixture was vortexed and 540 µL was transferred into a 5 mm NMR tube (Wilmad Lab Glass, Vineland, NJ, USA) to be analyzed.

4.3.2 Lipid extracts preparation

Lipids were extracted using the protocol described by Lamiquiz-Moneo *et al*. (Lamiquiz-Moneo *et al*., 2019). Three hundred microliters of BUME (butanol: methanol—2:1) was added to 100 µL serum aliquots in glass GC vials followed by 300 µL DIPE (diisopropyl ether: ethyl acetate—3:1) and 300 µL H_2O. Samples were vortexed for 1 minute after the addition of BUME and H_2O and incubated on a shaker for 10 minutes after DIPE addition to allow for lipid extraction. The samples were then centrifuged at 2,058 x*g* for 5 minutes, after which the top layer was transferred to clean vials, dried under liquid nitrogen at 37 °C and then resuspended in 600 µL solution of CDCl3:CD3OD:D_2O (chloroform-d:methanol-d:water-d; 16:7:1, v/v/v) containing 1.18 mM TSP. Five hundred and forty microlitres of this final solution were then transferred to a 5 mm NMR tube (Wilmad Lab Glass) for analysis.

4.3.3 NMR analysis

The NMR tubes, which contain the diluted serum were loaded on a 500 MHz Bruker Avance III HD NMR (Bruker BioSpin Corporation, Switzerland) spectrometer equipped with a triple-resonance inverse 1H probe head and x,y,z gradient coils, kept at a constant temperature of 37°C. One-dimensional proton 1H NMR spectra were acquired using different pulse sequences. A standard nuclear overhauser effect spectroscopy (NOESY) pulse sequence presat (noesygppr1d.comp) was used on both serum and lipid extract samples. On serum samples, NOESY was used to detect both signals of small metabolites and high-molecular-weight macromolecules such as lipoproteins. Additionally, a standard diffusion-edited (DIFF) pulse sequence (ledbpgppr2s1d) was used on the serum samples to detect only high-molecular-weight macromolecules, such as lipoproteins. Pooled samples from each severity group were used as a quality control sample and were included in each batch for qualitative assessment of repeatability by overlaying the raw spectra. The following adjustments were made to obtain reproducible data:

- Shimming to the TSP signal to correct the inhomogeneity of the magnetic field caused by variations of the applied magnetic field caused by interfering compounds in the sample or imperfections in the main magnet.
- To compensate for the magnetic field drift, the signal was locked to a pre-defined D_2O reference signal present in each sample.
- Tuning was done at 500.133 MHz and the pulse was calibrated to ensure a resonant frequency at 90°.

One hundred and twenty-eight scans were subjected to an excitation pulse of 90° for 8 µs followed by a relaxation delay of 4 seconds. The spectral width for the ^1H NMR was 6000 Hz.

a) Data Processing and Clean-Up

Data pre-processing steps were automatically completed by Bruker Topspin (version 3.5) software and included:

- Fourier transformation of the raw free induction decay signal to readable spectral peaks.
- Baseline phasing and correction.
- TSP calibration to exactly 0.00 ppm; and
- Pre-saturation/suppression of H_2O resonance at 4.72 ppm by single-frequency irradiation during the 4 s relaxation delay with 8 _s 90_ excitation pulse, using NOESY-presat pulse sequence program.
- The spectral resolution was inspected to ensure that shimming was done correctly by ensuring that the width of the TSP peak, at half the height of the peak, was <1 Hz. Data processing steps were then conducted using AMIX (version 3.9.14, Bruker, Rheinstetten, Germany), and the dataset normalized relative to the TSP, and the spectral data was quantified across 132 bins (variable-sized binning) to ensure that no spectral regions of noise were included in the statistical analyses as noise can have a negative impact on principal component analysis (Halouska and Powers, 2006).

NMR spectroscopy was used to quantify a panel of 38 signals. The peaks of the identified metabolites were fitted by combining a local baseline and Voigt functions based on the multiplicity of the NMR signal (Marshall et al., 1997). To validate the efficacy of the different deconvolution

models, the root-mean-square deviation was determined. The absolute concentration of each metabolite was calculated according to the previously reported equation (Serkova et al., 2005). The number of protons contributing to the unknown signals was imputed to 1. The concentration of carbohydrates was also estimated by considering the equilibrium between their cyclic forms.

b) Normalization

Data clean-up steps included log-transformation using natural shift log transformation (Wishart, 2008) (heteroscedasticity correction for non-gaussian variable distribution), as well as auto-scaling to align and correlate all variables (van den Berg et al., 2006), which were executed utilizing R Studio (version 1.1.456)

c) GlycA and GlycB signals were quantified by integrating the areas between 2.00 and 2.05 ppm and between 2.09 and 2.05 ppm, respectively, above a local baseline, aiming to remove the signal of lipoproteins.
d) The Liposcale test (Biosfer TesLab, Reus, Spain) was then used to determine lipoprotein parameters, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) and particle number, size, and lipid concentration of each subtype (Mallol *et al*., 2015).
e) Each DIFF spectrum in the range between 0.1 and 9.5 ppm, excluding the regions corresponding to the water signals between 4.40 and 5.00 ppm, was segmented into 0.001-ppm chemical shift bins, and the corresponding spectral areas under the curve, giving a total of 8800 variables.

4.4 Statistical analysis and marker identification

Statistical analysis and graphical illustrations of the data were generated in R (version 3.6.1) and R studio (version 1.1.456) software using scripts developed in-house. Wilcoxon and Kruskal-Wallis rank-sum test was used to compare differences in numerical covariates (e.g., age and metabolite concentration). Fisher's exact test was used to assess differences between categorical variables (e.g., gender). The Spearman's rank test was used to calculate the correlation coefficient (rho) between variables. Rho is a statistical tool that measures the strength of the linear relationship between two variables with values that range from – 1 to 1. Whereby, a rho of -1 describes a perfect

negative, and a rho of 1 describes a perfect positive. To account for multiple testing, a false discovery rate (FDR) of <10% was applied. FDR is the ratio of the number of false-positive classifications to the total number of positive classifications.

The KODAMA algorithm (Cacciatore *et al*., 2017) was used to facilitate the identification of patterns representing underlying metabolic phenotypes on all samples in the data set (Bray *et al*., 2017). The partition around medoids (PAM) clustering (Reynolds *et al*., 2006) was applied to the KODAMA scores using the silhouette algorithm 10 (Rousseeuw, 1987) to verify the obtained results. The silhouette median value was utilized to assess the ideal number of clusters, ranging from 2 to 10. Using partial least-squares (PLS) analysis, regression was performed on DIFF spectra metabolic profiles. Furthermore, 10-fold cross-validation was performed to evaluate the predictive efficacy of the model (Bertini *et al*., 2012). Both the goodness of fit parameter (R^2) and the predictive ability parameter (Q^2) were also calculated using standard formulas (Eriksson *et al*., 2003). The Q^2 value was calculated from p-values to assess the performance of the PLS regression model (Szymańska *et al*., 2012). For all the statistical analyses, a p-value < 0.05 was regarded as significant.

4.5 Results

4.5.1 Metabolite profile

To delineate the metabolic signatures of acute pancreatitis, Spearman's rank correlation test was performed to link metabolic values to the different AP groups in the following rank order: HC, MAP = 1, MSAP = 2, and SAP = 3. A total of 38 signals were quantified from the NMR spectra of serum samples and lipid extracts, including 27 metabolites, 9 lipid classes, inflammatory metabolic indicators, GlycA and GlycB, and 1 signal that correlated with protein concentration. The analysis of the metabolite concentrations in serum samples and lipid extracts is shown in **Table 4.1**. A strong positive correlation was observed with the concentration of the metabolites; phenylalanine (rho = 0.54; p-value < 0.001, FDR = 0.003), mannose (rho = 0.57; p-value < 0.001, FDR = 0.002), lactate (rho = 0.67; p-value <0.001, FDR = 0.001), acetoacetate (rho=0.63; p-value <0.001, FDR = <0.001, 3-hydroxybutyrate (rho = 0.46; p-value < 0.003, FDR = 0.013) and lipid alpha-CH2 (rho=0.45; p-value = 0.006, FDR= 0.013) which increased with AP severity. On the contrary, ascorbate (rho = 0.46; p-value < 0.003, FDR = 0.013), glutamine (rho = -0.55; p-value

<0.001, FDR = 0.002), methanol (rho = 0.46; p-value < 0.003, FDR = 0.013), ethanol (rho = 0.64; p-value < 0.001, FDR< 0.001), lipid=CH-CH2-CH= (rho = -0.55, p-value <0.001, FDR =0.002), lipid bet-CH2 (rho = -0.52; p-value < 0.001, FDR= 0.004), lipid-CH3 (rho = -0.44; p-value = 0.005, FDR = 0.016) and protein-NH (rho =-0.75; p<0.001, FDR <0.001) decreased in concentration with AP severity. Although not statistically significant, the inflammatory metabolic indicators GlycA and GlycB showed a positive correlation with the severity of the disease.

Table 4.1: Correlation of metabolites concentration with the disease severity of acute pancreatitis

Feature	rho	p-value	FDR
Formate	0.04	0.793	0.809
Unknown signal at 8.12 ppm	0.01	0.951	0.951
Unknown signal at 8.07 ppm	-0.08	0.630	0.684
Phenylalanine	0.54	0.0004	0.003
Tyrosine	-0.13	0.443	0.525
Unknown signal at 7.14 ppm	0.19	0.237	0.318
Histidine	-0.1	0.557	0.631
Glucose	0.24	0.140	0.238
Mannose	0.57	<0.001	<0.001
Unknown signal at 5.15 ppm	0.23	0.155	0.239
Unknown signal at 5.09 ppm	0.16	0.330	0.421
Unknown signal at 5.01 ppm	0.23	0.155	0.239
Ascorbate	-0.46	0.003	0.013
Threonine	-0.33	0.043	0.104
Lactate	0.67	<0.001	<0.001
Creatinine	0.2	0.218	0.309
Creatine	0.3	0.064	0.143
Glycine	0.06	0.717	0.746
Methanol	-0.46	0.003	0.013
Unknown signal at 2.55 ppm	0.25	0.130	0.229
Citrate	-0.16	0.330	0.421
Glutamine	-0.55	<0.001	0.002
Pyruvate	0.2	0.230	0.317
Glutamate	0.25	0.126	0.229
Acetoacetate	0.63	<0.001	<0.001
Acetate	0.28	0.0893	0.169
Alanine	-0.29	0.0773	0.158
Unknown signal at 1.45 ppm	0.44	0.005	0.016
Unknown signal at 1.43 ppm	0.23	0.155	0.239
3-Hydroxybutyrate	0.46	0.003	0.013
Ethanol	-0.64	<0.001	0.236
Unknown signal at 1.16 ppm	-0.07	0.686	0.729
Unknown signal at 1.14 ppm	0.31	0.005	0.124
Unknown signal at 1.11 ppm	0.47	0.003	0.013
Unknown signal at 1.06 ppm	0.4	0.011	0.032
Valine	-0.14	0.411	0.511
Isoleucine	0.22	0.184	0.276
Leucine	0.11	0.487	0.564
2-Hydroxybutyrate	0.34	0.032	0.081
Protein NH	-0.75	<0.001	<0.001

Unsaturated lipid -CH=CH-	-0.35	0.029	0.077
Lipid alpha-CH2	0.45	0.003	<0.001
Cholesterol	-0.43	<0.001	0.017
Lipid =CH-CH2-CH=	-0.55	<0.001	0.002
Glycorol phospholipid	0.21	0.199	0.289
Phospholipid	-0.13	0.440	0.525
Lipid beta-CH2	-0.52	<0.001	0.004
Lipid CH2	0.09	0.599	0.664
Lipid CH3	-0.44	0.005	0.016
GlycB	0.28	0.085	0.167
GlycA	0.29	0.074	0.158

Rho: Spearman's correlation coefficient FDR: False Discovery rate

4.5.2 Metabolite patterns

An unsupervised KODAMA analysis was performed to visualize patterns between metabolic phenotypes (metabotype) and disease severity. This method was applied to the metabolic concentrations of the samples collected during the second day of sample collection. As shown in **Figure 4A**, healthy subjects used as controls had a distinct metabolic profile from AP patients. Notably, a clear separation was evident also between patients with MAP and SAP. Interestingly, 2 patients with SAP showed a metabolic profile like the MAP (blue cycled). Further analysis indicated that these patients may have a high level of the ratio of free cholesterol /cholesterol ester suggesting they may have cholestasis. Cholestasis is characterized by the presence of lipoprotein X (Lpx) could mask the SAP phenotype. These patients were excluded from subsequent analyses. The group of AP patients with intermediate severity (i.e., MSAP) shared metabolic profiles both with MAP and SAP patients. However, MSAP patients with a closer phenotype to SAP had a higher incidence of adverse events.

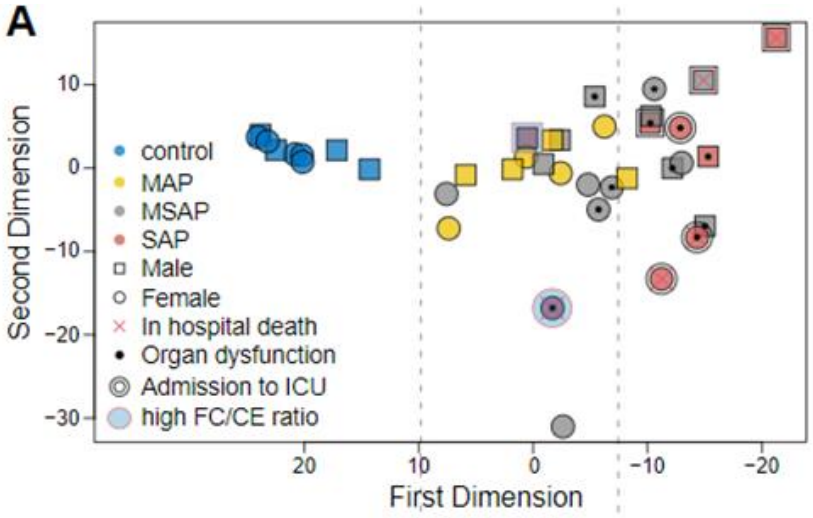


Figure 4A: Unsupervised analysis of the metabolites of HC, MAP, MSAP, and SAP. Two distinct clusters representing the metabolic phenotype of mild and severe AP separated with most of the MAP and MSAP patients in the first component while most of the SAP except for 2 SAP patients are in the second component.

Overall, our cohort of patients was separated into three different metabolic phenotypes. The first metabolic phenotype was characterized exclusively by controls. Separation into the other two metabolic phenotypes (MAP+MSAP, MSAP+SAP) was decided using the first dimension of KODAMA using as a threshold the value between MAP and SAP, as shown in **Figure 4B**.

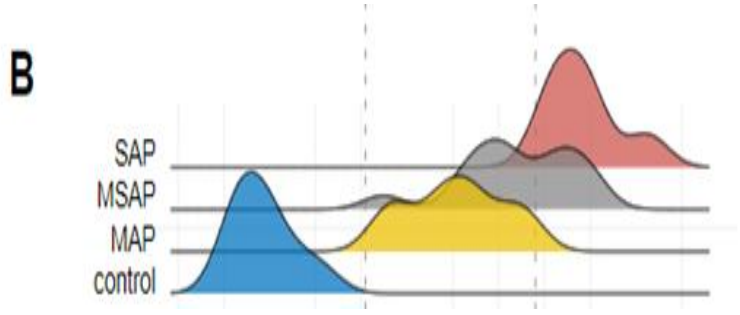


Figure 4B: The distribution of the different AP and HC groups, the distinct separation of the HC and SAP was observed at the left and right of the graph respectively. Both MAP and MSAP were observed in the middle with MAP at the left end and the SAP at the right end.

Some patients with MSAP were classified in the metabolic phenotype of MAP and others with the metabolic phenotype of SAP. Interestingly, those with the latter were characterized by a higher

incidence of adverse outcomes, such as local and systemic complications, organ dysfunction, and admission to the ICU **Figure 4C**.

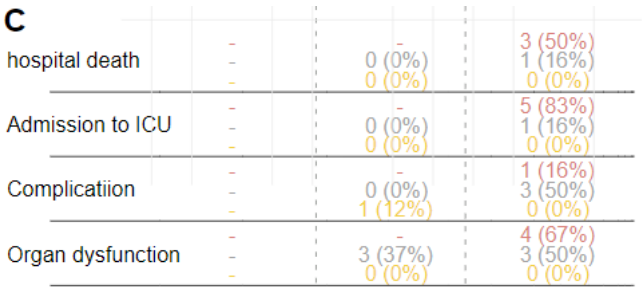


Figure 4C: Comparison of the incidence of the clinical features across severity groups. MAP had the least adverse effect and complications while SAP presented the most adverse effects including death. MSAP had the highest levels of organ dysfunction and complication in hospitalized patients.

A comparison of the clinical features and the metabolites demonstrated that the lipid profile distinctively separated the metabolic phenotypes of AP based on their severity which is shown in **Figure 4D**. The concentration of HDL particles seemed to be a characteristic difference between the metabolic phenotypes. Reduced levels of small HDL-Particle (HDL-P) numbers were observed with increased severity of AP **Figure 4E**. While the inflammatory marker, GlycA, increased across the groups and was at maximum level in the MAP patients with a slight decrease in MSAP and SAP patients **Figure 4F**.

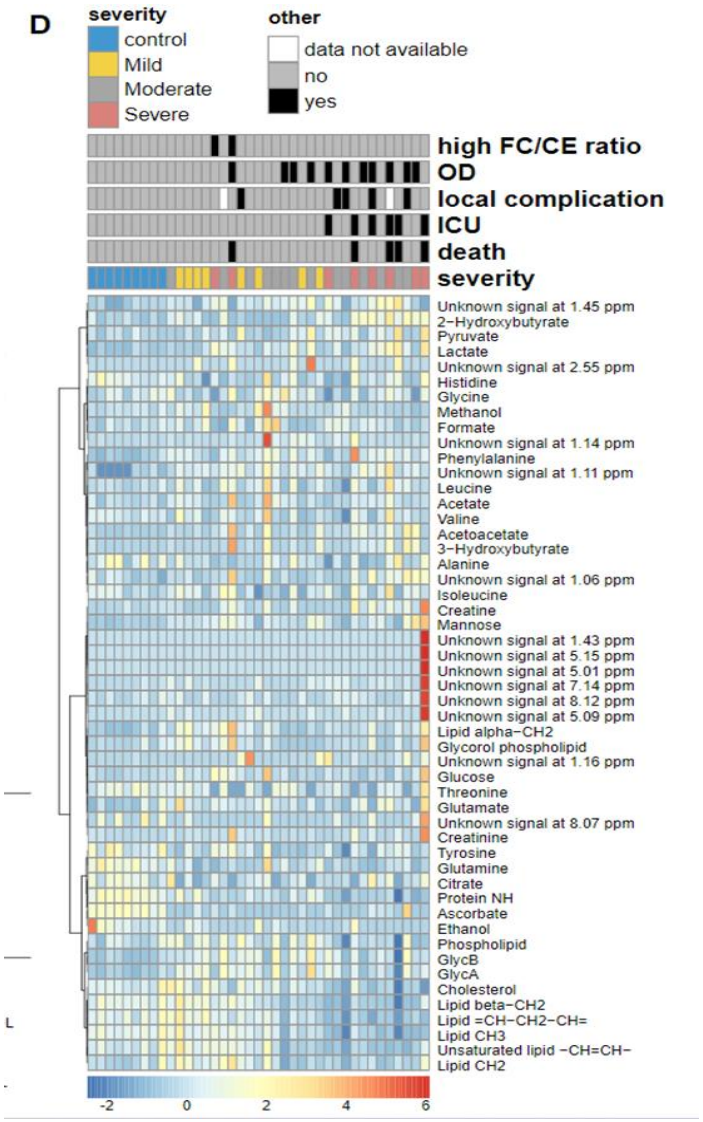


Figure 4D: Heatmap showing the comparison of the clinical features and the metabolites across the AP groups. Lipid profiles were observed to distinctly separate the AP groups as colour changes were observed from blue to red.
Notes: FC/CE - free cholesterol /cholesterol ester, OD- organ dysfunction, ICU- intensive care unit.

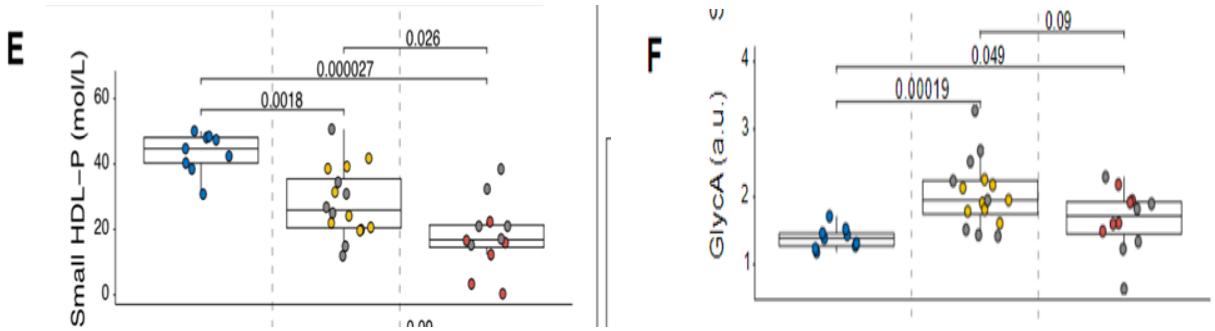


Figure 4E and F: Impact of sHDL-P and GlycA across the metabolic phenotypes of AP. **E** Decreased levels of small HDL-P with increased AP severity were observed. **F** GlycA increased at the mild pancreatic phenotype and a slight decline at the severe pancreatic phenotype. **Notes**: HDL- High-density lipoprotein.

The blood cholesterol decreased in the HDL in AP while the higher level of cholesterol in Intermediate Density Lipoprotein (IDL) and VLDL levels was observed in **Figure 4G**. HDL-C decreased in all the severity groups of AP while both VLDL-C and IDL-C were elevated, and a slight decrease in LDL was observed. Decreased HDL-C in AP might represent impaired anti-inflammatory function, reduced lipoprotein synthesis, and impaired immune functions.

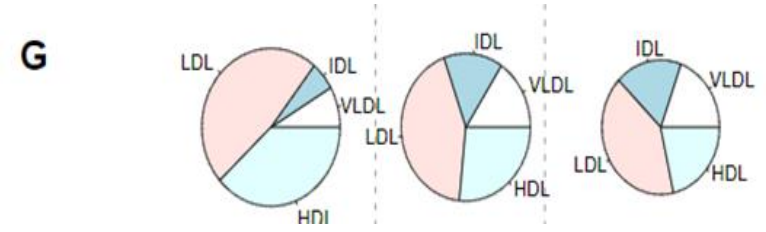


Figure 4G Distribution of cholesterol across the AP groups. HDL-C decreased from HC to SAP while both VLDL-C and IDL-C were elevated. A decrease in LDL was observed. The decreased HDL-C in AP might represent impaired anti-inflammatory function, reduced lipoprotein synthesis, and impaired immune functions. **Notes:** HDL- High-density lipoprotein HDL-C- high-density lipoprotein cholesterol, VLDL- very-low-density lipoprotein, IDL-C low-density lipoprotein, LpX - Lipoprotein X.

4.5.3 Trend analysis

To further understand the association of metabolites and lipoproteins with AP severity, KODAMA analysis was used. A comparison of the metabolites **Table 4.2** and lipoproteins **Table 4.3** with inverted first dimension KODAMA showed dysregulated metabolites such as mannose, ascorbate, phenylalanine and lipoproteins among others as we move from MAP to SAP.

Table 4.2 Correlation of metabolites with inverted first dimension of KODAMA

Feature	rho	p-value	FDR
Formate	-0.05	0.776	0.842
Unknown signal at 8.12 ppm	-0.11	0.531	0.639
Unknown signal at 8.07 ppm	0.05	0.747	0.828
Phenylalanine	-0.51	0.001	0.005
Tyrosine	0.26	0.116	0.211
Unknown signal at 7.14 ppm	-0.31	0.062	0.138
Histidine	0.19	0.266	0.357
Glucose	-0.04	0.834	0.868
Mannose	-0.46	0.005	0.017
Unknown signal at 5.15 ppm	-0.28	0.092	0.174
Unknown signal at 5.09 ppm	-0.17	0.306	0.401
Unknown signal at 5.01 ppm	-0.28	0.092	0.174
Ascorbate	0.44	0.006	0.020
Threonine	0.35	0.032	0.0898
Lactate	-0.62	<0.001	<0.001
Creatinine	0.01	0.934	0.934
Creatine	-0.31	0.062	0.138
Glycine	0.02	0.928	0.934
Methanol	0.39	0.018	0.053
Unknown signal at 2.55 ppm	-0.2	0.229	0.341
Citrate	0.2	0.242	0.341
Glutamine	0.57	<0.001	0.002
Pyruvate	-0.33	0.046	0.110
Glutamate	-0.16	0.329	0.420
Acetoacetate	-0.53	<0.001	0.004
Acetate	-0.13	0.425	0.529
Alanine	0.21	0.216	0.333
Unknown signal at 1.45 ppm	-0.45	0.005	0.018
Unknown signal at 1.43 ppm	-0.28	0.092	0.174
3-Hydroxybutyrate	-0.35	0.034	0.090
Ethanol	0.63	<0.001	<0.001
Unknown signal at 1.16 ppm	-0.1	0.566	0.656
Unknown signal at 1.14 ppm	-0.2	0.242	0.341
Unknown signal at 1.11 ppm	-0.52	<0.000	0.004
Unknown signal at 1.06 ppm	-0.33	0.044	0.110
Valine	0.22	0.200	0.320
Isoleucine	0.04	0.834	0.868
Leucine	-0.1	0.538	0.639
2-Hydroxybutyrate	-0.52	<0.001	0.004
Protein NH	0.82	<0.001	<0.001
Unsaturated lipid -CH=CH-	0.65	0.000	0.000
Lipid alpha-CH2	-0.28	0.089	0.174
Cholesterol	0.62	<0.001	<0.001
Lipid =CH-CH2-CH=	0.74	<0.001	<0.001
Glycorol phospholipid	0.07	0.695	0.788
Phospholipid	0.22	0.199	0.320
Lipid beta-CH2	0.77	<0.001	<0.001
Lipid CH2	0.19	0.247	0.341
Lipid CH3	0.81	<0.001	<0.001
GlycB	-0.23	0.176	0.309
GlycA	-0.22	0.189	0.320

Rho - Spearman's correlation coefficient, FDR – False discovery rate

These metabolites were also dysregulated over time as days progressed from days 1 – 7.

Table 4.3 Spearman correlation between lipoprotein parameters and inverted first dimension of KODAMA

Feature	rho	p-value	FDR
VLDL-C	0.26	0.116	0.216
IDL-C	0.32	0.050	0.131
LDL-C	-0.39	0.018	0.059
HDL-C	-0.63	<0.001	<0.001
VLDL-TG	0.23	0.171	0.278
IDL-TG	0.26	0.114	0.216
LDL-TG	0.21	0.204	0.294
HDL-TG	0.22	0.185	0.283
VLDL-P (nmol/L)	0.25	0.135	0.234
Large VLDL-P (nmol/L)	0.26	0.113	0.216
Medium VLDL-P (nmol/L)	0.14	0.409	0.466
Small VLDL-P (nmol/L)	0.27	0.111	0.216
LDL-P (nmol/L)	-0.4	0.015	0.059
Large LDL-P (nmol/L)	-0.18	0.283	0.369
Medium LDL-P (nmol/L)	-0.18	0.274	0.369
Small LDL-P (nmol/L)	-0.47	0.004	0.019
HDL-P (µmol/L)	-0.65	<0.001	<0.001
Large HDL-P (µmol/L)	0.02	0.895	0.895
Medium HDL-P (µmol/L)	-0.08	0.650	0.676
Small HDL-P (µmol/L)	-0.68	<0.001	<0.001
VLDL-Z (nm)	0.1	0.542	0.587
LDL-Z (nm)	0.34	0.0392	0.113
HDL-Z (nm)	0.68	<0.001	<0.001
Non-HDL-P (nmol/L)	-0.4	0.016	0.059
Total-P/HDL-P	0.16	0.338	0.418
LDL-P/HDL-P	0.14	0.412	0.466

Rho - Spearman's correlation coefficient, FDR – False discovery rate, VLDL-C – Very low-density lipoprotein cholesterol, IDL – Intermediate density lipoproteins, HDL- High density lipoprotein, HDL-P- High density lipoprotein - Particle, LDL- Low-density lipoprotein, VLDL-Very-Low Density Lipoprotein, TG - Triglyceride

The correlation of the concentration of metabolites with AP severity showed that GlycA (*p*-value = 0.004) was significantly altered **Figure 5A**, **Table 4.4**. In this pilot study, the ketoacidosis metabolite, 3-hydroxybutyrate (*p*-value = 0.003) was shown to be significantly linked to worse clinical outcomes in AP as shown in **Figure 5B**, **Table 4.5** and within the severe group this seem to increase with time from day 1 to day 7. Elevated levels of 3-hydroxybutyrate were observed in SAP patients compared to the other severity groups. A metabolite of interest, Lipid alpha-CH2 was observed to be dysregulated in AP patients with organ dysfunction **Figure 5C**. Elevated levels of alpha-CH2 (*p*-value = 0.007) were seen in SAP compared to MSAP and MAP **Table 4.6**.

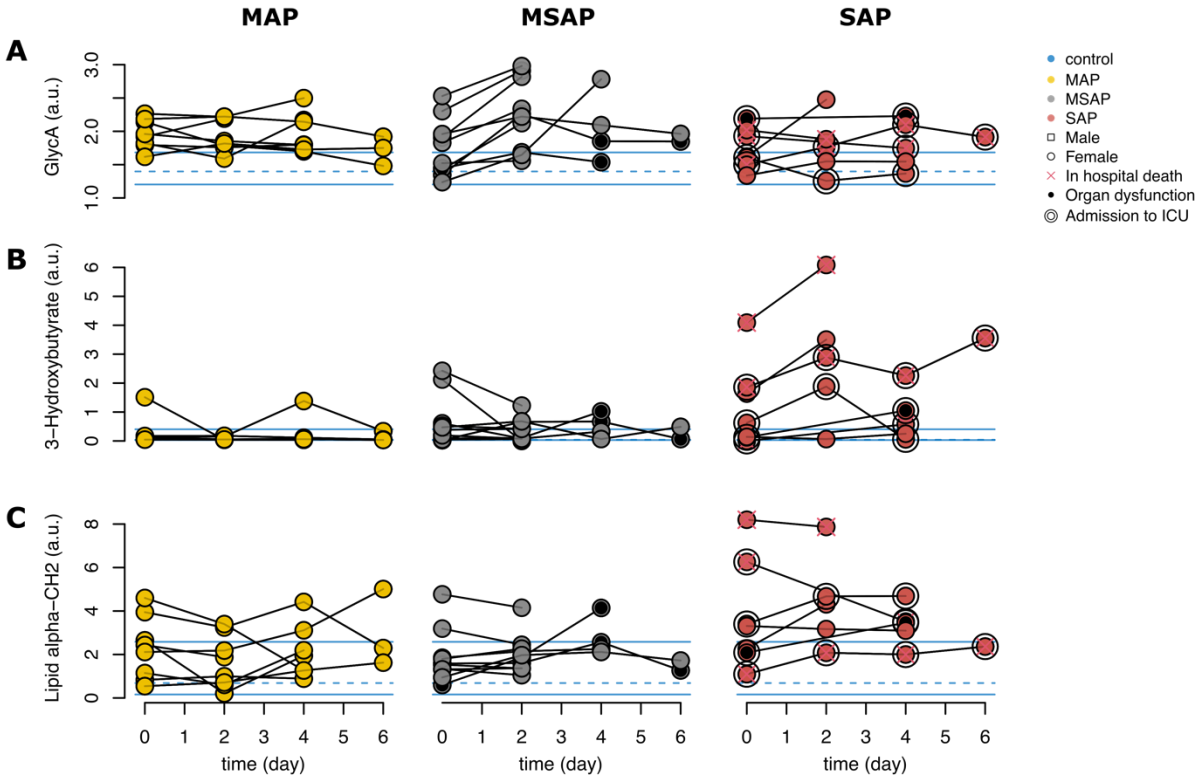


Figure 5: Trends in metabolic parameters over all time points. **A**; describes the pattern for GlycA in the severity groups of AP. **B**; the capability of 3-hydroxybutyrate to predict patients who died in the hospital or were admitted to ICU. **C**; describes the capability of lipid alpha-CH2 to predict which patients were likely to experience organ dysfunction.

Table 4.4 Comparison of Metabolites with severity in Acute Pancreatitis.

Feature	Mild	Moderate	Severe	p-value	FDR
Formate, median [IQR]	0.001 [-0.001 0.002]	0.004 [0.002 0.007]	0.002 [-0.002 0.004]	0.288	0.538
Unknown signal at 8.12 ppm, median [IQR]	0.001 [-0.003 0.005]	0.001 [0 0.003]	0.004 [-0.001 0.008]	0.673	0.838
Unknown signal at 8.07 ppm, median [IQR]	0 [0 0]	0 [0 0.003]	0.002 [0 0.003]	0.661	0.838
Phenylalanine, median [IQR]	-0.01 [-0.015 0.004]	0.004 [-0.01 0.015]	0.014 [0.008 0.018]	0.033	0.212
Tyrosine, median [IQR]	0.002 [-0.002 0.006]	0 [-0.007 0.003]	-0.001 [-0.003 0.004]	0.327	0.576
Unknown signal at 7.14 ppm, median [IQR]	0.021 [0 0.024]	0 [-0.001 0.015]	-0.003 [-0.009 0]	0.071	0.248
Histidine, median [IQR]	-0.005 [-0.009 0.003]	-0.001 [-0.008 0.011]	0.001 [-0.004 0.005]	0.491	0.716
Glucose, median [IQR]	-0.015 [-0.113 0.461]	0.133 [-0.042 0.476]	-0.425 [-0.693 0.155]	0.466	0.710
Mannose, median [IQR]	-0.002 [-0.013 0.007]	0.002 [-0.01 0.004]	0.001 [-0.007 0.012]	0.801	0.923
Unknown signal at 5.15 ppm, median [IQR]	0 [0 0]	0 [0 0]	0 [0 0]	0.276	0.538
Unknown signal at 5.09 ppm, median [IQR]	0.001 [0 0.013]	0 [-0.001 0.004]	0 [0 0.004]	0.548	0.740
Unknown signal at 5.01 ppm, median [IQR]	0 [0 0]	0 [0 0]	0 [0 0]	0.739	0.898
Ascorbate, median [IQR]	0 [0 0.004]	0 [0 0.003]	-0.004 [-0.005 0]	0.115	0.311
Threonine, median [IQR]	0.007 [0.003 0.028]	-0.003 [-0.008 0.009]	0.004 [-0.012 0.029]	0.473	0.710
Lactate, median [IQR]	-0.001 [-0.194 0.312]	0.213 [-0.064 0.42]	0.009 [-0.766 0.068]	0.220	0.487
Creatinine, median [IQR]	-0.001 [-0.005 0.006]	0.001 [-0.002 0.006]	-0.011 [-0.083 0.001]	0.204	0.473
Creatine, median [IQR]	0 [-0.004 0.001]	-0.011 [-0.026 0]	-0.004 [-0.036 -0.003]	0.241	0.512
Glycine, median [IQR]	0.01 [-0.001 0.02]	0.034 [0.001 0.042]	0.026 [0.002 0.041]	0.424	0.697
Methanol, median [IQR]	0.003 [-0.011 0.012]	0.002 [-0.004 0.036]	0.001 [-0.002 0.001]	0.393	0.668
Unknown signal at 2.55 ppm, median [IQR]	0 [0 0]	0 [0 0.006]	0 [0 0.036]	0.263	0.537
Citrate, median [IQR]	-0.003 [-0.011 0.006]	-0.001 [-0.024 0.006]	-0.002 [-0.014 0.005]	0.890	0.923
Glutamine, median [IQR]	-0.013 [-0.039 0]	0.026 [0.003 0.05]	0.028 [0.012 0.032]	0.098	0.311
Pyruvate, median [IQR]	-0.008 [-0.022 0]	0.004 [-0.014 0.013]	0.005 [-0.013 0.016]	0.470	0.710
Glutamate, median [IQR]	0.02 [0.003 0.046]	0.034 [0.018 0.063]	0.03 [-0.01 0.052]	0.829	0.923
Acetoacetate, median [IQR]	0.003 [-0.007 0.007]	-0.022 [-0.067 0.031]	0.145 [0.061 0.421]	0.007	0.092
Acetate, median [IQR]	-0.001 [-0.003 0.007]	0.002 [-0.001 0.006]	0.004 [-0.001 0.007]	0.819	0.923
Alanine, median [IQR]	0.006 [-0.036 0.046]	0.02 [-0.009 0.044]	0.012 [-0.023 0.027]	0.876	0.923
Unknown signal at 1.45 ppm, median [IQR]	0 [-0.005 0]	0.005 [-0.001 0.012]	0.004 [-0.007 0.017]	0.295	0.538
Unknown signal at 1.43 ppm, median [IQR]	0 [0 0]	0 [0 0]	0 [0 0]	1.000	1.000
3-Hydroxybutyrate, median [IQR]	0.002 [-0.015 0.003]	-0.023 [-0.213 0.016]	0.514 [0.187 0.781]	0.012	0.092
Ethanol, median [IQR]	0.003 [-0.003 0.008]	0 [0 0]	0 [0 0.009]	0.623	0.814
Unknown signal at 1.16 ppm, median [IQR]	-0.003 [-0.683 0.009]	-0.012 [-0.058 0.012]	0 [-0.448 0]	0.866	0.923
Unknown signal at 1.14 ppm, median [IQR]	0 [-0.003 0.012]	-0.007 [-0.019 0.012]	0 [0 0.015]	0.520	0.737
Unknown signal at 1.11 ppm, median [IQR]	-0.01 [-0.013 -0.005]	-0.002 [-0.01 0.003]	-0.002 [-0.004 0.004]	0.162	0.393
Unknown signal at 1.06 ppm, median [IQR]	-0.004 [-0.005 -0.001]	-0.007 [-0.012 0]	0.007 [0.004 0.015]	0.004	0.092
Valine, median [IQR]	-0.009 [-0.027 0.001]	0.002 [-0.009 0.024]	0.02 [0.011 0.054]	0.071	0.248
Isoleucine, median [IQR]	0.003 [-0.005 0.009]	0 [-0.002 0.012]	0.01 [0.002 0.013]	0.551	0.740
Leucine, median [IQR]	-0.006 [-0.011 0.001]	0.001 [-0.005 0.011]	0.01 [0.006 0.018]	0.073	0.248
2-Hydroxybutyrate, median [IQR]	-0.009 [-0.013 -0.003]	-0.005 [-0.025 0]	0.004 [-0.005 0.036]	0.116	0.311
Protein NH, median [IQR]	1.652 [-5.597 3.752]	1.062 [-6.484 14.877]	-2.663 [-5.193 3.942]	0.905	0.923
Unsaturated lipid -CH=CH-, median [IQR]	-0.226 [-0.746 -0.077]	0.12 [-0.457 1.535]	0.905 [0.578 1.35]	0.108	0.311
Lipid alpha-CH2, median [IQR]	-0.275 [-0.414 0.046]	0.018 [-0.128 0.213]	0.344 [-0.118 0.564]	0.148	0.377
Cholesterol, median [IQR]	-0.026 [-0.06 0.003]	0.02 [-0.052 0.106]	0.054 [0.044 0.176]	0.013	0.092
Lipid =CH-CH2-CH=, median [IQR]	-0.121 [-0.286 -0.01]	0.093 [-0.105 0.623]	0.469 [0.205 0.787]	0.013	0.092
Glycorol phospholipid, median [IQR]	-0.082 [-0.137 0.003]	-0.002 [-0.214 0.036]	0 [-0.201 0.102]	0.840	0.923
Phospholipid, median [IQR]	-0.202 [-0.358 0.008]	0.168 [-0.006 0.267]	0.06 [-0.027 0.146]	0.064	0.248
Lipid beta-CH2, median [IQR]	-0.138 [-0.426 -0.06]	-0.056 [-0.368 0.668]	0.453 [0.206 1.324]	0.061	0.248
Lipid CH2, median [IQR]	-2.675 [-5.831 -1.865]	1.226 [-2.266 7.464]	3.196 [1.422 7.154]	0.068	0.248
Lipid CH3, median [IQR]	-0.014 [-1.985 0.337]	0.589 [-0.394 2.7]	1.528 [0.599 1.627]	0.047	0.248
GlycB, median [IQR]	0 [-0.004 0.008]	0.045 [0.037 0.089]	0.014 [-0.001 0.038]	0.010	0.092
GlycA, median [IQR]	-0.021 [-0.069 0.039]	0.24 [0.149 0.337]	0.01 [-0.057 0.12]	0.004	0.092

While lipid alpha-CH2 was generally higher in the SAP group, within patients, it appeared to be consistent over time.

Table 4.5 Comparison of metabolites with worse clinical outcomes in AP.

Feature	FALSE	TRUE	p-value	FDR
Formate, median [IQR]	0.003 [0 0.004]	-0.001 [-0.003 0.004]	0.659	0.997
Unknown signal at 8.12 ppm, median [IQR]	0.001 [-0.001 0.004]	0.007 [-0.001 0.009]	0.395	0.821
Unknown signal at 8.07 ppm, median [IQR]	0 [0 0.004]	0 [0 0.002]	0.798	0.997
Phenylalanine, median [IQR]	0.003 [-0.013 0.014]	0.01 [0.006 0.014]	0.325	0.821
Tyrosine, median [IQR]	0.001 [-0.003 0.004]	-0.001 [-0.003 0.005]	0.973	1.000
Unknown signal at 7.14 ppm, median [IQR]	0 [0 0.021]	-0.006 [-0.012 -0.003]	0.019	0.238
Histidine, median [IQR]	-0.003 [-0.008 0.004]	0.001 [-0.006 0.005]	0.812	0.997
Glucose, median [IQR]	0.068 [-0.113 0.665]	-0.425 [-0.48 -0.045]	0.164	0.706
Mannose, median [IQR]	0.002 [-0.011 0.009]	-0.006 [-0.007 0.001]	0.709	0.997
Unknown signal at 5.15 ppm, median [IQR]	0 [0 0]	0 [0 0]	0.057	0.450
Unknown signal at 5.09 ppm, median [IQR]	0 [0 0.009]	0 [0 0]	0.414	0.821
Unknown signal at 5.01 ppm, median [IQR]	0 [0 0]	0 [0 0]	0.904	0.997
Ascorbate, median [IQR]	0 [0 0.004]	-0.004 [-0.005 0]	0.166	0.706
Threonine, median [IQR]	0.003 [-0.006 0.023]	0.004 [0 0.016]	0.919	0.997
Lactate, median [IQR]	0.151 [-0.193 0.375]	0.009 [-0.719 0.046]	0.126	0.644
Creatinine, median [IQR]	0.001 [-0.005 0.007]	-0.025 [-0.141 -0.004]	0.053	0.450
Creatine, median [IQR]	-0.003 [-0.015 0.001]	-0.022 [-0.05 -0.003]	0.262	0.785
Glycine, median [IQR]	0.022 [-0.001 0.04]	0.026 [-0.021 0.026]	0.292	0.785
Methanol, median [IQR]	0.002 [-0.001 0.018]	-0.002 [-0.002 0.001]	0.096	0.544
Unknown signal at 2.55 ppm, median [IQR]	0 [0 0]	0 [0 0]	0.732	0.997
Citrate, median [IQR]	-0.001 [-0.02 0.008]	-0.002 [-0.008 0.002]	0.709	0.997
Glutamine, median [IQR]	0.012 [-0.019 0.04]	0.013 [0.012 0.028]	1.000	1.000
Pyruvate, median [IQR]	-0.001 [-0.018 0.011]	0.005 [-0.016 0.007]	0.919	0.997
Glutamate, median [IQR]	0.034 [0.013 0.062]	0.015 [-0.036 0.03]	0.209	0.710
Acetoacetate, median [IQR]	0.002 [-0.032 0.018]	0.145 [0.074 0.259]	0.004	0.123
Acetate, median [IQR]	0.002 [-0.002 0.007]	0.002 [-0.005 0.008]	0.865	0.997
Alanine, median [IQR]	0.012 [-0.02 0.05]	0.012 [-0.03 0.026]	0.519	0.879
Unknown signal at 1.45 ppm, median [IQR]	0 [-0.004 0.009]	0.004 [-0.008 0.012]	0.919	0.997
Unknown signal at 1.43 ppm, median [IQR]	0 [0 0]	0 [0 0]	1.000	1.000
3-Hydroxybutyrate, median [IQR]	0.002 [-0.055 0.009]	0.514 [0.231 0.628]	0.003	0.123
Ethanol, median [IQR]	0 [0 0.007]	0 [0 0]	0.692	0.997
Unknown signal at 1.16 ppm, median [IQR]	0 [-0.063 0.011]	0 [-0.895 0]	0.534	0.879
Unknown signal at 1.14 ppm, median [IQR]	0 [-0.014 0.014]	0 [-0.001 0]	0.919	0.997
Unknown signal at 1.11 ppm, median [IQR]	-0.004 [-0.012 0.001]	-0.003 [-0.005 -0.002]	0.973	1.000
Unknown signal at 1.06 ppm, median [IQR]	-0.004 [-0.007 0.003]	0.007 [0.004 0.009]	0.011	0.184
Valine, median [IQR]	-0.003 [-0.015 0.028]	0.017 [0.005 0.02]	0.435	0.821
Isoleucine, median [IQR]	0 [-0.005 0.011]	0.01 [0.003 0.012]	0.209	0.710
Leucine, median [IQR]	0 [-0.007 0.012]	0.008 [0.003 0.01]	0.359	0.821
2-Hydroxybutyrate, median [IQR]	-0.006 [-0.02 0]	-0.003 [-0.007 0.004]	0.395	0.821
Protein NH, median [IQR]	2.231 [-6.081 10.547]	-3.854 [-6.531 -2.663]	0.209	0.710
Unsaturated lipid -CH=CH-, median [IQR]	-0.154 [-0.404 1.137]	0.667 [0.489 0.905]	0.519	0.879
Lipid alpha-CH2, median [IQR]	-0.03 [-0.286 0.1]	0.344 [-0.166 0.492]	0.476	0.866
Cholesterol, median [IQR]	0.005 [-0.052 0.066]	0.049 [0.038 0.148]	0.083	0.530
Lipid =CH-CH2-CH=, median [IQR]	-0.01 [-0.139 0.513]	0.232 [0.177 0.469]	0.292	0.785
Glycorol phospholipid, median [IQR]	-0.028 [-0.139 0.02]	0 [-0.332 0.051]	0.865	0.997
Phospholipid, median [IQR]	0.031 [-0.164 0.214]	0.06 [-0.077 0.061]	0.760	0.997
Lipid beta-CH2, median [IQR]	-0.111 [-0.267 0.472]	0.386 [0.025 0.453]	0.396	0.821
Lipid CH2, median [IQR]	-0.403 [-3.014 3.979]	2.495 [0.349 4.314]	0.564	0.898
Lipid CH3, median [IQR]	0.366 [-0.619 1.49]	0.679 [0.518 1.528]	0.435	0.821
GlycB, median [IQR]	0.035 [0.002 0.049]	0.014 [-0.015 0.026]	0.262	0.785
GlycA, median [IQR]	0.126 [0.009 0.267]	-0.045 [-0.068 0.01]	0.062	0.450

FDR – False discovery rate, IQR- Interquartile range

Table 4.6 Comparison of Metabolites with Organ dysfunction in Acute Pancreatitis

Feature	FALSE	TRUE	p-value	FDR
Formate, median [IQR]	0.002 [0 0.004]	0.003 [-0.002 0.004]	0.978	0.997
Unknown signal at 8.12 ppm, median [IQR]	0.002 [-0.001 0.005]	0.001 [-0.001 0.006]	0.637	0.792
Unknown signal at 8.07 ppm, median [IQR]	0 [0 0]	0.001 [0 0.003]	0.113	0.339
Phenylalanine, median [IQR]	-0.005 [-0.014 0.013]	0.008 [0.003 0.014]	0.212	0.491
Tyrosine, median [IQR]	0.001 [-0.003 0.005]	0 [-0.003 0.004]	0.718	0.852
Unknown signal at 7.14 ppm, median [IQR]	0 [-0.001 0.021]	0 [-0.005 0.013]	0.597	0.780
Histidine, median [IQR]	-0.005 [-0.008 0.003]	0.004 [-0.003 0.006]	0.063	0.248
Glucose, median [IQR]	-0.053 [-0.291 0.266]	0.068 [-0.33 0.502]	0.524	0.722
Mannose, median [IQR]	-0.001 [-0.014 0.005]	0.002 [-0.007 0.012]	0.332	0.604
Unknown signal at 5.15 ppm, median [IQR]	0 [0 0]	0 [0 0]	0.462	0.722
Unknown signal at 5.09 ppm, median [IQR]	0 [-0.001 0.001]	0.003 [0 0.008]	0.283	0.535
Unknown signal at 5.01 ppm, median [IQR]	0 [0 0]	0 [0 0]	0.492	0.722
Ascorbate, median [IQR]	0 [0 0.004]	0 [-0.005 0]	0.192	0.466
Threonine, median [IQR]	0.004 [-0.006 0.029]	0.003 [-0.003 0.014]	0.978	0.997
Lactate, median [IQR]	0.019 [-0.197 0.365]	0.068 [-0.15 0.158]	1.000	1.000
Creatinine, median [IQR]	-0.003 [-0.011 0.005]	0 [-0.007 0.007]	0.978	0.997
Creatine, median [IQR]	-0.004 [-0.017 0]	-0.003 [-0.021 0]	0.934	0.997
Glycine, median [IQR]	0.013 [-0.003 0.028]	0.035 [0.026 0.045]	0.081	0.257
Methanol, median [IQR]	0.002 [-0.005 0.029]	0.001 [-0.001 0.002]	0.524	0.722
Unknown signal at 2.55 ppm, median [IQR]	0 [0 0]	0.004 [0 0.058]	0.027	0.140
Citrate, median [IQR]	-0.007 [-0.016 0.002]	0.001 [-0.015 0.008]	0.421	0.693
Glutamine, median [IQR]	-0.003 [-0.043 0.043]	0.019 [0.012 0.028]	0.390	0.663
Pyruvate, median [IQR]	-0.01 [-0.022 0.002]	0.006 [0 0.022]	0.071	0.257
Glutamate, median [IQR]	0.025 [0 0.05]	0.029 [0.018 0.063]	0.524	0.722
Acetoacetate, median [IQR]	0.001 [-0.022 0.008]	0.094 [0.043 0.231]	0.007	0.121
Acetate, median [IQR]	0 [-0.003 0.007]	0.003 [0 0.005]	0.978	0.997
Alanine, median [IQR]	0.015 [-0.032 0.05]	0.002 [-0.014 0.027]	0.637	0.792
Unknown signal at 1.45 ppm, median [IQR]	0 [-0.005 0.006]	0.002 [-0.005 0.014]	0.524	0.722
Unknown signal at 1.43 ppm, median [IQR]	0 [0 0]	0 [0 0]	0.175	0.447
3-Hydroxybutyrate, median [IQR]	0.001 [-0.046 0.005]	0.275 [0.033 0.599]	0.011	0.134
Ethanol, median [IQR]	0 [0 0.009]	0 [0 0]	0.264	0.518
Unknown signal at 1.16 ppm, median [IQR]	0 [-0.082 0.023]	0 [-0.055 0]	0.652	0.792
Unknown signal at 1.14 ppm, median [IQR]	0 [-0.011 0.017]	0 [-0.003 0.004]	0.889	0.997
Unknown signal at 1.11 ppm, median [IQR]	-0.01 [-0.014 -0.002]	-0.002 [-0.004 0.003]	0.142	0.380
Unknown signal at 1.06 ppm, median [IQR]	-0.003 [-0.006 0.003]	0.003 [-0.006 0.008]	0.255	0.518
Valine, median [IQR]	-0.003 [-0.02 0.014]	0.019 [-0.003 0.045]	0.127	0.360
Isoleucine, median [IQR]	0 [-0.007 0.01]	0.011 [0.002 0.014]	0.081	0.257
Leucine, median [IQR]	-0.002 [-0.008 0.007]	0.009 [0.002 0.02]	0.038	0.159
2-Hydroxybutyrate, median [IQR]	-0.007 [-0.015 0]	-0.003 [-0.016 0]	0.560	0.751
Protein NH, median [IQR]	1.147 [-6.205 6.871]	-1.031 [-5.977 7.473]	0.890	0.997
Unsaturated lipid -CH=CH-, median [IQR]	-0.247 [-0.625 0.088]	0.786 [0.505 1.611]	0.018	0.134
Lipid alpha-CH2, median [IQR]	-0.271 [-0.361 0.03]	0.418 [0.045 0.603]	0.002	0.106
Cholesterol, median [IQR]	-0.004 [-0.061 0.039]	0.052 [0.03 0.156]	0.021	0.136
Lipid =CH-CH2-CH=, median [IQR]	-0.113 [-0.215 0.051]	0.351 [0.151 0.636]	0.018	0.134
Glycorol phospholipid, median [IQR]	-0.11 [-0.279 0]	0.033 [-0.002 0.138]	0.025	0.140
Phospholipid, median [IQR]	-0.003 [-0.313 0.135]	0.063 [-0.008 0.206]	0.255	0.518
Lipid beta-CH2, median [IQR]	-0.149 [-0.514 0.041]	0.42 [0.112 1.023]	0.014	0.134
Lipid CH2, median [IQR]	-2.623 [-5.925 0.397]	5.321 [0.886 9.455]	0.007	0.121
Lipid CH3, median [IQR]	0.117 [-1.375 0.627]	1.104 [0.486 2.753]	0.038	0.159
GlycB, median [IQR]	0.012 [0 0.037]	0.042 [0.028 0.056]	0.233	0.517
GlycA, median [IQR]	0.019 [-0.034 0.18]	0.132 [0.037 0.279]	0.360	0.633

FDR – False discovery rate, IQR- Interquartile range

4.6 Discussion

AP is an inflammatory disease with many variations, the mild cases can self-resolve, however, in 20-30% of cases, patients may develop serious complications which may lead to death (Leppäniemi *et al*., 2019). The disease progression in AP is usually due to systemic inflammatory response syndrome and subsequent organ failure or an infection in the pancreas (Garg and Singh, 2019). It is important to effectively identify high-risk patients as soon as they present to assist clinicians to improve the clinical prognosis. The challenges with some existing metabolic indicators are lack of specificity and the fact that some of these metabolic indicators are short-lived in the body (Walkowska *et al*., 2022) In this chapter, the physiological metabotype in AP disease was able to distinguish between the mild and the severe groups of patients. Based on the metabolite concentration of the AP groups, two distinct phenotypes which were mild and severe were detected. NMR has been used to investigate the urinary and plasma metabolic phenotype of AP patients to determine the diagnostic and prognostic potential of metabolites as biometabolic indicators by Villasenor (Villaseñor *et al*., 2014). However, they were only able to distinguish between gallstones and colonic inflammation using the metabolic profiles but were unable to distinguish between the other causes of AP such as antiretroviral drugs, ERCP, or hypertriglyceridemia (Villaseñor *et al*., 2014). The study aimed to identify potential prognostic biometabolic indicators for AP and stratification of patients based on severity.

4.6.1 Impact of dysregulated metabolites on AP severity

Hypermetabolism due to AP results in disturbances in the metabolism of proteins, carbohydrates, and lipids. These alterations are characterized by an increased rate of metabolic activity which results in diseases such as hypertriglyceridemia, and insulin-resistance diseases such as diabetes mellitus (Kota *et al*., 2013). Therefore, predicting the course of AP in patients on admission is important, and consequently knowing the role of metabolites in AP progression. The detection of the metabolites could potentially help in understanding the pathophysiological conditions of AP which could aid in the early diagnosis of the disease (Ma *et al*., 2012). The presence of pancreatic inflammation could lead to an alteration in the level of metabolites (Xiao *et al*., 2017). Protein concentration decreases with increased severity in this study confirming that AP promotes protein catabolism which involves the breakdown of proteins into amino acids (Singh *et al*., 2012).

Phenylalanine an amino acid that is converted to tyrosine by phenylalanine hydrolase (Matthews, 2007) increased with severity, and levels of tyrosine were reduced. The two main ketone bodies, acetoacetate, and 3-hydroxybutyrate positively correlated with the severity of AP. The ketone bodies including acetone are synthesized in the liver from acetyl-Coenzyme A by 3-hydroxybutyrate dehydrogenase (Dhillon and Gupta, 2022). They are used as an alternative energy source in the absence of glucose and can be seen during starvation, in alcoholism, and in conditions such as type I diabetes (Dhillon and Gupta, 2022). During ketogenesis, the pH of blood in the body drops and this affects internal body conditions such as the electrolyte balance and leads to morbidity and mortality (Kanikarla-Marie and Jain, 2016). Oxidation of ethanol by alcohol dehydrogenase results in the increase of nicotinamide adenine dinucleotide hydrogen (NADH) and creates an imbalance favoring free fatty acids conversion into ketones (Kanikarla-Marie and Jain, 2016).

The metabolite, 3-Hydroxybutyrate (3-HB) is a predictive marker for SAP (Xiao *et al*., 2017; Xiao *et al*., 2020), hence its increase in severity in this study is not surprising. Furthermore, 3-HB was shown to be elevated after ERCP in the serum of AP patients indicating modification in pancreatic functions (Lusczek *et al*., 2013). Oxygen deficiency and increased energy consumption enhance anaerobic glycolysis which occurs in the early phase of AP ultimately leading to elevated levels of lactate (Li *et al*., 2014). Increased lactate concentration has been associated with persistent organ failure in AP (Valverde-López *et al*., 2017), hence the positive correlation with the severity of AP in our group of patients. The metabolic profiles of the patient in this study revealed elevations of biochemical molecules such as lactate dehydrogenase shown in **Chapter 2**, **Table 2.2**, an enzyme involved in the conversion of lactate to pyruvate. This enzyme has been found in AP patients on NRTIs (Mahto *et al*., 2018).

The regimen used in the treatment of HIV such as a nucleoside reverse transcriptase inhibitor (NRTI) has been implicated in some metabolic abnormalities in AP patients such as lactic acidosis (Oliveira *et al*., 2014). Patients with increased lactate levels are at an increased risk for morbidity and mortality in many other diseases (Bou *et al*., 2017). Glutamine, an abundant amino acid in the body, had a negative correlation to severity. Glutamine can become deficient due to infection, trauma, cancer, and critical illness. In SAP, intravenous glutamine has been shown to improve the prognosis in SAP patients by reducing the length of hospital stay, complications, and death (Arutla

et al., 2019). Changes in glutamine can reflect disease severity, thus, its supplementation can reduce oxidative stress and organ function but not infected necrosis (Arutla *et al*., 2019; Dong *et al*., 2022). Oxidative stress was shown to cause pancreatic injury in AP hence reducing levels of ascorbate, which is an antioxidant, that alleviates pancreatic injury (Esrefoglu, 2012).

4.6.2 Lipid profile may determine AP severity.

In this study, the lipid profiles distinctively separated AP groups based on their severity. Serum lipid levels are associated with the severity of AP (Khan *et al*., 2013; Zhang *et al*., 2017). Small HDL-P is known to play a vital role in ATP binding Cassette transporter A 1 (ABCA-1) mediated cholesterol efflux from macrophages which leads to the deployment of intracellular cholesterol to the plasma membrane (Du *et al*., 2015). HDL-C decreased from HC to SAP while IDL and VLDL increased in this study. Recent studies demonstrated that decreased HDL-C levels are an independent prognostic factor of adverse outcomes such as persistent organ failure and death in AP (Zhang *et al*., 2017). HDL possesses anti-inflammatory properties, hence decreased amounts could be latent for AP severity (Murphy and Woollard, 2010). Oxidized VLDL is a marker of oxidative stress which inhibits lipoprotein lipase responsible for triglyceride metabolism (Jong *et al*., 2000). Excessive triglycerides due to a diet high in carbohydrates and alcohol may lead to an increase in VLDL in the blood. This is true for our study population which had overweight and obese individuals which are risk factors for severe acute pancreatitis (Hong *et al*., 2018).

4.6.3 Role of Inflammation on AP severity

Although not statistically significant, the inflammatory metabolic indicators GlycA and GlycB showed a positive correlation with the severity of the disease in **Table 4.1**. The results in **Figure 4F** also demonstrated that GlycA marker levels were elevated for the mild pancreatitis phenotype when compared to the HC. A slight increase was observed with the severe pancreatitis phenotype when compared to the HC. AP is an immune disorder characterized by the release of either anti or proinflammatory cytokines (Zhang *et al*., 2017). The impaired anti-inflammatory function has been linked to reduced HDL-C levels in AP leading to increased free fatty acids which produce an acidic microenvironment (Zhang *et al*., 2017). HDL-C also possesses an antioxidant ability, which is mediated by the paraoxonase-1 activity in AP (Yuksekdag *et al*., 2019). However, in the

presence of inflammation and oxidative stress, HDL-C promotes the inflammatory response (Navab *et al*., 2009).

4.6.4 Specificity of the dysregulated metabolites in AP

Although the perturbed metabolites/lipids such as low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein (VLDL-C) triglycerides (TG) are not directly linked to AP, there is further evidence that altered LDL-C, VLDL-C and TG, are specific to biliary acute pancreatitis (Hong *et al*., 2018). The metabolites such as acetoacetate, and 3-hydroxybutyrate positively correlated with the severity of AP. The metabolite, 3-Hydroxybutyrate (3-HB) is a predictive marker for SAP (Xiao *et al*., 2017; Xiao *et al*., 2020). Protein concentration decreased with increased severity in this study confirming that AP a sudden inflammation of the pancreas which is responsible for exocrine function and produces enzymes which are responsible for digestion, thus, promotes protein catabolism which involves the breakdown of proteins into amino acids. This study demonstrated that metabolites such as phenylalanine, mannose, lactate, glutamine, and ascorbate were altered during AP, however, they are not specific to AP.

4.7 Conclusion

This study demonstrated that proteins, lipids, and metabolites such as phenylalanine, mannose, lactate, acetoacetate, 3-hydroxybutyrate, glutamine, and ascorbate were altered during AP. An unhealthy diet may contribute to the increase in lactate due to lactic acidosis and the increase in ketone bodies due to excessive alcohol consumption as well as VLDL and HDL-C alterations in the blood. All these molecules must be investigated further as prognostic metabolic indicators that can stratify AP patients into three severities: MAP, MSAP, and SAP using NMR spectroscopy. Although not specific to AP, some altered metabolites will help with early stratification of AP, which will help the clinicians to quickly administer the treatment to patients, especially in MSAP and SAP patients who need specialized care.

CHAPTER 5 CORRELATION STUDIES BETWEEN THE CLINICAL DATA AND LABORATORY-BASED INVESTIGATIONS

5.1 Introduction

According to the 2012 Atlanta classification, AP is grouped into mild, moderately severe, or severe pancreatitis. MAP has a good prognosis with rapid recovery, while MSAP and SAP are characterized by the presence of complications, exacerbation of comorbidities, and organ failure that is transient or persistent. Given the challenges in stratifying AP patients for adequate care due to limitations in current biochemical parameters and scoring systems, there is a need to identify biometabolic indicators that can supplement or replace existing ones. In this study, we aimed to find the link between the immune response, apoptosis, and oxidative stress to the metabolomic profile of AP patients and their potential for monitoring AP disease severity. The challenge with the metabolic indicators which are currently being used is that they lack specificity to the pancreas, are short-lived in the blood, make use of many parameters that make their use difficult, and are ill-suited for assessing patients during admission or in the early phase of disease which is usually from the onset of pain to day 7 post epigastric pain. This results in delays in getting the necessary diagnosis and affects the timely treatment of the patients.

5.2 Objective

To provide a summary of the findings and correlations from the clinical and laboratory-based results to find links between the immune responses, apoptosis, oxidative stress, and prominent metabolites and to provide insights for the translational application of the findings.

5.3 Methods

The clinical parameters and results generated from the previous chapters were used to determine relationships and associations between the measured parameters. This was done for selected variables, specifically those which had prominent variations in the different AP severity. A summary of the prominent changes is shown in **Table 5.1**.

5.4 Statistical analysis

The analysis was done using STATA 15 software. Spearman's rank test was used to calculate the correlation coefficient between variables.

Table 5.1: Summary of the parameters that were measured in this study.

Parameter measured	HC	MAP	MSAP	SAP
Lymphocyte ratio (4 MAP, 2MSAP, 1 SAP, 6 HC)	No changes, except for 2 participants	↑ over time	↑ at d5	↑ d3+d5; ↓ d7
Caspase 2 (13 MAP, 14 MSAP, 7 SAP,6 HC)	No changes	↓ over time	↑ at d5+d7	↓ d5+ d7
Caspase 8(13 MAP, 14 MSAP, 7 SAP,6 HC)	No changes	Remain unchanged	↓ at d7	↑ at d5
Caspase 3(13 MAP, 14 MSAP, 7 SAP,6 HC)	No changes	↑ at day 3	↓ at d7	↑ at d5 + d7
Caspase 7- significantly significant (13 MAP, 14 MSAP, 7 SAP,6 HC)	No changes	No changes	↑at d3+d5	↑ at d5 + d7
Hydroperoxides levels (serum) (10 MAP, 7 MSAP, 5 MAP, 10 HC)	No changes	No changes	↑at d3 + d5 ↓ d7	↑ at d3, ↓ d5+ d7
Hydroperoxides levels (plasma) (10 MAP, 7 MSAP, 5 MAP, 10 HC)	No changes	↑ at d5	↑ at d3	↑at d5 + d7
Metabolic changes (8 MAP, 14 MSAP,8 NMR, 10 HC)				
[HDL-p]		↓ vs HC but ↑ vs Severe (↓ with severity)		
HDL-c		↓ vs HC but ↑ and Severe (↓ with severity)		
VLDL		↑ mild ↑↑ Severe (↑ with severity)		
IDL		↑ mild ↑↑ Severe (↑ with severity)		
GlycA		↑ vs HC and Severe		
Phenylalanine		↑ with severity		
Mannose				
Lactate				
Acetoacetate				
3-Hydroxybutyrate				
Lipid-alpha CH2				
Ascorbate		↓ with severity		
Glutamine				
Methanol				
Ethanol				
Protein-NH				
lipid=CH-CH2-CH=				
Lipid beta-CH2				
Cholesterol				
Lipid CH3				

5.5 Results

The correlation coefficient measures the strength of the linear relationship between two variables with values that range from – 1 to 1. Whereby, a rho of -1 describes a perfect negative, and a rho of 1 describes a perfect positive. The spearman's correlation of the hydroperoxides, CD4, CD8, CD4:CD8, and *caspase 7* with the metabolites found in AP samples of different severities was performed. The research parameters below had perfect positive correlations with some of the metabolites in **Table 5.2**. The correlation of the clinical parameters used in the diagnosis of AP with the metabolites are also shown in **Table 5.3.**

Table 5.2: Spearman's correlation showing the perfect positive correlations of the hydroperoxides, CD4, CD8, CD4:CD8, and *caspase 7* with the metabolites found in AP samples of different severities.

	ROS	CD4	CD8	CD4CD8	Caspase 7	Phenylalanine	Mannose	Ascorbate	Lactate	Methanol	Glutamine
ROS	--------										
CD4	-0.5000	-------									
CD8	0.5000	0.5000	--------								
CD4:CD8	-1.0000	0.5000	-0.5000	--------							
Caspase 7	-0.5000	1.0000*	0.5000	0.5000	--------						
Phenylalanine	-1.0000	0.5000	-0.5000	1.0000*	0.5000	-------					
Mannose	0.5000	0.5000	1.0000*	-0.5000	0.5000	-0.5000	--------				
Ascorbate	0.8660	-0.8660	0.0000	-0.8660	-0.8660	-0.8660	0.0000	--------			
Lactate	-0.5000	-0.5000	-1.0000	0.5000	-0.5000	0.5000	-1.0000	0.0000	--------		
Methanol	1.0000*	-0.5000	0.5000	-1.0000	-0.5000	-1.0000	0.5000	0.8660	-0.5000	--------	
Glutamine	0.5000	-1.0000	-0.5000	-0.5000	-1.0000	-0.5000	-0.5000	0.8660	0.5000	0.5000	--------
Pyruvate	-0.5000	0.5000	-1.0000	0.5000	-0.5000	0.5000	-1.0000	0.0000	1.0000*	-0.5000	0.5000
Acetoacetate	-1.0000	0.5000	-0.5000	1.0000*	0.5000	1.0000*	-0.5000	0.8660	0.5000	-1.0000	-0.5000
3-hydroxybutyrate	-1.0000	0.5000	-0.5000	1.0000*	0.5000	1.0000*	-0.5000	-0.8660	0.5000	-1.0000	-0.5000
protein-NH	0.5000	0.5000	1.0000*	-0.5000	0.5000	-0.5000	1.0000*	0.0000	-1.0000	0.5000	-0.5000
lipid alpha-CH2	-0.5000	1.0000*	0.5000	0.5000	1.0000*	0.5000	0.5000	-0.8660	-0.5000	-0.5000	-1.0000
Cholesterol	-0.5000	-0.5000	-1.0000	0.5000	-0.5000	0.5000	-1.0000	0.0000	1.0000*	-0.5000	0.5000
LipidCHCH2CH	-1.0000	0.5000	-0.5000	1.0000*	0.5000	1.0000*	-0.5000	-0.8660	0.5000	-1.0000	-0.5000
lipid beta-CH2	0.5000	0.5000	1.0000*	-0.5000	0.5000	-0.5000	1.0000*	0.0000	-1.0000	0.5000	-0.5000
lipid CH3	-0.5000	1.0000*	0.5000	0.5000	1.0000*	0.5000	0.5000	-0.8660	0.5000	0.5000	-1.0000
GlycB	-1.0000	0.5000	-0.5000	1.0000*	0.5000	1.0000*	-0.5000	-0.8660	0.5000	-1.0000	-0.5000
GlycA	-0.5000	1.0000*	0.5000	0.5000	1.0000*	0.5000	0.5000	-0.8660	-0.5000	-0.5000	-1.0000

- ***Caspase 7*** *with* ***CD4***, ***lipid CH3***, ***Lipid-alpha-CH2*** *as well as with* ***GlycA***.
- ***ROS*** *with* ***methanol***.
- ***CD4*** *with* ***lipid alpha-CH2***, ***lipid-CH3***, *and* ***GlycA***.
- ***CD8*** *with mannose, protein-NH and lipid beta-CH2.*
- ***C4:CD8*** *with* ***phenylalanine***, ***acetoacetate***, *and* ***3-hydroxybutyrate***, *and* ***GlycB***.

Table 5.3: The correlation of the clinical parameters used in the diagnosis of AP with the metabolites.

	Amylase	Lipase	WBC	CRP	Alanine transaminase (ALT)	Aspartate transaminase (AST)	Alkaline phosphatase (ALP)	Platelets
Phenylalanine	0.1429	0.2000	0.4857	0.7143	0.1429	0.4286	-0.7714	1.0000
Mannose	-0.6000	0.4286	-0.0857	0.0857	-0.6000	-0.0857	-0.6000	0.4857
Ascorbate	-0.3928	0.3928	-0.6547	0.1309	-0.3928	-0.1309	-0.3928	0.1309
Lactate	0.2571	-0.0857	-0.1429	0.2571	0.2571	-0.1429	-0.0857	-0.1429
Methanol	0.0286	-0.7143	-0.2571	0.7714	0.0286	0.3143	0.1429	-0.3143
Glutamine	0.0286	0.3143	0.6000	0.6000	0.0286	0.3143	-0.5429	0.8857*
Pyruvate	-0.0286	-0.1429	0.6571	0.0286	-0.0286	-0.4286	-0.0286	-0.1429
Acetoacetate	0.4286	0.0857	0.0286	0.7714	0.4286	0.5429	-0.6000	0.7714
3-Hydroxybutyrate	0.1429	0.2000	-0.1429	0.6000	0.1429	0.4857	-0.6571	0.7714
Ethanol	-0.6547	-0.1309	0.3928	0.6547	-0.6547	-0.3928	-0.1309	-0.1309
ProteinNH	-0.4857	-0.2000	0.1429	0.3714	-0.4857	0.0286	-0.6000	0.3143
Lipid alpha-CH2	-0.6000	0.4286	0.6000	0.0286	-0.6000	-0.6000	-0.1429	0.0857
Cholesterol	0.4857	-0.8286*	0.1429	0.3143	0.4857	0.7714	-0.0857	0.2000
Lipid=CH-CH2-CH=	0.2571	-0.7714	0.0857	0.5429	0.2571	0.6000	0.0286	0.0286
Lipid beta-CH2	-0.0286	-0.6571	0.2571	0.6000	-0.0286	0.3714	-0.1429	0.0857
Lipid CH3	0.2571	-0.7714	0.0857	0.5429	0.2571	0.6000	0.0286	0.0286
GlycB	-0.1429	0.3143	0.0286	0.5429	-0.1429	0.2571	-0.8286*	0.8286*
GlycA |	-0.3143	0.1429	0.0857	0.2571	-0.3143	0.1429	-0.8857*	0.7143

- ***Cholesterol*** *had a positive correlation with* ***amylase***, ***ALT***, *and* ***AST*** *and a strong negative correlation with* ***lipase***
- ***Lipid CH3***, ***Lipid beta-CH2***, *and* ***Lipid=CH-CH2-CH=*** *negatively correlated with* ***lipase*** *and positively correlated with* ***CRP***, *while* ***Lipid=CH-CH2-CH=*** *had a positive correlation with* ***AST*** *as well.*
- ***GlycB*** *had a strong positive correlation with* ***CRP*** *and positive strong correlation with the* ***platelets*** *and a strong negative correlation with* ***ALP***.
- ***GlycA*** *strong negative correlation with* ***ALP*** *and a positive correlation with the* ***platelets***.
- ***Phenylalanine*** *had a positive correlation with* ***WBC***, ***CRP***, *and* ***platelets*** *and negatively correlated with* ***ALP***.
- ***Mannos****e had a positive correlation with the* ***platelets*** *and negatively correlated with* ***amylase***, ***ALT***, *and* ***ALP***.
- ***Ascorbate*** *had a negative correlation with the* ***WBC***.
- ***Methanol*** *had a negative correlation with* ***lipase***.
- ***ProteinNH*** *negative correlations with* ***amylase***, ***ALT***, *and* ***ALP***
- ***Lipid alpha-CH2*** *negatively correlated with* ***amylase***, ***ALT***, *and* ***AST*** *and positively correlated with the* ***WBC***

5.5 Discussion

AP is an inflammatory disorder of the exocrine pancreas. There have been increasing incidents of AP during the last decades, however little is known about the molecular mechanisms involved in the initiation of AP, and the effects remain unknown (Mukherjee *et al*., 2016; Szatmary *et al*., 2022). Some theories have been described, among them are the release and activation of pro- and anti-inflammatory cytokines, oxidative stress, mitochondrial dysfunction, and the types of cell death involved (Jiang *et al*., 2020; Kang *et al*., 2014). In our study, we aimed to investigate the metabolic changes that take place in the early phase of AP. There was an association between elevated CRP levels and LFTs. CRP levels above 150mg/L are used to predict severity in AP patients. Elevated ALT and ALP enzymes indicate a biliary obstruction and are associated with a biliary aetiology of AP and longer hospitalization (Güngör *et al*., 2011; Thiyagarajan *et al*., 2020). Thus, high CRP levels in SAP are likely to be from biliary-associated AP and these are patients who will need to undergo surgical procedures to remove the obstructions in the ducts, which usually result in longer hospitalization.

In the AP patient cohort, there were overall higher *caspase* expression levels in MAP and the healthy controls compared to the MSAP and SAP groups, indicative of higher apoptosis levels. Apoptosis is a type of cell death that is mediated by the *caspases* and during apoptosis, there is no destruction of tissues and cell membranes (Kang *et al*., 2014). A strong positive correlation between *caspase 7* and CD4 means that the patients experienced apoptosis which protects against the immune responses in MAP patients and healthy controls. CD4 counts may also be elevated in PLWH when they are on ART treatment (Mrudula *et al*., 2012). We also found high ROS levels in SAP patients, hence a strong positive correlation with methanol. Alcohol has been shown to induce oxidative stress, moreover, excessive alcohol consumption results in pancreatic damage due to ROS through the metabolism of alcohol in the body (Apte *et al*., 2016). The correlation of lipids to the immune cells was also noted, lipids are central to metabolism and are involved in the functions of T lymphocytes, thus alterations in lipids affect the immune system (Howie *et al*., 2018). CD4 and CD8 T cell subsets are heavily dependent on various aspects of lipid synthesis, catabolism, and storage (Howie *et al*., 2018).

The CD4:CD8 ratio also had a correlation with phenylalanine, a primary amino acid that is an intermediate in the Krebs cycle. The Krebs cycle is used by living organisms to generate energy

during respiration and carbohydrates, amino acids, and lipids are oxidized (Alabduladhem and Bordoni, 2022). Mitochondrial dysfunction as a result of AP affects Krebs's cycle function. Obesity, excessive production of ROS, increased apoptosis and inflammation shows mitochondrial alteration (Manna and Jain, 2015; Pérez-Torres *et al*., 2021). Additionally, the alterations in the Krebs cycle have been noted in many cancers (Cardaci and Ciriolo, 2012). Phenylalanine and GlycB, an inflammatory marker, had a positive correlation with CRP.

Detection of the metabolites could be potential in understanding the pathophysiological conditions of AP which could aid in the early diagnosis of the disease (Nagana Gowda *et al*., 2008). The presence of pancreatic inflammation could lead to an alteration in the level of metabolites (Xiao *et al*., 2017). AP undergoes hypermetabolic condition which dysregulates amino acid, lipid, and glucose metabolism. Protein concentration decreased with increased severity in this study confirming that AP promotes protein catabolism which involves the breakdown of proteins into amino acids (Singh *et al*., 2012). Metabolites such as phenylalanine, lactate, mannose, and 3-hydroxybutyrate (3-HB) increased in concentration with AP severity in this cohort as demonstrated in previous studies (Silva-Vaz *et al*., 2021; Xiao *et al*., 2017). Thus, further understanding of the metabolic state and pathophysiology of AP can drive new drug development.

Phenylalanine has been previously associated with aggressiveness in pancreatic diseases and in our study shown to be linked with the severity of AP (Xiao *et al*., 2017). However, GlycA, another inflammatory marker, had a strong negative correlation with ALP. The metabolites associated with diabetic ketoacidosis (DKA), a serious complication of diabetes that develops when your body doesn't have enough insulin to allow blood sugar into your cells for use as energy and can be life-threatening i.e., 3- hydroxybutyrate and acetoacetate, and glycolysis, i.e. lactate had a positive correlation with the severity of AP while elevated levels of ALP and ALT were observed in our study population and indicate liver damage or the blocked bile duct in biliary pancreatitis (Lala *et al*., 2022). These metabolic indicators are currently being used to distinguish between the two main severities of AP in other countries (Çağlayan, *et al*., 2011). Increased lactate concentration has been associated with persistent organ failure in AP (Valverde-López et al., 2017). Lactate was elevated in our study population, a marker that is also used to predict poor prognosis in AP patients as well as sepsis and sudden cardiac arrest in many other diseases (Bou Chebl et al., 2017; Fuller and Dellinger, 2012; Shu et al., 2019).

Taken together the data supports the inflammatory profile associated with AP (↑Phe, GlycA, and B) which is characterized by high energy demands and sourcing of alternate fuel substrates for energy (ketone production). Inflammation persists irrespective of stage or treatment. This profile is mirrored by increased lactic acidosis (spurred on by lifestyle/ART). It also confirms the milder (MAP/MSAP) groups mainly undergo apoptosis (caspase gene expression) and experience oxidative stress (higher hydroperoxides levels, especially evident in plasma samples). In the milder, non-progressive groups, glutamine is not depleted as in the case of AP disease progression suggesting the cells of such cohorts to still be immunocompetent (supported by relatively high CD4/CD8 ratios). Since the MAP profile mainly resembles HC, the best group for prognosis is likely MSAP although sampling in this group may have to happen at an earlier time point to minimize overlap with SAP. The detection of ketones suggests that sampling at day 3 may be too late as the use of ketones as a source of energy usually suggests advancement/progression in various pathologies. While many of the parameters trend similarly in MSAP and SAP, there may have to be a concentration-dependent cut-off for distinguishing prognostic indicators (early less perturbed group/cohort) vs diagnostic indicators (advanced group/cohort). So it may be that in mild you have ↑ and severe you have ↑↑. Specificity of metabolic indicators for diagnosis to be tested against other diseased controls with persistent inflammation.

5.6 Conclusion

The relationships between the immune response, apoptosis, oxidative stress, and metabolites can predict the clinical course of AP. The biochemicals which either had strong positive or negative correlations to other study parameters such as the inflammatory metabolic indicators, lipids as well as metabolites such as phenylalanine and the ketone bodies need to be further investigated in a larger patient cohort. The “biometabolic indicators” found in this study due to the alterations in the immune system, lipids, and proteins can provide a conceptualization of new targets for predicting the severity and treatment early in AP patients using a non-invasive technique such as NMR.

CHAPTER 6: OVERALL RECOMMENDATIONS, STUDY LIMITATIONS, AND CONCLUSIONS

Acute pancreatitis (AP) is induced by inflammation due to the autodigestion of the pancreas and the sudden release of digestive enzymes before they reach the small intestine. AP clinically presents with nausea, vomiting, abdominal pain, and an increase in pancreatic enzymes which leads to a systematic immune response syndrome. The challenge with the metabolic indicators which are currently being used is that they lack specificity to the pancreas, do not predict severity, use many parameters that make their use difficult and are ill-suited for assessing patients during admission. This results in delays in getting the necessary diagnosis and affects the timely treatment of the patients. The main aim of this study was to find the link between the immune response, type of cell death (apoptosis), and oxidative stress in the blood cells of pancreatitis patients in relation to the metabolites and their potential for monitoring disease in different severities of acute pancreatitis.

6.2 Overall Conclusions

Our preliminary findings assessed the aetiology and demographic characteristics of 55 AP patients recruited from CHBAH. Among the 33 MAP, 14 MSAP, and 8 SAP patients we found the main aetiologies to be biliary and alcohol-induced pancreatitis. The adoption of the western diet of eating high-cholesterol foods and excessive alcohol consumption has played an enormous role in the increase of AP in the South African population of African descent. This diet leads to people being overweight and obese, especially older females. These factors contribute to the increase in costly surgical procedures such as laparoscopic cholecystectomies which are performed to remove the stones or any blockages in the pancreas in biliary-associated pancreatitis. AP associated with alcohol was found mostly in young males. Although not common in our population, the recurring attacks of alcoholic acute pancreatitis progress to chronic AP, with a 20-times more chance for the development of pancreatic cancer, a disease with a grim prognosis. The commonest comorbidities in our patient cohort were HIV and type II diabetes.

The excessive inflammation in response to pancreatic injury by the infiltrating immune cells in the pathogenesis of AP also influences disease severity. Thus, the activated immune system affects

the dysregulation of the immune cells and correlates to the severity of acute pancreatitis. In this study, whole blood was collected from seven AP patients to determine the CD4:CD8 ratio and the findings compared to the ratios with the healthy controls. The results showed that MSAP and SAP groups had low CD4 percentages and high CD8 percentages, hence a lower CD4:CD8 ratio than the normal range. Hence, there were alterations in the CD4:CD8 ratio of the diseased individuals compared to the healthy controls. Therefore, the dysregulation of the immune cells correlates to the severity of AP. From the thirty-four PBMCs of AP patients, we found that the MAP and MSAP patients generally experienced the highest level of *caspase* expression compared to the other severity groups and the healthy controls although the amount of gene expression decreased over time. The activation of *caspases,* which leads to apoptosis in MAP patients provides protection from the SIRS that most severe cases of AP patients experience. ROS expression in serum and plasma samples was compared to the healthy controls. Twenty-two AP patient samples of different severities were assayed and overall SAP and MSAP patients experienced higher levels of ROS expression compared to the MAP patients and the healthy controls. Oxidative stress plays a role in the pathogenesis of many diseases. It is no surprise to find high oxidative stress levels in MSAP and SAP patients compared to healthy individuals as infection, amongst other factors contributes to the development of oxidative stress. The other factors which contribute to ROS include infection, exposure to toxins, inflammation, stress, alcohol, and smoking. However, ROS can also be produced and released during an inflammatory response by the circulating neutrophils. The neutrophils are involved in the events during the pancreatic injury and the systematic complications of AP. Additionally, studies have also shown that the generation of ROS may play a protective role during AP since acinar cells under conditions of stress respond to increases in mitochondrial ROS by initiating the apoptotic cell death pathway thereby avoiding necrosis.

Our preliminary findings are the first metabolomics analysis on AP in our population. Although the major limitation of the study was the small sample size, similarities to literature findings suggest that they are noteworthy. A similar study by Silva-Vaz *et al*., (2021) identified metabolites that may predict the onset as well as the progression of gallstone AP severity. They found major alterations in phenylalanine, lipids, ROS, and antioxidants relating to AP severity. Our results highlight the alterations in the metabolites as well as in proteins, lipids, and cholesterols. A positive correlation was observed with the concentration of the metabolites such as phenylalanine, mannose, lactate, acetoacetate, and 3-hydroxybutyrate, which increased with AP severity. On the

contrary, ascorbate, glutamine, and proteins showed a negative correlation with disease severity. Some lipids had a positive and others had a negative correlation with the severity of AP. A comparison of the clinical features and the metabolites demonstrated that the lipid profile distinctively separated the metabolic phenotypes of AP based on their severity. There was a distinct separation of the MAP from MSAP and SAP and those severe patients who presented the most adverse effect such as complications, organ dysfunction, and admission to ICU. Reduced levels of small HDL-P were observed with increased severity of AP. HDL-C decreased across the metabolic phenotype of AP when compared with the HC while elevated IDL-C and VLDL-C levels were observed. All the biomolecules which were altered need to be validated in an independent larger cohort of AP.

Future work

Targeted metabolomics focuses on amino acids and proteins as well as lipids as well as correlations between the cytokines/ chemokines with the highlighted metabolites. A larger cohort of participants looking at MAP and MSAP patients for 30 days (early phase as well as the late phase of AP). We also need to determine the concentrations at which identification metabolic indicators serve a prognostic role versus the diagnostic role and develop libraries to identify unknown signals and metabolite identification of unknowns. It is important to note that while the metabolic signatures are indicative of severity, they may not be specific to AP. To get specific biomarkers of AP, one would have to include diseased controls (other inflammatory diseased samples and do similar analysis to check whether a specific marker will only show for AP). The metabolic signatures are not the only markers used for AP but also other scoring tools including physical examination for AP diagnosis. It may happen that the results are not different when disease controls are included.

Study limitations Although not completely specific to AP, the altered metabolites and molecules will help with early stratification of AP into severity groups. The major limitation of this study was the sample size. Our sampling period coincided with the COVID-19 pandemic and due to the COVID-19 regulations, patient recruitment for research studies was prohibited. Some SAP patients were demised during the study period. There was restricted access to the ICU and there were patients on life support whom we could not get consent from. Furthermore, delays with the presentation to the hospital meant sampling on earlier days e.g., days 1 and 3 was not possible for

some patients. Despite these challenges, the data suggests that an integration of the dysregulated immune parameters, metabolomic profiles, and clinical parameters may inform treatment modalities and help differentiate the complex immunometabolic status of different AP severities and may guide management. Given the challenges in obtaining sufficient SAP samples, future work will involve validation studies using models of AP.

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APPENDIX I

ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Miss Jeanet Mazibuko

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190407

NAME: (Principal Investigator)	Miss Jeanet Mazibuko
DEPARTMENT:	Department of Surgery North West University Chris Hani Baragwanath Hospital Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Apoptosis inducing metabolites in early acute pancreatitis
DATE CONSIDERED:	2019/4/26
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr P Fonteh, Dr A Williams and Dr J Dewar
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	14/08/2019

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **April** and will therefore be due in the month of **April** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 16/08/2019

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX II

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

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

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

E-mail: Mwangalan@gmail.com

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

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

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

DATE: 05/06/2018

REF: *R14/49*

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

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

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

MSWorks2000/Iain0007/Clearscan.wps

APPENDIX III
INFORMATION SHEET: EXPERIMENTAL SAMPLE

Lymphocyte ratio, oxidative stress, cell death and metabolic profiling in acute pancreatitis: implications for disease monitoring

Good day,

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

You are invited to take part in this study. This research study is looking for at least 10 participants diagnosed with acute pancreatitis of different severity to donate 2½ teaspoons (about 12 ml) of blood. The blood will be drawn from a vein in your arm using a syringe at the Hepatopancreaticobiliary Unit of the academic hospital in which you are admitted, and the sample transported safely protected in a secondary container to the University of the Witwatersrand where the research will be done. Transport will be by private means or through the NHLS transport services. From the blood, the liquid portion called plasma and serum will be separated and used for the study. Some of these samples cannot be used immediately and will be frozen at -80ºC until needed (within a month to 5 years). The results from these samples will be compared to those of participants who lack a history of pancreatic diseases. Sampling will be part of routine management procedures and you may be subjected to some pain or discomfort caused by the needle, but a qualified and experienced medical practitioner (nurse or doctor) has been recruited to perform the procedure and to minimize the risks. The procedure will only take about 5 minutes and blood will be obtained from you once you consent. 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 120ºC, and disposed of in bio-hazardous/ medical waste boxes.

For further information/ reporting study-related adverse effects/complaints contact:
Researchers:
Ms. Jeanet Mazibuko (Research student)
Tel: 0117172479/0815646618 Email: Jeanet.Mazibuko@wits.ac.za
Dr Pascaline Fonteh (Supervisor)
REC Administrators - Ms Zanele Ndlovu/ Mr Rhulani Mkansi
Tel 011 717 2700/2656/1234/1252, Email: HREC-Medical.ResearchOffice@wits.ac.za

APPENDIX IV

CONSENT FORM FOR PATIENTS

Dear Sir/Madam,

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

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

Whilst we are not currently involved in research that requires us to use any information now but rather is interested in collecting a blood sample from you, this may change in the future when you may have already been discharged. We would like to obtain your consent to use information from your file for research, subject to the aforementioned conditions. We anticipate that such information will be accessed up to 5 years after sample collection. If you choose not to give consent, this will not compromise your treatment in any way. If at any time you choose to withdraw consent, you are free to do so and will not be prejudiced in any way. Should you wish to contact us at any stage regarding consent, phone: Jeanet Mazibuko at (011) 717-2479, or Prof. 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 V

Information sheet for Healthy controls

Lymphocyte ratio, oxidative stress, cell death and metabolic profiling in acute pancreatitis: implications for disease monitoring

Good day,

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

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

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

Participating in this study will not result in any "out of pocket' expenses. Refusal to participate will involve no penalty. Blood samples will be collected anonymously to ensure confidentiality. Personal information may only be disclosed if required by law or organizations that inspect and/or copy research records for quality assurance and data analysis such as the Research Ethics Committee (REC). At the end of the study, leftover blood will be discarded in 10% bleach, sterilized by heating to 120 degrees Celsius and disposed in bio-hazardous/ medical waste boxes.

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

Researchers:
Miss Jeanet Mazibuko (Research student)
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REC Chairperson – Prof Cleaton-Jones: peter.cleaton-jones1@wits.ac.za
REC Administrators - Ms Zanele Ndlovu/ Mr Rhulani Mkansi/ Mr Lebo Moeng
Tel 011 717 2700/2656/1234/1252, Email: HREC-Medical.ResearchOffice@wits.ac.za

APPENDIX VI
CONSENT FORM: USE OF BLOOD SAMPLE AS HEALTHY CONTROL

Dear Sir/Madam,

If you are reading this form, it means you have responded to a request to be recruited as part of the study on acute pancreatitis. Before signing this document, the researcher would have related to you the details of the study in which you are participating.

You are required to donate to this study approximately 2½ teaspoons of blood. This sample will only be collected once. The information generated from this study is subject to the following:

1. Approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand.
2. The 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 to maintain anonymity.

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

If you choose not to give consent, this will not compromise you in any way. If at any time you choose to withdraw consent, you are free to do so and will not be prejudiced in any way. Should you wish to contact us at any stage regarding consent, you can phone Ms. Jeanet Mazibuko at (011) 717-2479, Prof. 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 VII



DATASHEET FOR ACUTE PANCREATITIS DIAGNOSIS

DEMOGRAPHICS

Sample No: _____ Study No: _____

Sex: M F Age: _____ Cause of pancreatitis: _____

Severity of Pancreatitis: Mild Moderately severe Severe

Cause of acute pancreatitis:

Height _____m Weight _____kg

DETERMINANTS OF SEVERITY

WCC _____ 109 /L Glucose _____mmol /L LDH _____ IU/L

C - reactive protein _____mg/L Procalcitonin (PCT) _____ng/mL

Hematocrit_____%

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

Na _____ mg/dL or mmol/L

K_____ mg/dL or mmol/L

Creatinine_____ mg/dL or mmol/L

FiO2: _____

Base deficit _____ mmol/L

C - reactive protein _____ mg/L

Platelets: _____ 109 /L

RR_____ bpm

Neutrophils_____ 10^9 /L

APPENDIX VIII
DATASHEET FOR CONTROL SAMPLES FOR THE ACUTE PANCREATITIS STUDY

BEFORE ANSWERING THIS FORM PLEASE READ THE INFORMATION SHEET AND SIGN THE CONSENT FORM

Control Sample No: _____

Sex: M ☐ F ☐

Age: _____

Do you have history of pancreatic disease?

Yes ☐ No ☐ Don't know ☐ (please tick relevant box)

To the best of your knowledge do you have any diseases that may make you immunocompromised?

Yes ☐ No ☐ Don't know ☐ (please tick relevant box)

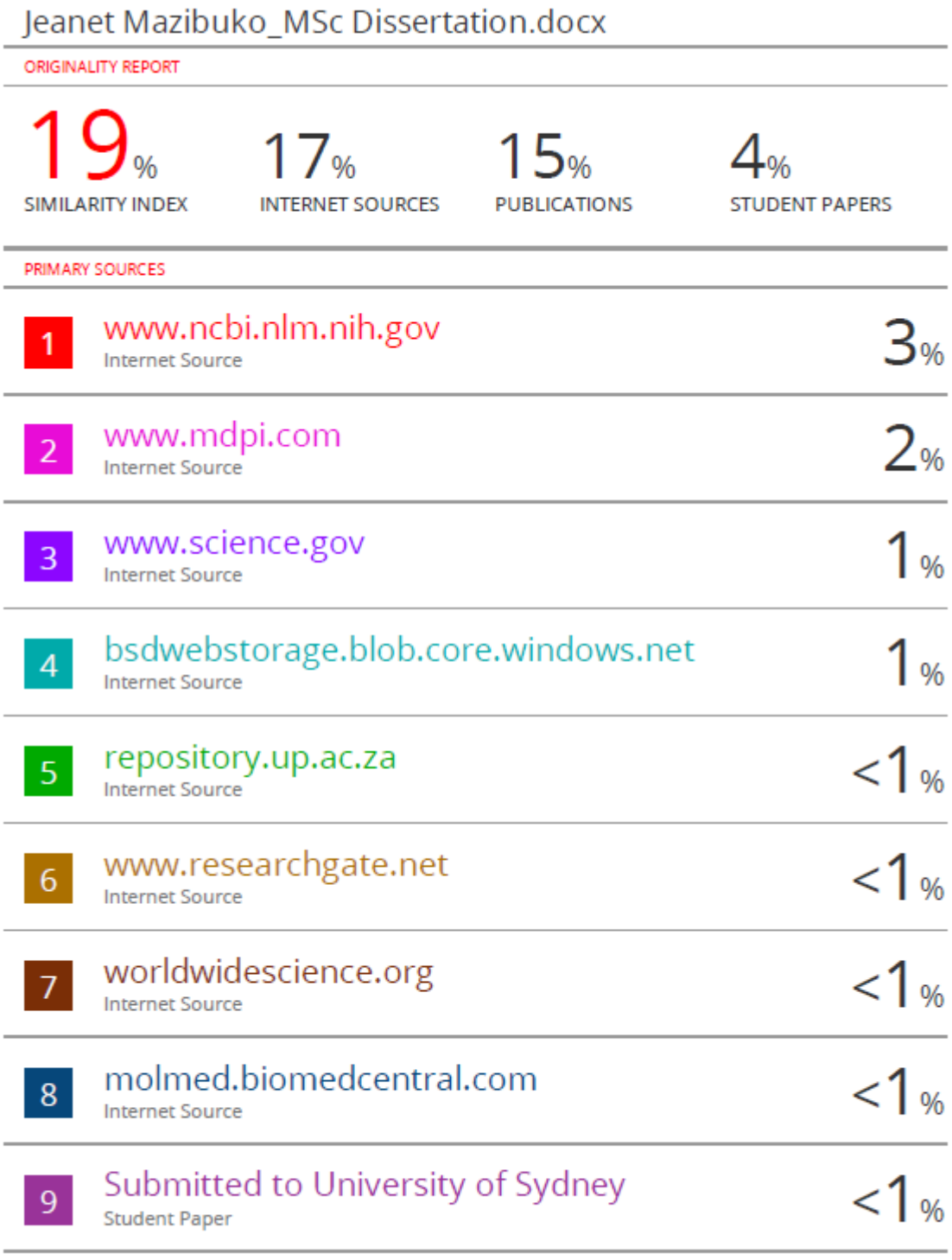
Height _____m Weight _____kg

Name: _____

Signature: _____

Date: _____

APPENDIX IX



Jeanet Mazibuko_MSc Dissertation.docx

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