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EDITORIAL

Bacterial translocation in patients undergoing major gastrointestinal surgery and its role in postoperative sepsis

Christos Doudakmanis, Konstantinos Bouliaris, Christina Kolla, Matthaios Efthimiou, Georgios D Koukoulis

ORCID number: Christos Doudakmanis 0000-0001-8242-0452; Konstantinos Bouliaris 0000-0001-8403-8177; Christina Kolla 0000-0002-0177-1240; Matthaios Efthimiou 0000-0002-3644-6069; Georgios D Koukoulis 0000-0002-6958-6447.

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Christos Doudakmanis, Konstantinos Bouliaris, Christina Kolla, Matthaios Efthimiou, Georgios D Koukoulis, Department of General Surgery, General Hospital of Larissa, Larisa 41221, Greece

Corresponding author: Georgios D Koukoulis, MD, MSc, PhD, Adjunct Professor, Doctor, Surgeon, Department of General Surgery, General Hospital of Larissa, Tsakalof 1, Larisa 41221, Greece. georgios.koukoulis@gmail.com

Abstract

Bacteria of the human intestinal microflora have a dual role. They promote digestion and are part of a defense mechanism against pathogens. These bacteria could become potential pathogens under certain circumstances. The term "bacterial translocation" describes the passage of bacteria of the gastrointestinal tract through the intestinal mucosa barrier to mesenteric lymph nodes and other organs. In some cases, the passage of bacteria and endotoxins could result in blood stream infections and in multiple organ failure. Open elective abdominal surgery more frequently results in malfunction of the intestinal barrier and subsequent bacterial translocation and blood stream infections than laparoscopic surgery. Postoperative sepsis is a common finding in patients who have undergone non-elective abdominal surgeries, including trauma patients treated with laparotomy. Postoperative sepsis is an emerging issue, as it changes the treatment plan in surgical patients and prolongs hospital stay. The association between bacterial translocation and postoperative sepsis could provide novel treatment options.

Key Words: Bacterial translocation; Major gastrointestinal surgery; Postoperative sepsis; Intestinal permeability; Microbiota

Core Tip: Increased intestinal permeability can potentially induce intestinal flora dysbiosis. Bacterial translocation, attributed to intestinal barrier impairment, may lead to systematic infection in the postoperative period. The definitive correlation between translocation and postoperative sepsis is yet to be proven, but the latter is an emerging issue for patients undergoing major gastrointestinal surgeries.

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INTRODUCTION

The incidence of postoperative sepsis has increased in the past decades, with the proportion of severe sepsis cases rising to unprecedented levels. Cases of sepsis are noted both after elective and emergency surgeries, but in the cases of elective surgeries, mortality is not respectively affected[1]. Gastrointestinal perforation is the most common surgical condition requiring immediate surgical intervention. More specifically, colonic perforation may cause peritonitis through the spread of bacteria from the intestines, and, therefore, there is a high risk for further bacterial spread *via* blood flow[2].

Gut microbiota affects the host decisively in both states of health and illness. The human gut microbiota consists of numerous bacteria that coexist and play a beneficial role in normal functions of the intestine. In normal conditions, bacteria of the gut assist in the absorption of nutrients. In illness, there are vast changes that alter the balance of these bacteria, leading to proliferation of potentially dangerous bacteria, capable of causing infections[3]. Diseases like colorectal cancer, inflammatory bowel disease, and diseases of the liver could alter the relationship between bacteria of the gut and the host.

In addition to bacterial dissemination due to mechanical disruption of the continuity of the intestinal barrier, as in the case of perforation, another potential mechanism proposed is bacterial translocation. Bacterial translocation is the movement of bacteria or their products from the intestinal lumen through the mucosa layer to a normally sterile tissue[4]. The most common routes for bacterial passage from the intestine to the systemic circulation and eventually to distant organs are the lymphatic route and the vascular route[5].

Major abdominal surgeries are procedures that promote an imbalance in intestinal bacteria. Patients undergoing major abdominal surgery are considered at high risk of developing postoperative infections as a result of bacterial translocation. Those undergoing emergency surgery are at even higher risk[6]. An increase in morbidity and mortality has been shown in cases of ascertained translocation to locoregional mesenteric lymph nodes[7,8].

ROLE OF THE INTESTINAL BARRIER

The intestinal barrier interacts with the contents of the intestinal lumen at immunological and chemical levels, besides being a physical barrier. It is composed of a single layer of columnar epithelial cells, which have diverse functions, such as absorptive, secretory and immune functions. The majority of intestinal epithelial cells are absorptive enterocytes. Other types of intestinal epithelial cells are secretory goblet cells, Paneth cells, and enteroendocrine cells. All these cells are under constant renewal by intestinal epithelial stem cells located in the bases of mucosal crypts[9].

Commensal bacteria found in the intestinal lumen prevent the proliferation of potential pathogens through regulating intestinal pH and decreasing the nutrients required by those pathogens. On the surface of the lumen, a layer of water, the glycocalyx, and the mucus layer containing immunoglobulin A (IgA) create a first defensive line, preventing adhesion of pathogenic bacteria to the epithelium and diminishing interaction between pathogen and epithelial cells. In addition, antimicrobial agents secreted by epithelial cells attract monocytes and assist in the opsonization of macrophages. Immunoglobulins and cytokines are secreted by cells of the lamina propria, as those cells are part of the innate and acquired immune system and play a vast role in immunological regulation in the intestine[10]. Besides having a role as a physical barrier, the mucus layer of the intestine contains an abundance of secretory IgA and antimicrobial proteins. There is a substantial difference in the composition of the mucus layer between the small and large intestine. This layer in the small intestine is penetrable by bacteria, while the large intestine has both a penetrable

outer mucus layer and an impenetrable inner mucus layer. Intestinal epithelial cells create a defense barrier below the layers of mucous inside the lumen of the intestine [11]. A barrier formed by mucins between the intestinal lumen and intestinal epithelial cells can regulate expression of tolerogenic and inflammatory cytokines[12].

INTESTINAL PERMEABILITY

Intestinal epithelial cells are connected to each other with tight junctions[9,10]. Tight junctions are an assembly of multiple proteins located on the apical part of neighboring epithelial cells and affect paracellular permeability, as they selectively regulate permeability. Tight junctions are fundamental in maintaining intestinal barrier function. They act as adhesive and mechanical mediators, maintaining barrier function, but do not seal the paracellular space. There are two functional protein categories, namely integral transmembrane proteins that form a network between adjacent cell membranes and peripheral membranes. Four integral transmembrane proteins are occludin, claudin, junctional adhesion molecule, and tricellulin[13,14]. In certain conditions of intestinal inflammation, it is shown that these tight junctions dysfunction, increasing permeability. The repair process of the epithelial cells affects intestinal motility and is considered an important factor in intestinal barrier function[9, 10,15].

Intestinal permeability is the condition during which soluble molecules and fluids are exchanged between the intestinal lumen and tissues. In normal conditions, intestinal barrier homeostasis acts to prevent this exchange, but both permeability and barrier function are dynamic states[16]. Dysfunction of the mucosal barrier can be found in both stress-associated conditions and in a diverse group of conditions in otherwise healthy people. It has been shown that there is increased intestinal permeability in patients with gastroenterological diseases correlated with intestinal inflammation, especially in those with inflammatory bowel disease. In addition, healthy relatives of these patients are at high risk of developing increased intestinal permeability[17,18]. Use of non-steroidal anti-inflammatory drugs may alter the structural normality of the intestinal lumen, thereby impairing the barrier and potentially increasing permeability[19]. More importantly, studies have shown that in a number of patients undergoing abdominal surgery, bacterial DNA was detected as early as a few hours postoperatively, indicating a relation between surgery and translocation[6].

INTESTINAL MICROBIOTA IN GASTROINTESTINAL DISEASES

The human intestinal microbiota plays a main role in intestinal metabolism and in immunological response of the intestines[20]. Balance of the intestinal microbiota is a prerequisite for a healthy intestinal environment. Imbalance of microbiota and of the host immune system is present in intestinal diseases. Altered concentrations of commensal intestinal bacteria depends on disease activity, and this can easily be noted when patients are compared to healthy individuals[21].

Studies in patients with colorectal cancer have shown that alterations in microbiota are also associated with tumorigenesis. These alterations are characterized by the dominance of certain bacteria species. In the spotlight are *Fusobacterium nucleatum*, *Escherichia coli*, and *Bacteroides fragilis*[22]. Analyses of intestinal microbiota are performed using 16S ribosomal RNA techniques. When the aforementioned bacteria species are increased, other bacteria are depleted. Gram-positive bacteria are vastly affected, and *Clostridia* species are also decreased in these patients[23-26]. *Bacteroides fragilis* colonizes the intestine and has a prominent place in the microbiota. Although *Escherichia coli* is considered a commensal bacteria, some of its species are potential pathogens, promoting intestinal inflammation and producing oncogenic toxins. This phenomenon, when there is an imbalance in intestinal flora, is called dysbiosis. Dysbiosis may characterize inflammatory gastrointestinal diseases and colorectal cancer but may also be explained by the changes in dietary habits that have occurred over the past decades. The importance of metabolites and their products to intestinal inflammation have led to increased concern for the impact of metabolic diseases on microbiota[23,27].

Bowel obstruction has both local and systemic effects. Fecal retention promotes bacterial overgrowth. Besides changes in bowel motility, moderate inflammation is a probable finding. This inflammatory response may lead to systemic responses, with

sepsis and septic shock being the most serious. The causative factor for these systemic responses is bacterial translocation[28].

BACTERIAL TRANSLOCATION, ABDOMINAL SURGERY, AND POSTOPERATIVE SEPSIS

Bacterial translocation, attributed to increased intestinal permeability, can be present as early as 2 h after abdominal surgery. The grade and the prognosis depend on the severity of surgical trauma and the presence of intestinal ischemia. Studies have proposed that the potential mechanism involves visceral vasoconstriction due to surgical trauma, anesthetic agents, intestinal ischemia, and blood loss. In addition, production of vasoactive agents and release of inflammatory cytokines promote visceral vasoconstriction and therefore intestinal ischemia. Postoperative bacterial translocation is associated with systematic infection and systematic inflammatory response[6] (Figure 1).

In order to assess and confirm bacterial translocation, cultures from mesenteric lymph nodes are taken. Furthermore, blood cultures are collected from patients in the postoperative period. These samples are assessed using real-time polymerase chain reaction techniques to identify bacteria. In the case of bacterial translocation, positive cultures of samples from mesenteric lymph nodes have been reported to have slightly higher specificity[29-31]. The most common isolated bacterium associated with translocation is *Escherichia coli*[32]. A feasible method proposed to assess and monitor the progress of bacterial translocation is the evaluation of levels of D-lactate. D-lactate is a product of bacteria normally found in the intestinal lumen and is not metabolized by the human body. Levels of plasma D-lactate are used as a postoperative indicator of dissemination of these bacteria from the intestinal tract to the mesenteric lymph nodes, liver, spleen, and bloodstream[33].

Infections in the postoperative period are found to be more common in patients with identified bacterial translocation. A positive result in cultures taken from mesenteric lymph nodes is a more accurate prognostic factor than cultures from surgical site, intra-abdominal fluid collection, or peripheral blood samples. In other words, mesenteric lymph nodes act as beacons for progression of the infection[34]. This fact raises concerns regarding prophylactic use of antibiotics in patients undergoing abdominal surgery. Elective surgeries are performed under better circumstances and with better precautions taken. Emergency surgeries and surgeries for trauma are considered high-risk for the development of bacterial translocation, thus requiring use of antibiotics in the perioperative period[35]. Patients with advanced colorectal and gastric cancer, potentially associated with cachexia, are also in need of prophylactic use of antibiotics due to immunological imbalance induced by the progressed disease[36,37]. Prophylaxis against bacterial translocation seems to be associated with better survival rates in cancer patients who undergo surgery[7]. However, while gastric and colon resections are correlated with augmented rates of translocation, the use of antibiotics does not seem to prevent the occurrence of translocation[38,39].

The definite significance of bacterial translocation is yet to be determined, although there is evidence suggesting a causative role for sepsis. In some cases of sepsis, the causative factor was determined to be bacteria found in the intestine. In critically ill and frail patients undergoing major abdominal surgeries, those bacteria cause sepsis and even septic morbidity[40]. Sepsis is a diverse syndrome of varying severity. Late diagnosis and treatment could lead to more severe illness, even septic shock. In some cases, it may cause multi-organ failure. Severe sepsis is characterized by the presence of hypoperfusion or hypotension and by the failure of at least one organ[41]. However, this is hard to verify in most cases, as in cases of multi-organ failure occurring early postoperatively, and it is probably due to the inflammatory response causing endothelial cell activation. In contrast, late-onset multi-organ failure may be attributed to bacterial translocation, as it creates an imbalance between proinflammatory and anti-inflammatory cytokines[42]. When the septic condition in surgical patients is so severe that it causes a state of immunosuppression, multi-organ failure is responsible, with high mortality rates (reaching 50%-80%). This fact supports the theory of gut-induced sepsis[43].

Postoperative sepsis was found to be most common among men and among older and low-income populations. Besides these characteristics, other factors regarding hospitalization are also crucial. Larger hospital bed size, urban hospital location, and non-teaching status were associated with higher postoperative sepsis rates.

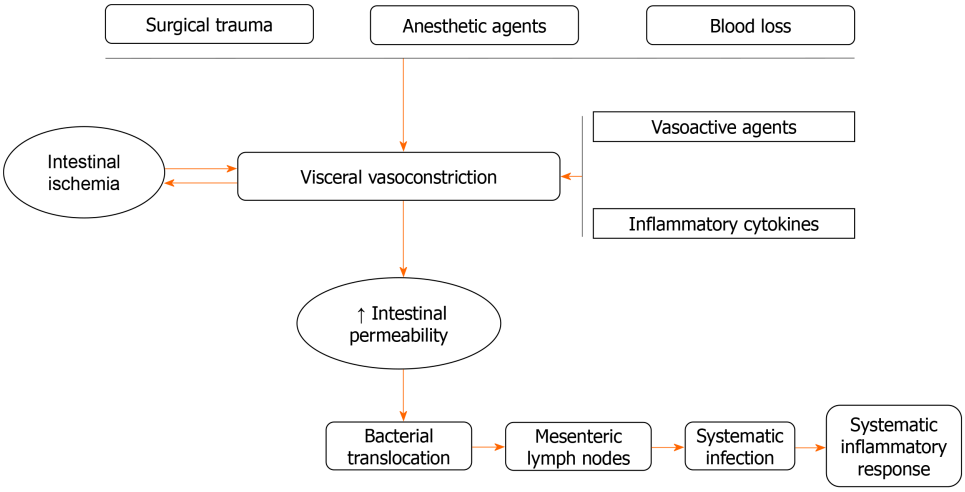

Figure 1 Mechanism of bacterial translocation after major gastrointestinal surgery.

Comorbidities like diabetes, chronic renal disease, cardiovascular disease, and hypertension increase the risk for postoperative sepsis. Patients' preoperative status and lifestyle choices contribute to modifying the relative risk. In addition, the type of surgery also has an impact, as gastric, biliary and colorectal surgeries were associated with relatively higher rates of postoperative sepsis, when at the same time esophageal surgery had the lowest risk of postoperative sepsis[44-46] (Figure 2). Although the incidence is rising, especially in elderly patients, mortality rates are decreasing[47].

As sepsis progresses, the release of proinflammatory cytokines triggers the production of toxic mediators that damage the endothelium, thus leading to increased capillary leakage. In addition, the release of agents that act as vasodilators, resulting in hypotension, indicates that evolution of sepsis to septic shock and subsequently to multi-organ failure requires vigilance. Early detection and therapeutic intervention could improve outcome and prognosis. Diagnosis is based on both clinical assessment and taking into consideration other factors, such as impaired consciousness and severe underlying diseases. Hypotension, oliguria, and acute altered mental status are indicative signs of severe sepsis[48]. As this condition continues to cause concern, efforts are being made to create a predictive score that will help physicians to assess probability of postoperative sepsis and mortality and to intervene sooner[49].

The cornerstone of treatment is fluid resuscitation to address hypovolemia, hypotension, and hypoperfusion. Hemodynamic stability could be restored using vasopressors when fluids alone are not adequate to maintain blood pressure. Furthermore, broad-spectrum intravenous antibiotics should be administered within the first hour. The choice of antibiotics should be guided by the suspected causative factors. Response of patients to treatment must be monitored closely, because in cases where there is no improvement, surgical intervention may be needed[50]. Novel treatments have been proposed for postoperative sepsis due to bacterial translocation, such as the use of probiotics and prebiotics. These are considered living microorganisms, which can be beneficial. *Lactobacillus* and *Bifidobacterium* are the most commonly used. They act through competition with pathogens for binding sites and nutrients. Probiotics also induce immunological response and reduce inflammation. Prebiotics are non-digestible food ingredients that promote the growth and the increase in activity of certain intestinal bacteria. These treatments have been studied well in patients with sepsis in intensive care units, with results being promising, as prophylactic use of probiotics has been shown to reduce infections, sepsis, and mortality. Another potential treatment is fecal microbiota transplantation. This is a technique that attempts to restore commensal bacteria in the intestinal epithelium. It also acts as an immunomodulatory tool, as it assists intestinal crypts to express immunological pathways. This being said, this technique prevents severe inflammation and dysregulation of intestinal lumen homeostasis[51].

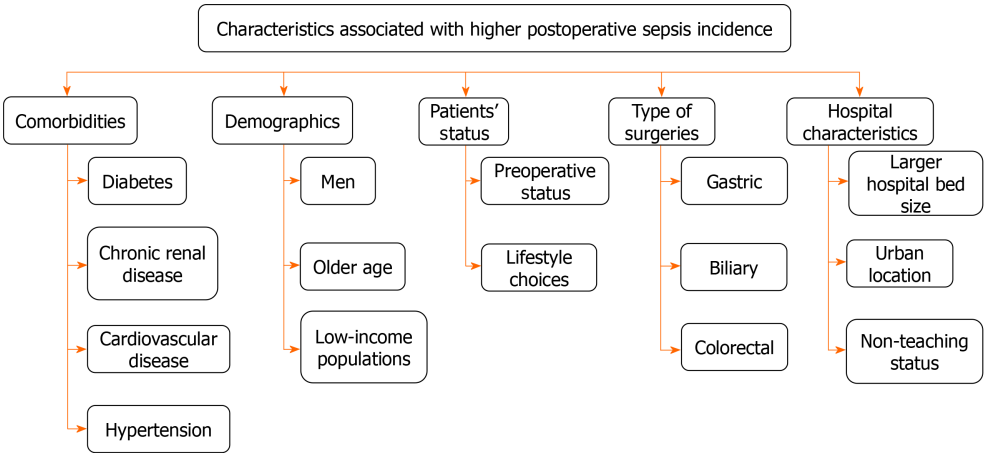

Figure 2 Characteristics associated with higher postoperative sepsis incidence.

LAPAROTOMY *VS* LAPAROSCOPIC SURGERY AND THEIR IMPACT IN BACTERIAL TRANSLOCATION

The effect of increased intra-abdominal pressure on bacterial translocation has been under investigation. Abdominal surgeries are associated with increased intra-abdominal pressure. Studies have shown that bacterial translocation usually occurs at pressure levels above 14 mmHg[52]. Patients undergoing laparoscopic surgeries should be monitored, as pneumoperitoneum significantly increases intra-abdominal pressure. Randomized control trials regarding patients with colorectal cancer have concluded that there is an increase in intra-abdominal pressure, systemic endotoxemia, and bacterial translocation during both open and laparoscopic resection but without a statistically significant difference between the two groups[53]. The effect of pneumoperitoneum in translocation was also studied in animal models. It was found to provoke alterations in the inflammatory response, with milder inflammation and quicker restoration. However, there was no evidence supporting the premise that laparoscopic surgery is related to higher incidence of bacterial translocation[54-56].

CONCLUSION

Postoperative sepsis is an emerging issue that can be present as soon as a few hours postoperatively and requires immediate treatment. It may cause severe disease and result in high mortality rates, especially in frail and elderly surgical patients. Bacterial translocation is proposed as a causative factor of postoperative sepsis. This fact suggests that intestinal microbiota combined with altered homeostasis in the intestinal barrier could create a chain of events leading to sepsis, as commensal bacteria translocate to usually sterile tissues. Bacterial translocation has been noted both in laparotomy and in laparoscopic surgeries, with no significant differences regarding incidence. Proper management and early intervention are needed, based on the fundamentals of sepsis treatment. Over the past few years, data regarding novel treatments using probiotics, which assist classic treatments, have been developed. More randomized studies will be needed to clarify the role of these treatments in postoperative sepsis in the years to come.

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ORIGINAL ARTICLE

Clinical and Translational Research

Chemokine receptor 8 expression may be linked to disease severity and elevated interleukin 6 secretion in acute pancreatitis

Mwangala Nalisa, Ekene Emmanuel Nweke, Martin D Smith, Jones Omoshoro-Jones, John WS Devar, Rebecca Metzger, Tanya N Augustine, Pascaline N Fru

ORCID number: Mwangala Nalisa 0000-0001-7736-1718; Ekene Emmanuel Nweke 0000-0003-4026-1697; Martin D Smith 0000-0003-3470-7576; Jones Omoshoro-Jones 0000-0002-1071-298X; John WS Devar 0000-0001-7526-9985; Rebecca Metzger 0000-0001-6623-0843; Tanya N Augustine 0000-0002-3985-317X; Pascaline N Fru 0000-0002-3631-1893.

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Mwangala Nalisa, Ekene Emmanuel Nweke, Martin D Smith, Jones Omoshoro-Jones, John WS Devar, Pascaline N Fru, Department of Surgery, School of Clinical Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg 2193, Gauteng, South Africa

Martin D Smith, Jones Omoshoro-Jones, John WS Devar, Department of Surgery, Chris Hani Baragwanath Academic Hospital, Johannesburg 1864, Gauteng, South Africa

Rebecca Metzger, Institut für Immunologie, Ludwig-Maximilians-Universität München, München 80539, Germany

Tanya N Augustine, School of Anatomical Sciences, Faculty of Health Science, University of the Witwatersrand, Johannesburg 2193, Gauteng, South Africa

Corresponding author: Pascaline N Fru, BSc, MSc, PhD, Senior Scientist, Department of Surgery, School of Clinical Medicine, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, Johannesburg 2193, Gauteng, South Africa.
pascaline.fru@wits.ac.za

Abstract

BACKGROUND

Acute pancreatitis (AP) is an inflammatory disease, which presents with epigastric pain and is clinically diagnosed by amylase and lipase three times the upper limit of normal. The 2012 Atlanta classification stratifies the severity of AP as one of three risk categories namely, mild AP (MAP), moderately severe AP (MSAP), and severe AP (SAP). Challenges in stratifying AP upon diagnosis suggest that a better understanding of the underlying complex pathophysiology may be beneficial.

AIM

To identify the role of the chemokine receptor 8 (*CCR8*), expressed by T-helper type-2 Lymphocytes and peritoneal macrophages, and its possible association to Interleukin (IL)-6 and AP stratification.

METHODS

This study was a prospective case-control study. A total of 40 patients were recruited from the Chris Hani Baragwanath Academic Hospital and the Charlotte Maxeke Johannesburg Academic Hospital. Bioassays were performed on 29

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patients (14 MAP, 11 MSAP, and 4 SAP) and 6 healthy controls as part of a preliminary study. A total of 12 mL of blood samples were collected at Day (D) 1, 3, 5, and 7 post epigastric pain. Using multiplex immunoassay panels, real-time polymerase chain reaction (qRT-PCR) arrays, and multicolour flow cytometry analysis, immune response-related proteins, genes, and cells were profiled respectively. GraphPad Prism™ software and fold change (FC) analysis was used to determine differences between the groups. $P<0.05$ was considered significant.

RESULTS

The concentration of IL-6 was significantly different at D3 post epigastric pain in both the MAP group and MSAP group with $P = 0.001$ and $P = 0.013$ respectively, in a multiplex assay. When a FC of 2 was applied to identify differentially expressed genes using RT^{2}Profiler, *CCR8* was shown to increase steadily with disease severity from MAP (1.33), MSAP (38.28) to SAP (1172.45) median FC. Further verification studies using RT-PCR showed fold change increases of *CCR8* in MSAP and SAP ranging from 1000 to 1000000 times when represented as Log_{10}, compared to healthy control respectively at D3. The findings also showed differing lymphocyte and monocyte cell frequency between the groups. With monocyte population frequency as high as 70% in MSAP at D3.

CONCLUSION

The higher levels of *CCR8* and IL-6 in the severe patients and immune cell differences compared to MAP and controls provide an avenue for exploring AP stratification to improve management.

Key Words: Acute Pancreatitis; Severity; Stratification, Interleukin-6; Chemokine Receptor 8; Lymphocytes; Monocytes

Core Tip: Chemokine receptor 8 (*CCR8*) is a chemokine receptor that is highly expressed on monocytes and cells of T helper type-2 (Th2) lineage including innate lymphoid cells group 2 and 3 (ILC2 and 3). This study shows possible linkages between increasing *CCR8* expression and severity in mainly moderately severe acute pancreatitis (MSAP) patients when compared to mild acute pancreatitis (MAP). Differing lymphocyte and monocyte cell frequencies suggest that in MAP, interleukin (IL)-6 was highly expressed in lymphocytes, and in the severe patients [MSAP and severe acute pancreatitis (SAP)] were highly expressed by monocytes. The findings open doors for future work, which could include an in-depth look at IL-6 producing cells such as Th2 Lymphocytes, monocytes, and innate ILC2 to determine cell-associated cytokine as a novel approach in prognosticating AP disease severity.

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease that presents with epigastric pain and is clinically diagnosed by amylase and lipase levels three times the upper limit of normal[1]. The disease is localized to the pancreas and is triggered by the premature release of digestive enzymes resulting from damaged pancreatic acinar cells[2-3]. Through activation of the immune system, patients develop a systemic inflammatory response syndrome (SIRS) and subsequently, single or multiple organ failure leading to high mortality[4]. This disease is one of the most common cause of hospital admissions and has an annual incidence of 80 in 100000 people worldwide[5-7].

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The severity of acute pancreatitis is classified as mild, moderate, or severe[1]. Mild AP (MAP) presents with no organ failure and no local complications. Moderately severe acute pancreatitis (MSAP) only differs from severe AP (SAP) in that the patients have transient organ failure (OF) within 48 h and possibly pancreatic necrosis[1,8-9]. If OF persists for more than 48 h the patient is classified as severe[1]. MSAP is further defined by specified local complications or exacerbation of the co-morbid disease. Local complications include pancreatic fluid collections, pancreatic and peripancreatic necrosis (sterile or infected), pseudocyst, and walled-off necrosis (sterile or infected) [1]. Due to the complications and subsequent high mortality observed with increasing disease severity, the need arises for early stratification of the disease through the understanding of the pathogenesis of the disease and its systemic inflammatory response[4,7,10].

It is generally accepted that the premature release or activation by trypsin of proenzymes (including trypsinogen) is the initial trigger of pancreatitis[11]. Under normal conditions, trypsin and other proteolytic enzymes are blocked from activation by serine protease inhibitor, Kazal type 1, which is secreted by acinar cells[11]. AP is characterized by the activation of trypsin and other events such as obstruction and passage of gallstones in the bile duct (in the case of acute biliary pancreatitis), which in turn blocks the transport of trypsin to the small intestine[7,12]. This leads to premature activation of lipase and elastase causing intracellular damage of cells and subsequently inflammation and thrombosis. Damaged acinar cells are unable to regulate trypsin activity leading to further inflammation and eventual tissue damage through excessive amounts of activated enzymes within the pancreas. Lipase in particular, induces necrosis in fat cells within the pancreas leading to local recruitment of proinflammatory markers including cytokines[7,13].

Identifying prognostic markers of AP would ensure early patient stratification. Markers such as C-reactive protein (CRP), nuclear factor kappa B (NF-κB), and IL-6 have been identified as potential prognostic markers in AP. CRP, an acute-phase reactant produced by the liver and induced by IL-6, is well described as an inflammatory marker for the disease. It has been demonstrated as an effective prognostic marker of AP severity at 48 h after admission, although other studies found that its strength as a prognostic marker is prominent only at 72 h after admission[11,14-15]. NF-κB, on the other hand, is a transcription factor involved in cell proliferation[13]. This molecule is responsible for cellular responses to free radicals such as reactive oxygen species, production of inflammatory cytokines (IL-2, IL-6, TNF-α, IL-1β, and IL-8), and excess production of calcium within acinar cells, which results in premature activation of trypsinogen[13]. NF-κB is also responsible for activating the cytokine cascade that manifests as SIRS[13,6-17].

Considering that AP is an inflammatory disease, continuous efforts to fully understand its immunopathogenesis are critical to potentially improve management. This is due to the underlying complex pathophysiology associated with the disease [18]. For autoimmune diseases, the excessive recruitment of inflammatory mediators and subsequent increase in the production of cytokines and chemokines after an insult is responsible for inflammation[19]. This condition is further aggravated by the continued recruitment and infiltration of macrophages, neutrophils, and lymphocytes to the site of injury[19-21]. The resulting inflammation from the tissue injury, as a result of damage to the pancreas due to either obstruction or passage of gallstones, in biliary AP, can be attributed to damage-associated molecular patterns, which may result in necrosis of the pancreas in more severe forms of AP[20]. These inflammatory molecules are then recognized by pattern recognition receptors of the innate immune system. This process mobilizes the recruitment of neutrophils, macrophages, dendritic cells, and mast cells in the peripheral blood and at the site of injury, which in turn produces cytokines including IL-1, IL-6, and TNF-α[20-21]. This results in inflammation at the site of injury and phagocytosis by macrophages and neutrophils[21]. Phagocytosis activates antigen-presenting cells (APCs), which include macrophages, dendritic cells, and B cells[21]. Another cell type involved in innate immunity is natural killer (NK) cells, which help activate the adaptive immune system (AIS) by increasing the production of interferon-gamma (IFN-γ), a recognized initiator of the AIS[21-22]. This presentation process of the AIS activates T cell proliferation[22]. Naïve T cells will differentiate into cytotoxic T cells (CD8+) or T helper (Th) cells (CD4+ cells). CD8+ cells eliminate the threat of infected cells and tumorous cells[21]. Once the threat is eliminated, another group of T cells, T regulatory cells, suppress the immune response to achieve homeostasis[19].

Natural Killer cells belong to a group of cells known as innate lymphoid cells (ILCs). These cells are responsible for regulating immune responses and are mainly found within the tissues[21-22]. The ILCs have been described as mirrors of the T helper cells

but within the innate immunity[23]. Three groups of ILCs produce the same cytokines as T helper cells, *i.e.*, ILC group 1 (ILC1) produces Th1 cytokines; ILC2 produces Th2 cytokines and ILC3 produces Th17 cytokines[21-22]. As detailed in Supplementary Table 1, the group 1 ILCs produce IFN-γ and require T-box transcription factor for their proliferation; group 2 ILCs require transcription factor *GATA-3* and *ROR-α* to develop and will produce Th2 cytokines, such as IL-4, IL-5, and IL-13[24]. Group 3, ILCs depend on the transcription factor, *ROR-γt*, for their development and produce IL-17 and IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF)[22]. ILCs have also been reported to act as antagonists of both innate and adaptive immune cells[25], by mimicking the activity of T regulatory cells in achieving immune homeostasis[22].

CCR8, a chemokine receptor, is highly expressed on monocytes and cells of Th2 Lineage including ILC2 and ILC3[26]. This chemokine is also expressed on peritoneal macrophages in tissue and lymphocytes of Th2 Lineage[27]. Studies demonstrate that NF-κB is suppressed in *CCR8* deficient mice and that macrophage chemotaxis in the peritoneal cavity, which includes the pancreas, is Chemokine (C-C motif) ligand 1 (CCL1), which is the ligand of *CCR8* is dependent[27]. *CCR8* and its ligand, CCL1, are known to recruit and activate macrophages in type 1 diabetes[28-29]. This study is the first to describe *CCR8* in AP and its possible linkages to lymphocyte and monocyte cell frequencies.

This study utilized patients' samples at different severities (MAP, MSAP, and SAP) to profile inflammatory genes, and proteins (including *CCR8* and IL-6) and identified those that were distinctly upregulated or downregulated. White blood cell populations were characterised and assessed and linkages to gene and protein expression are proposed as potential prognostic markers for AP. The findings also provide insights that are more recent and contribute to the scarce literature on the prevalence, demographics, and etiology of AP in an African setting.

MATERIALS AND METHODS

Patient recruitment and sample collection

Ethics approval for this study was obtained from the Human Research Ethics Committee Medical of the University of the Witwatersrand (Ethics No. M180133). All patients included in the study were duly informed and written consent was received before blood samples were taken. Using the Revised Atlanta Classification (RAC) for AP[1], patients were recruited from the Hepatopancreatobiliary Unit of the Chris Hani Baragwanath Academic Hospital (CHBAH) and the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) in Johannesburg, South Africa, from August 2018 to September 2019. The total number of patients recruited was 40 (21 MAP, 14 MSAP, and 5 SAP) and 6 healthy volunteers were recruited as controls after being age and sex-matched to recruited patients. Blood samples were collected on Day 1, 3, 5, and 7 post epigastric pain using three BD vacutainer® purple blood collection tubes (BD Biosciences, New Jersey, United States) with 4 mL of blood each. Patients on average presented at the hospital approximately after 72 h of pain (day 3 of post epigastric pain). Clinicians within the Gastrointestinal Unit of the respective hospitals diagnosed patients and classified them into the three groups (MAP, MSAP, and SAP). The stratification of severity was determined using the RAC guidelines.

Sampling and processing: Overview

The different aspects of the study included different numbers of patients as illustrated (Figure 1). From the 40 patients, plasma and cell samples were processed in the laboratory within 4 h of phlebotomy. Plasma from a total of 31 out of 40 patients was analysed using the Th1/Th2/Th17 cytometric bead array (CBA) kit in an initial exploratory study. Based on this analysis, plasma samples from 23 patients were randomly selected for analysis of selected Th17 related cytokines including IL-6 using the MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit (Millipore™, Massachusetts, United States).

RNA was extracted from peripheral blood mononuclear cells (PBMCs) using the TriReagent® (Sigma Aldrich, Missouri, United States) method from 13 patients for screening of genes with the human innate and adaptive RT² Profiler 96-well PCR array plates (QIAGEN, Hilden, Germany). Findings showed dose-dependent expression of the *CCR8* gene with disease severity, prompting further analysis in 29 patients using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) to verify its roles. To characterize cell types into monocytes, lymphocytes, and

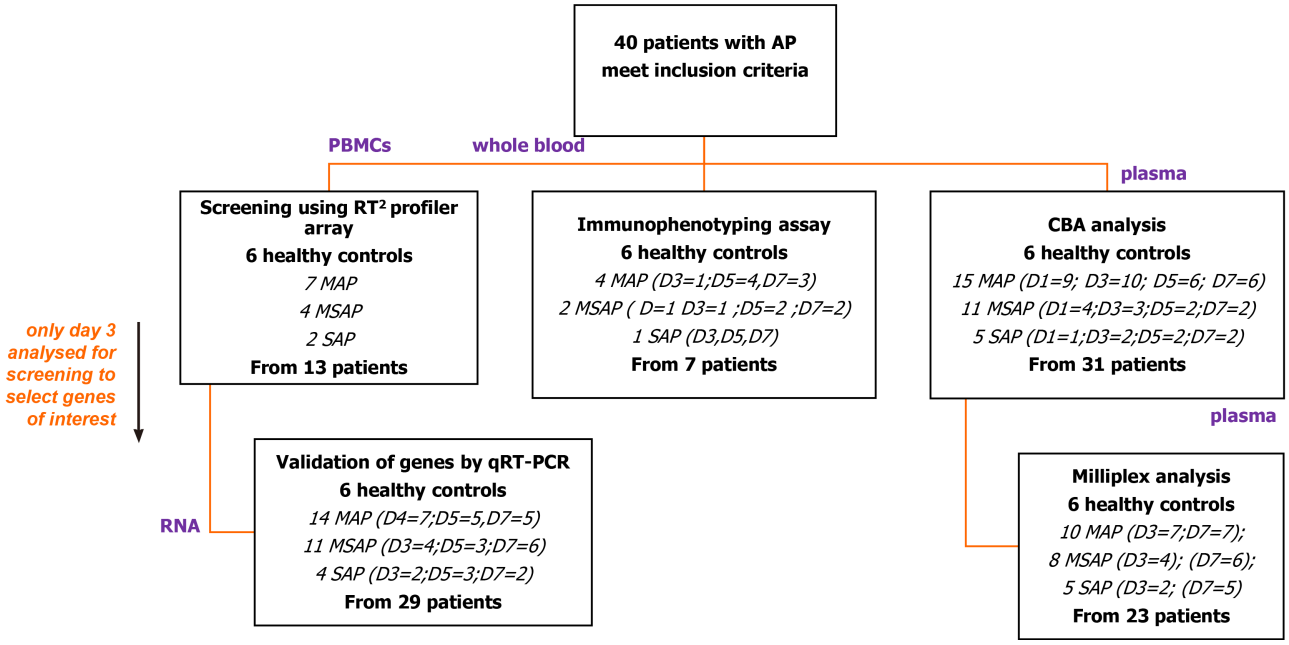

Figure 1 Flow diagram of patient recruitment. From the 40 patients and 6 healthy controls recruited over the study period, peripheral blood mononuclear cells, whole blood and plasma were used for the various study assays as shown. Peripheral blood mononuclear cells (PBMCs) from 13 patients with Day 3 data were used to do a screening study of innate and adaptive immune cell genes using RT^2 Profiler Array (Qiagen, Hilden, Germany). *CCR8* was selected as a target gene and further verification studies done in 29 patients as depicted. For immunophenotyping, 12 antibodies were selected to discriminate monocytes, lymphocytes, and granulocytes and their subpopulations from blood samples of seven patients. An exploratory study of seven Th1/Th2/Th17 cytokines was done on 31 patient samples and 23 of these randomly selected for further analysis using the MILLIPLEX® assay. PBMCs: Peripheral blood mononuclear cells; RT^2: Reverse transcriptase square; D: Day; MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; *CCR8*: Chemokine receptor 8; Th1/Th2/Th17: T helper type 1/2/17.

granulocytes, seven patients were included in an antibody specific multicolour immunophenotyping flow cytometry experiment.

Blood processing

From the blood samples, plasma was isolated by gravity separation for 45 min at room temperature followed by centrifugation at 1500 r/min for 30 min. Plasma samples were aliquoted (200 μL) in single use vials and stored at -80 °C until needed.

Using Ficoll-Paque™ (GE Healthcare, Illinois, United States) separation method, as per the manufacturer's instructions, PBMCs were separated and stored in single use aliquots in liquid nitrogen in a freezing medium (10% dimethyl sulphoxide in fetal bovine serum, Sigma Aldrich, Missouri, United States) until required. Samples were only thawed once to preserve integrity.

Cytokine expression analysis

Protein expression analysis was performed using two methods as depicted in Figure 1. The first was a BD BioSciences cytometric bead array Th1/Th2/Th17 kit that served as an exploratory step to determine the concentration of interleukin (IL-2), IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A cytokines. The assay was done on 31 AP patients (15 MAP, 11 MSAP, and 5 SAP) and 6 healthy control donor samples on days 1, 3, 5, and 7 post epigastric pain (see the supplementary section for detailed protocol). The second analysis was done using a MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit (Millipore™, Massachusetts, United States).

Using the MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit

In the MILLIPLEX® assay, preselected cytokines, based on the performance of the CBA analysis and based on literature and previous work from the research group were used [30-32]. These cytokines were; IL-17A, IL-21, and IL-6, IFN-γ, IL-23, IL-28λ, and TNF-β measured from 23 randomly selected AP patient samples (10 MAP, 8 MSAP, and 5 SAP) from the pool of 31 patient samples tested in the CBA assay on days 3 and 7 post epigastric pain. Six healthy controls were included.

A solid 96 well plate was prepared using the manufacturer's instructions. Plates were run on BioPlex® 2200 system (BioRAD, California, United States) and data were

collected and analysed using BioPlex® Manager 5.0 software (BioRad, California, United States). All samples and controls were measured in duplicate to minimize errors. Controls included quality control (QC) 1 samples (low level) and QC2 samples (high level) as well as standards with the lowest dilution at 4:1. The observed concentration of cytokines was determined by excluding outliers and values extrapolated beyond the standard range. Values designated by an asterisk as per the BioPlex® Manager 5.0 software, were inputted as zero while values labeled as Out of Range were not considered in the analysis.

Total RNA extraction

Total RNA was extracted using the TriReagent® (Sigma Aldrich, Missouri, United States) protocol, according to the manufacturer's instructions, from the isolated PBMCs on Day 3, 5, and 7 samples. However, initial screening was performed on 13 (MAP, n = 7; MSAP, n = 4; SAP, n = 2) Day 3 samples only. The quality of RNA was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fischer Scientific, Massachusetts, United States), and samples with an A260/280 ratio > 1.8 were observed across all samples[33-34].

Complementary DNA synthesis and PCR array analysis

Complementary DNA synthesis (cDNA) was performed from 250 ng/μL of total RNA using the RT^2 First Strand Kit (QIAGEN, Hilden, Germany), according to the manufacturers' instructions. A genomic DNA elimination mix was first prepared and incubated for 5 min at 42 °C in a SimpliAmp™ thermocycler (ThermoFischer Scientific, Massachusetts, United States), which was subsequently placed on ice for 1 min. Following this, a 20 μL cDNA synthesis reaction was prepared and run at 42°C for 15 min followed by incubation at 95 °C for 5 min. From the cDNA, 102 μL was added to the PCR mixture and loaded onto the human innate and adaptive RT^2 Profiler 96-well PCR array plates (QIAGEN, Hilden, Germany). The mixture was amplified on Quant Studio 1 Real-Time System (Thermo Fischer Scientific, Massachusetts, United States) the PCR reaction was run for 40 cycles including a 10 min hot start at 95 °C for 1 cycle; 95 °C for 15 s and 60 °C for 1 min. The human innate and adaptive RT^2 Profiler array includes 96 genes, 5 of which are reference genes and 3 reverse-transcription controls, 3 positive PCR controls, and 1 human genomic DNA control. Using the QIAGEN GeneGlobe online tool (https://geneglobe.qiagen.com/za/analyze/), a fold-change of 2 was applied as the cut-off for differential analysis comparing the expression level of genes in the 3 severity groups to healthy control.

Verification of selected gene targets using Real-time PCR

After screening of Day 3 samples for early immune markers with the RT^2 Profiler PCR Array Human Innate and Adaptive Immune Responses (QIAGEN, Hilden, Germany) the *CCR8* gene was selected for further analysis. Twenty-nine patients (MAP = 14, MSAP = 11, SAP = 4) were included in this assay as stated in Figure 1 and Table 1. The TaqMan® Fast Advanced Master Mix (Thermo Fischer, Massachusetts, United States) was used to perform duplex qRT-PCR. The PCR reaction was run for 40 cycles including a 2 min hold at 95°C for 1 cycle; 95°C for 1 s and 60 °C for 20 s. Normalisation was done using RPL13A on VIC (assay ID *Hs04194366_g1*, Thermo Fischer Scientific, Massachusetts, United States) as the reference gene. This gene is well established in AP disease models as a reference gene[35]. The target gene was *CCR8* on FAM (assay ID: *Hs00174764_m1,* Thermo Fischer Scientific, Massachusetts, United States). The Quant Studio™ 1 Real-Time System (Thermo Fischer Scientific, Massachusetts, United States) was used to run the RT-qPCR reactions. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression[36].

Immunophenotyping using multicolor flow cytometry analysis

Selected blood samples from days 3, 5, and 7 of onset of AP symptoms were analysed using multicolour flow cytometry to determine immune cell frequency levels to make a correlation to protein production or expression. The sampled patients included 4 in the MAP group, 2 patients from the MSAP group; 1 patient from the SAP group. Six healthy participant samples were used as controls. While the numbers here are small, given the well characterized levels of monocytes, lymphocytes, and granulocytes in AP patients from the literature[18,20,37-38], inferences from this preliminary data will be discussed with reference to the literature.

A 12-colour panel was established to characterize heterogeneous cell populations in the three risk categories of AP. Using the lyse/wash method, whole blood was used to isolate white blood cells from 100 μL of blood from an EDTA blood tube within 6 h of

Table 1 Demographic characteristics of the acute pancreatitis patients included in the gene expression analysis study

Parameter	Value [*n*, %]
AP patient demographics	*n* = 29
MAP	14 (48)
MSAP	11 (38)
SAP	4 (14)
Age (yr), [median (IQR)]	41 (23, 76)
Male (*n*, %)	17 (49)
Female (*n*, %)	12 (51)
AP etiology/risk factor	
Biliary (*n*, %)	13 (45)
Alcohol (*n*, %)	13 (45)
ERCP (*n*, %)	1 (3)
Antiretroviral (*n*, %)	2 (7)
Healthy control Demographics	
Age (yr), [median (IQR)]	36.5 (23, 55)
Male (*n*, %)	3 (50)
Female (*n*, %)	3 (50)

AP: Acute pancreatitis; MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; IQR: Interquartile range; ERCP: Endoscopic retrograde cholangiopancreatography.

phlebotomy. Antibodies were optimized by titration to optimally stain lymphocytes populations and subpopulations using CD3 BD Horizon Brilliant™ Ultraviolet (BUV); CD4 Alexa flour; CD8 Brilliant Violet™ 605; CD56 PE Phycoerythrin Cyanine 7 (PECy7), CD16 PECy5) and monocyte populations using CD16PECy5 and CD14 Peridinin-Chlorophyll-protein cyanine 5.5 (PerCPCy5.5), and CD14PerCP Cy5.5 and human leukocyte D related (HLA-DR BV650). Other antibodies that were included in the 12 colour panel but not reported in the study are listed in Supplementary Table 2. All antibodies were from BD Biosciences, (New Jersey, United States).

Cells were prepared both as fully stained samples and as unstained samples. Fully stained samples were suspended in BD Horizon brilliant buffer (BD Biosciences, New Jersey, United States) and stained with selected antibodies (see Supplementary Table 2). The cells were then incubated in the dark for 20 min and thereafter fixed with 2 mL of diluted BD FACS Lyse (BD Biosciences, New Jersey, United States) and incubated for 12 min with intermittent mixing with a pipette. The cells were then washed with diluted Dulbecco's Phosphate Buffered Saline (Sigma Aldrich, Missouri, United States) at 150 x *g* for 5 min. Approximately 100000 cells were acquired on BD LSRFortessa™ II flow cytometer (BD Biosciences, New Jersey, United States) for each sample at a threshold of 5,000 after the necessary quality controls using FACSDiva™ software version 5 (BD, Biosciences, New Jersey, United States). The controls included voltages optimization using single stains, compensation for spillover was done using CompBeads (Anti-Mouse Ig, κ/Negative Control Compensation Particles Set; BD Biosciences, New Jersey, United States) and 8 peak beads (BD Biosciences, New Jersey, United States) were used to determine linearity in fluorescence detection channels on every sample run.

Data was further analysed using FlowJo LLC version 10 (BD, Biosciences, New Jersey, United States) with previously linked compensation controls from FACSDiva™ software. Cells were gated as singlets, then further as granulocytes, lymphocytes, and monocytes using forward scatter and side scatter properties as well as fluorescent antibody stains for specific subsets. Doublets were excluded using Forward scatter height (FSC-H) and FSC area (FSC-A), then FSC and side scatter (SSC) were used to discriminate white blood cells namely lymphocytes, granulocytes, and monocytes. All populations were represented as percentages of parent populations. Of the 12 antibodies used for cell differentiation, analysis was done for *CCR8* associated cell

populations. These include lymphocytes and monocytes[39]. These populations were lymphocyte subpopulations ($CD3^{-}CD16^{+}$ $CD56^{+/-}$, $CD3^{-}CD16^{+}CD57^{+/-}$) and monocyte populations and subpopulations ($CD14^{+/-}$ $CD16^{+/-}$ and $CD14^{+}HLA\text{-}DR^{+/-}$).

Statistical and Data analysis

The cytokine data and qRT-PCR data were analysed using GraphPad Prism™ software version 8 (GraphPad Software Inc, California, United States). A Shapiro-Wilk test was used to test for normality. Once data was determined to be non-parametric, a Kruskal Wallis test was used to determine significant differences between the healthy control groups and between the MAP, MSAP, and SAP groups. The *P* values were considered significant at $P < 0.05$. A Dunn's Multiple Comparison Test was used to perform a post hoc analysis to eliminate type 1 errors. Immunophenotyping data focused on lymphocytes and monocytes as they relate to *CCR8* expression[39] and were presented as percentages and ratios. The statistical methods of this study were reviewed by Mr. Glory Chidumwa from the Division of Epidemiology and Biostatistics, School of Public Health, Faculty of Health Sciences, University of the Witwatersrand.

RESULTS

Patient demographics

A total of 40 patients were included in the overall study using prescribed inclusion criteria from 1 August 2018 to 22 August 2019 from CHBAH and CMJAH in Johannesburg, South Africa. Of these 40 patients, 29 were reported in the gene expression studies (Figure 1). The gender distribution of the 29 patients was 41% females and 59% males. The most common etiologies of AP were alcohol and biliary-related with each category consisting of 45% of the recruited patients (Table 1). The median age of the patients was 36.5 years. The MSAP group age range was between 26 to 76 years and that of the SAP group was between 40 and 69 years old.

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

In the exploratory CBA assay, data were expressed as Mean Fluorescent Intensity (MFI) as shown in Supplementary Figure 1. In the analysis of the data, only the MFI of IL-6 revealed changes between patient plasma samples at Day 3. On Day 1, the MAP group had a high expression of IL-6 at above 5000 MFI, which was significantly different from healthy controls ($P = 0.015$). At Day 3 in the MAP a significant difference was reported with $P = 0.004$ when compared to the healthy control. In the MSAP group, there was a significant difference on Day 3 ($P = 0.004$) and 7 ($P = 0.029$). IL-6 MFI was in the region of 5000 for the SAP patient.

The results from the MILLIPLEX® data showed visible trends between severities over time as well as between groups. The mean concentration of IL-6 in the MAP group was 20 ± 4.9pg/mL on Day 3 and dropped to 2.9 ± 1.7pg/mL on Day 7. A similar trend was seen in the MSAP group with a drop in mean concentration from 13 ± 4 pg/mL on Day 3 to 10 ± 7.7pg/mL on Day 7. The IL-6 concentration was significantly different at D3 for MAP ($n = 7$) and MSAP ($n = 4$) compared to healthy controls with $P = 0.001$ and $P = 0.013$ respectively (Figure 2). The concentration of the SAP group was not significantly different at both Days 3 ($n = 2$) and 7 ($n = 5$) compared to healthy controls with $P = 0.094$ and $P = 0.186$ respectively. However, the mean concentration of IL-6 in the SAP group was higher compared to the MAP and MSAP groups. The concentration at Day 3 was 50 pg/mL (this included two patients with individual IL-6 concentrations of 0.13 pg/mL and 100 pg/mL). The mean concentration at Day 7 was 65 ± 62pg/mL as shown in Figure 2.

Differential gene expression in the different acute pancreatitis severity groups

A fold change (FC) of 2 was applied to identify differentially expressed genes in the patient groups (7 MAP, 4 MSAP, and 2 SAP) at Day 3 compared to healthy controls from the RT^{2}First Strand Kit (QIAGEN, Hilden, Germany) assay as summarized in Table 2. Of the 96 genes analysed (represented by the heat map in Figure 3), a total of 31 genes were downregulated while 9 genes were upregulated in the MAP group with *CXCL8* (fold change = -45.26) and *CD14* (FC = -21.58) being the most downregulated compared to the healthy control samples. The chemokine receptor *CCR6* was also downregulated in the MAP group (FC = -21.05). In the MSAP patients, 68 genes were upregulated and 4 were downregulated. The downregulated genes included *CCL5* (FC

Table 2 List of selected genes and their fold changes in mild, mild acute pancreatitis, moderate, moderately severe acute pancreatitis, and severe acute pancreatitis patients when compared to healthy controls

Gene symbol	MAP (*n* = 7)	MSAP (*n* = 4)	SAP (*n* = 2)
CCL5	-2.97	-3.76	-15.22
CCR8	1.33	38.28	1172.45
IL10	-1.30	58.62	-1.47
FOXP3	3.90	137.02	96.27
IL13	-1.92	83.66	19.53
IL17A	1.72	116.93	2.56
IL23A	-5.60	18.07	6.57
IL4	-1.13	108.64	36.83
IL5	1.33	192.59	1.21
NOD1	-8.93	-14.62	64.21
MPO	1.33	91.77	11.8

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; *CCL5:* Chemokine (C-C Motif) Ligand 5; *CCR8*: Chemokine receptor 8; *IL:* Interleukin-(4; 5; 10; 13; 17A; 23A); FOXP3: Forkhead box P3; *NOD1*: Nucleotide-binding oligomerization domain-containing protein 1; *MPO*: Myeloperoxidase.

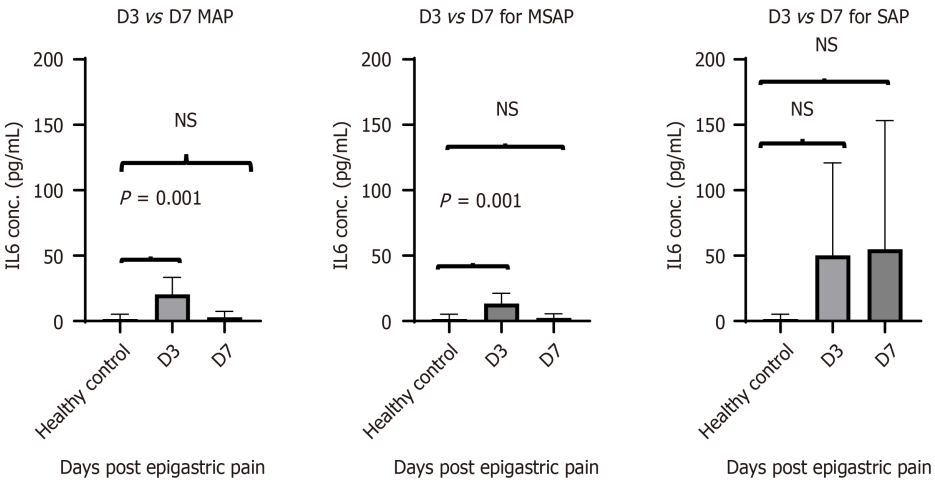

Figure 2 IL-6 secretion levels acquired using the MILLIPLEX® MAP Human Th17 Magnetic Bead Panel kit. Analysis was done on 23 patients sampled on Day 3 and Day 7 and 6 healthy controls were included. The concentration of interleukin (IL)-6 was highest in the SAP group 50 ± 50 pg/mL and 65 ± 61 pg/ml on D3 (*n* = 2) and D7 (*n* = 5) respectively. The MAP group IL-6 levels were 13 ± 8 pg/mL (*n* = 7) and MSAP 20 ± 13 pg/mL groups (*n* = 4) on D3. Significant differences were observed between the healthy controls (*n* = 6) and MSAP at D3 (*n* = 4) with *P* = 0.014 and *P* = 0.013 respectively. A Dunn's multiple comparison test was used as a post hoc to adjust *P* values. D: Day of the specific severity group; *n*: number; MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; IL-6: Interleukin-6.

= -3.76) and the upregulated genes included *FOXP3* (FC = 137.02) and *APCS* (FC = 262.91) being the most downregulated and overexpressed, respectively. Importantly, moderately severe patients had the highest number of upregulated genes, specifically those involved in inflammation such as *IL4* (FC = 108.64), *IL5* (FC = *192.59)*, *IL23A* (FC = 18.07), *GATA-3* (FC = 11.58), and *CRP* (FC = 177.42), as shown in the heat map in Figure 3. A total of 34 genes were upregulated in the SAP patients while 25 were downregulated. *CCR8* (FC = 1172.45) and *CD8A* (FC = -74.26) were the top upregulated and downregulated genes, respectively in the SAP group. Notably, *CCR8* increased steadily with disease severity producing the highest fold change across all groups. Other genes that increased with severity were *GAPDH, NOD1, TRL 1* and *TICAM 1, TBX21,* and *CASP1,* which are all genes closely associated with *CCR8* (Figure 3).

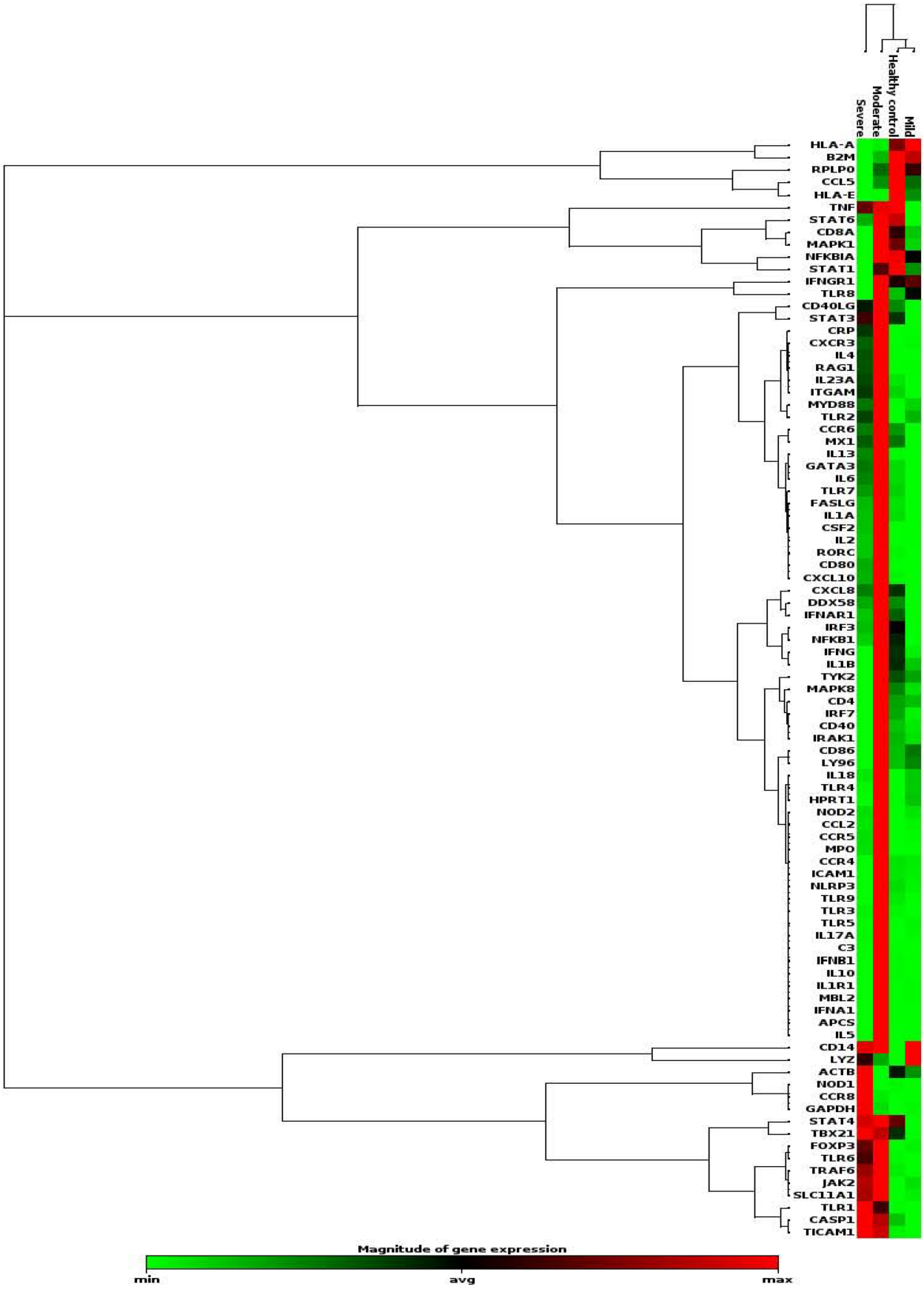

Figure 3 A heat map showing gene dysregulation in mild moderate and severe acute pancreatitis patients compared to healthy controls. Hierarchical cluster of all the genes across patient severities are shown. Red colour represents upregulated genes, green is downregulated and black is unchanged. Chemokine receptor 8 *(CCR8)* was shown to increase with severity and so were *GAPDH, NOD1, TRL 1, TICAM 1, TBX21,* and *CASP1,* which are associated with *CCR8* expression. *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase; *NOD1*: Nucleotide-binding oligomerization domain-containing protein 1; *TRL 1*: Toll like

receptor 1*; TICAM 1:* Toll Like Receptor Adaptor Molecule 1*; TBX21*: T-Box Transcription Factor 21*; CASP1:* Caspase 1; apoptosis-related cysteine peptidase; *CCR8:* Chemokine receptor 8; MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis.

CCR8 expression and the severity of AP by Real-time PCR

The real-time PCR verification findings were plotted as Log10 of fold change ($2^{-\Delta\Delta CT}$), shown in Figure 4. The results show that at Day 3 post epigastric pain the fold change of *CCR8* for the MAP group compared with the healthy control group was almost 1 to 1 (Figure 4A). Whereas the MSAP is 1000 times more than the healthy control for the same day (Figure 4B). The SAP group was 10000000 times that of the healthy control at Day 3 (Figure 4C). This was due to an individual sample that can be considered as an outlier. This group had an FC of a 1090632 ± 1090631 (Figure 4C). On Day 5 and Day 7 the fold change dropped to almost 1:1 ratio with the healthy control in the MAP and SAP group. In the MSAP group, the FC on Day 5 was consistent with Day 3 Levels and dropped slightly to 800 ± 846 on Day 7 as observed in the comparisons of the FC of *CCR8* within groups on different days (Table 3).

NK subsets in an MSAP patient and immune suppression

In one of the sampled patients from the MSAP group, NK cell frequencies of $CD3^-CD16^+CD56^-$ doubled from 12% to 27%, and those from the $CD3^-CD16^+CD56^-$ subsets increased in percentage from 19.8% to 49.6% from Day 3 to Day 5. NK cell subsets, which were $CD57^+$ increased by over 30% for MSAP patients from Day 3 to Day 5 (Supplementary Figure 2).

Monocyte cell populations and severity

In the immunophenotyping analysis by flow cytometry, 7 patients were recruited, 4 MAP, 2 MSAP, and 1 SAP as shown in Figure 1. Cells known to express *CCR8*, namely lymphocytes (including NK cells belonging to ILC1) and monocytes were assessed as part of a multicolour panel using flow cytometry[39]. Classical monocyte subpopulations (CD14+CD16-) were higher in more severe patients with the MSAP patient having as much as 71.6% of the parent population on day 3, dropping to undetectable levels on day 5 (Figure 5A and B). In the SAP patients, the classical monocyte population consistently increased by more than 7% from Day 3 to 5 (Figure 6A and B). In the MSAP patient, the percentage of HLA-DR+ monocyte increased by 43% from Day 3 to Day 5 (Figure 5C and D). Whereas the percentage of HLA-DR+ monocytes increased from 4.2% on Day 3 to 13.5% on Day 5 in the SAP patient (Figure 6C and D).

DISCUSSION

In this preliminary study, the demographics of 29 AP patients, the role of *CCR8*, IL-6, and the frequency of cells expressing these biomolecules were explored. Patient demographics were as expected with older patients falling into more severe groups[1, 40]. The study demonstrated that the increase in IL-6 Levels maintained an upward trend in the SAP group up to Day 7, compared to the healthy control group, the MAP, and MSAP group (Figure 2). The consistency in the concentration of IL-6 protein levels in the SAP group in the peripheral blood is likely as a result of the observed activated monocytes and hence *CCR8* expression on these cells. CRP is a well-defined severity marker in acute pancreatitis and is initiated by elevation of IL-6[14,38]. Elevated levels of *CRP*, an acute phase reactant, in the pooled sample of the MSAP and SAP group (Table 2, Supplementary Table 3) may be due to increased monocyte cell populations [37]. Although, IL-6 was not shown as a useful independent marker to distinguish different risk categories of AP in this study, cells producing IL-6 such as monocytes (Figure 5A and B, Figure 6A and B) and NK cells (Supplementary Figure 2), which are part of group 1 ILCs, increased in frequency at Day 3 and 5 in the MSAP and SAP group[24,39]. A possibility exists in exploring the potential prognostic value of a lymphocyte to monocyte ratio based on the resulting difference in frequency in MAP compared to MSAP and SAP.

Our findings further show the presence of HLA-DR dim to negative monocyte subsets in an SAP patient suggesting downregulation (Figure 6C and D). This supports findings from a study that found that the presence of monocytes that do not express HLA-DR correlates with organ dysfunction in AP[37]. An important observation was that in the MSAP patient at Day 3 (Figure 5C), HLA-DR was downregulated but upregulated by Day 5 (Figure 5D) showing resolve in organ failure, supportive of the

Table 3 Gene expression levels (fold change $2^{\wedge-\Delta\Delta CT}$) for chemokine receptor 8 gene in acute pancreatitis patients.

Severity	Mean fold change ($2^{\wedge-\Delta\Delta CT}$)
MAP D3	0.8 ± 0.22
MAP D5	0.9 ± 0.25
MAP D7	1.0 ± 0.26
MSAP D3	1386 ± 1372
MSAP D5	68.0 ± 67
MSAP D7	848 ± 846
SAP D3	1090632 ± 1090631
SAP D5	1.1, NA
SAP D7	1.3 ± 0.46

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis. D: Day; D3: Day 3 post epigastric pain; *CCR8*: Chemokine receptor 8; $2^{\wedge-\Delta\Delta CT}$: Fold change, is used to measure change in the expression level of a gene[32]; NA: Not available due to single data points.

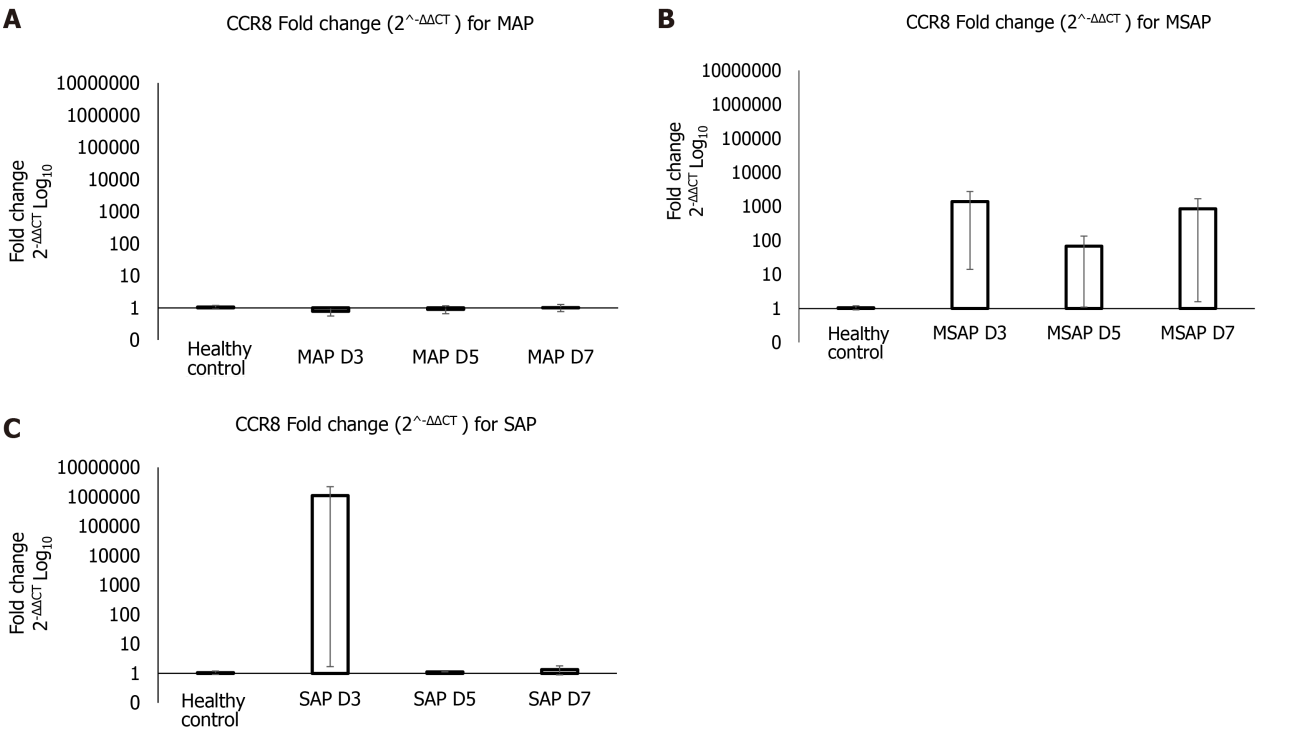

Figure 4 Gene expression analysis for chemokine receptor 8 in different severities at Day 3, 5 and 7 post epigastric pain due to acute pancreatitis for mild acute pancreatitis, moderately severe acute pancreatitis, and severe acute pancreatitis. The data is shown on a Log10 transformed scale of the fold change ($2^{-\Delta\Delta CT}$) normalized using *RPL13A* gene on VIC fluorescent dye (*Hs04194366_g1*, Thermo Fischer Scientific) as reference gene. A: The FC of *CCR8* was less than 1 at D3 and D5, then increased slightly to 1 at D7 for MAP group compared to healthy controls. B: At D3 and D7 the FC for the MSAP group was 1000 times more than in the healthy controls. C: In the SAP group the FC at D3 was 1000000 times more than in the healthy controls and the D5 and D7 was 1 time more. FC: fold change. D: D3 of the specific severity group; MAP: Mild acute pancreatitis: MSAP: Moderately severe acute pancreatitis; SAP: Severe acute pancreatitis; *CCR8*: Chemokine receptor 8.

MSAP classification[1]. The presence of immunosuppressive NK cell subsets, which are $CD57^+$ (Supplementary Figure 2) may also play an important role in this[41]. $CD3^-CD16^-CD57^+$ cell subsets have a protective function in autoimmune disease[41]. This further supports the hypothesis of a possible linkage between monocyte and lymphocyte frequencies to severity based on the observed decrease of classical monocytes from Day 3 to 5 in the MSAP patient who experienced transient organ failure. These preliminary results may indicate possible links between monocytes and NK cells in the stratification of the MSAP group of patients.

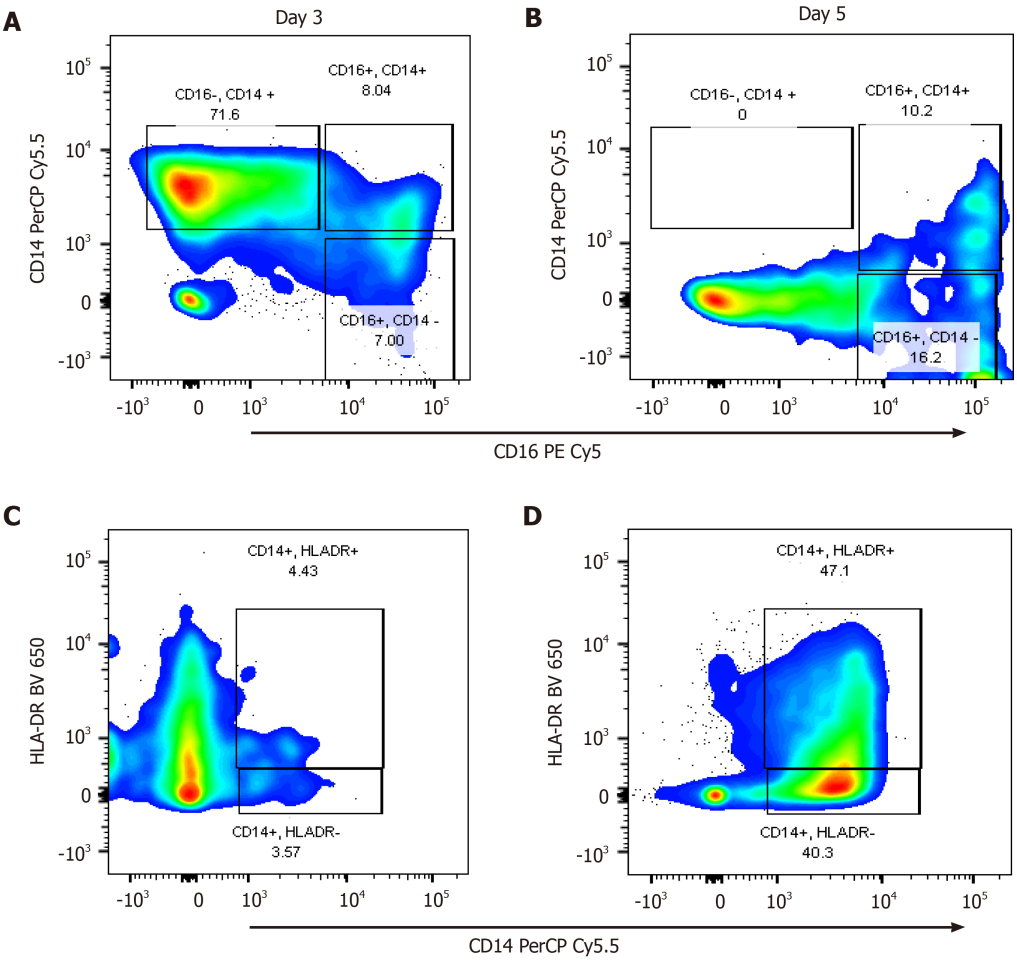

Figure 5 Representative moderately severe acute pancreatitis profile of the monocyte subpopulation. Cells were gated into intermediate (CD14+CD16+), classical (CD14+CD16-), non-classical monocytes (CD16+CD14-) and HLA-DR+/- monocytes. A and B: They showed CD14 PerCP Cy5.5 and CD16 PECy5 plot for Day 3 and Day 5 respectively. Classical monocyte subpopulations (CD14+CD16-) were higher in more severe patients with the MSAP patient having as much as 70% of the parent population on Day 3, which subsequently dropped to undetectable levels on Day 5. C and D: They showed a plot of CD14 PerCP Cy5.5 and HLA-DR BV650 for Day 3 and 5 respectively. The percentage of HLA-DR+ monocytes increased from 4% on Day 3 to 47% on Day 5. BUV: BD Horizon Brilliant™ Ultraviolet; Cy: Cyanine; BV: Brilliant Violet™; HLA DR: Human leukocyte D related; PE: Phycoerythrin; PerCP: Peridinin-Chlorophyll-protein; CD: Cluster of differentiation.

This study investigated expression patterns of several inflammatory and immune response-related molecules at the early stages of AP. We further describe a hypothetical model, which is deduced from this preliminary study and literature (Figure 7).

CCR8, a chemokine receptor, is known to be highly expressed on monocytes and cells of Th2 lineage including innate lymphoid cells group 2 (ILC2) and ILC3 cells[26,42]. Cells of the ILC1 population that are $CD56^-$ are found abundantly in peripheral blood in the disease state[17,18]. These cells are known to suppress autoimmune diseases[41]. This may explain the reason why organ failure is resolved in the MSAP patient compared to the NK cell-deficient SAP patient[1,36]. Acinar cell injury and elevation of trypsin in pancreatic tissue are followed by excessive recruitment of monocytes, neutrophils, and ILCs, to the local site of injury[42-44]. These ILCs include NK cells (ILC1), ILC2, and ILC3 cells. Once the pancreatic tissue is damaged due to AP, monocytes, and macrophages are responsible for the maintenance of inflammation [37,45]. Thus, the upregulation of *CCR8* observed in this study may be due to increased levels of activated monocytes in peripheral blood. The main agonist of *CCR8* is its own ligand *CCL1*[46]. *CCL1* in the peripheral blood is highly expressed on classical, non-classical, and intermediate monocytes[39]. In other autoimmune diseases such as cancers of the renal system, *CCR8* positive cells, namely monocytes and granulocytes were the most abundant in the bloodstream and contributed to prolonged inflammation within patients[47]. *CCR8* is also expressed on peritoneal macrophages in tissue and lymphocytes of Th2 Lineage[27]. Oshio *et al*[27] demonstrated that NF-κB is suppressed in *CCR8* deficient mice and that macrophage chemotaxis in the peritoneal cavity, which includes the pancreas, is *CCL1* dependent.

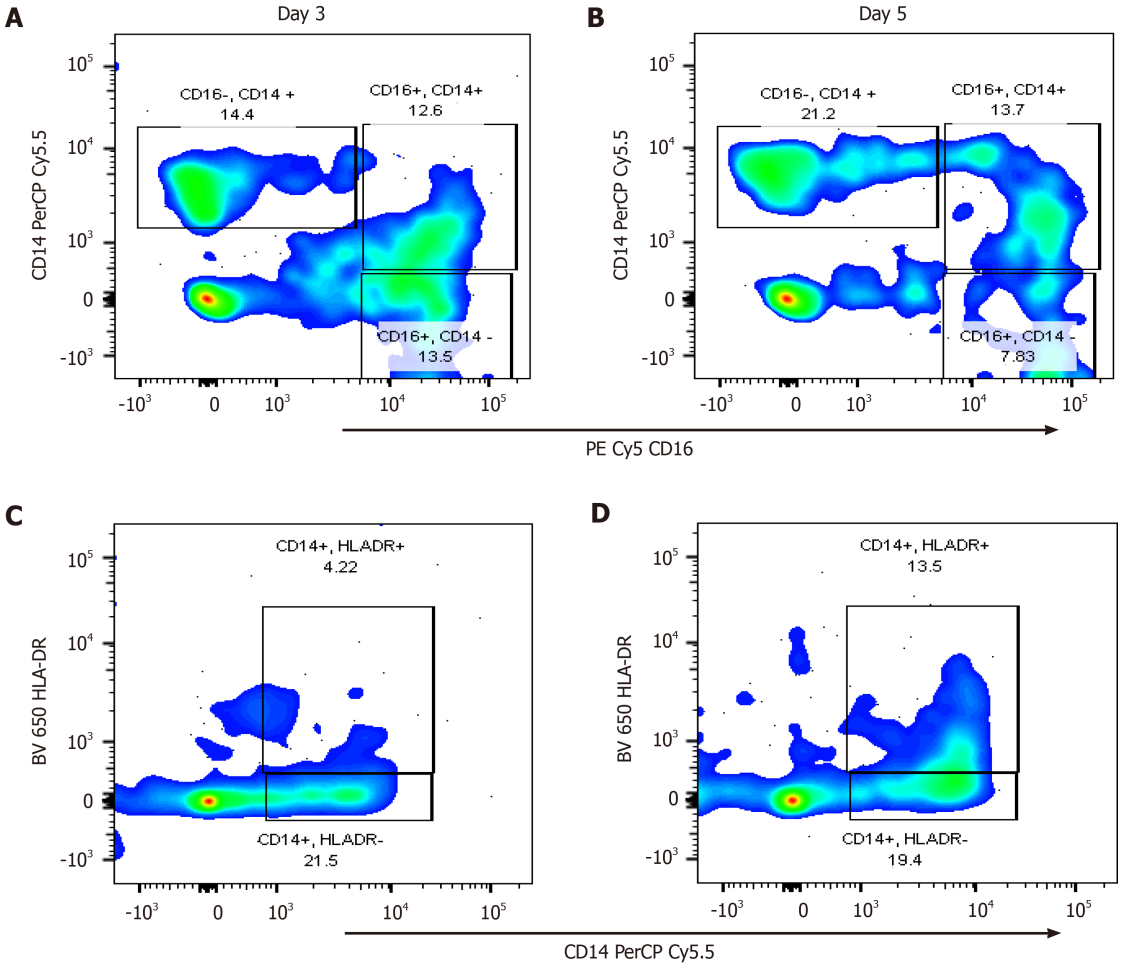

Figure 6 A plot generated from FlowJo™ version 10 (Oregon, United States) of an severe acute pancreatitis patient for the monocyte subpopulations. Cells were gated into intermediate ($CD16^+CD14^+$), classical ($CD16^-CD14^+$) and non-classical monocytes ($CD16^+CD14^-$). A and B: They showed CD14CD16 plot for Day 3 and Day 5 respectively. C and D: They showed a plot of CD14HLA-DR for Day 3 and 5 respectively. The percentage of $HLA\text{-}DR^+$ monocytes increased from 4% on Day 3 to 13% on Day 5. BUV: BD Horizon Brilliant™ Ultraviolet; CD: Cluster of differentiation; Cy: Cyanine; BV: Brilliant Violet™; HLA DR: Human leukocyte D related; PE: Phycoerythrin; PerCP: Peridinin-Chlorophyll-protein; CD: Cluster of differentiation.

This provides a possible link between monocyte expression and upregulation of *CCR8* in more severe patients observed in this study.

CCR8 gene was concomitantly upregulated with *TLR1*, *NOD1*, *CASP1*, and *GAPDH* (Table 2, Supplementary Table 3). These genes are all expressed on activated monocytes[39] and were observed in the analysis of the pooled samples (Table 2 and Supplementary Figure 3). Studies looking at inflammation in pancreatic injury have shown that continued release of proinflammatory cytokines by macrophages, increased number of neutrophils, and excess levels of nitric oxide impaired tissue regeneration and contributed to organ tissue damage[48]. This suggests that the observed increase of *CCR8* levels in MSAP patients, and to an extent the SAP patients, could be associated with macrophages and monocytes levels. The 1090631 fold upregulation of *CCR8* in the SAP group was due to one sample and was observed in the results of the pooled sample in the RT^2 profiler analysis (Figure 4C). This means that the *CCR8* expression levels in the SAP group may not necessarily be representative due to the limited number in this group suggesting the need for further research.

Several genes associated with Th2 Lymphoid cells were upregulated in the MSAP group. The upregulation of the transcription factor, *GATA-3*, and the *IL4, IL5, and IL13* genes in Supplementary Table 3 may indicate a stronger type-2 response in MSAP patients compared to the SAP group, which is a result of excessive recruitment of macrophages and monocytes in pancreatic tissues and the bloodstream respectively [22,49]. On the other hand, upregulation of proinflammatory genes such as *IL6*, *CRP*, and *FOXP3* (Supplementary Table 3) associated with *CCR8/CCL1* in the MSAP group may be attributed to ILC3 and or Th17 cells. Overexpression of *FOXP3 via* the *STAT3* pathway was directly proportional to the observed increase in fold changes for *IL-17A*

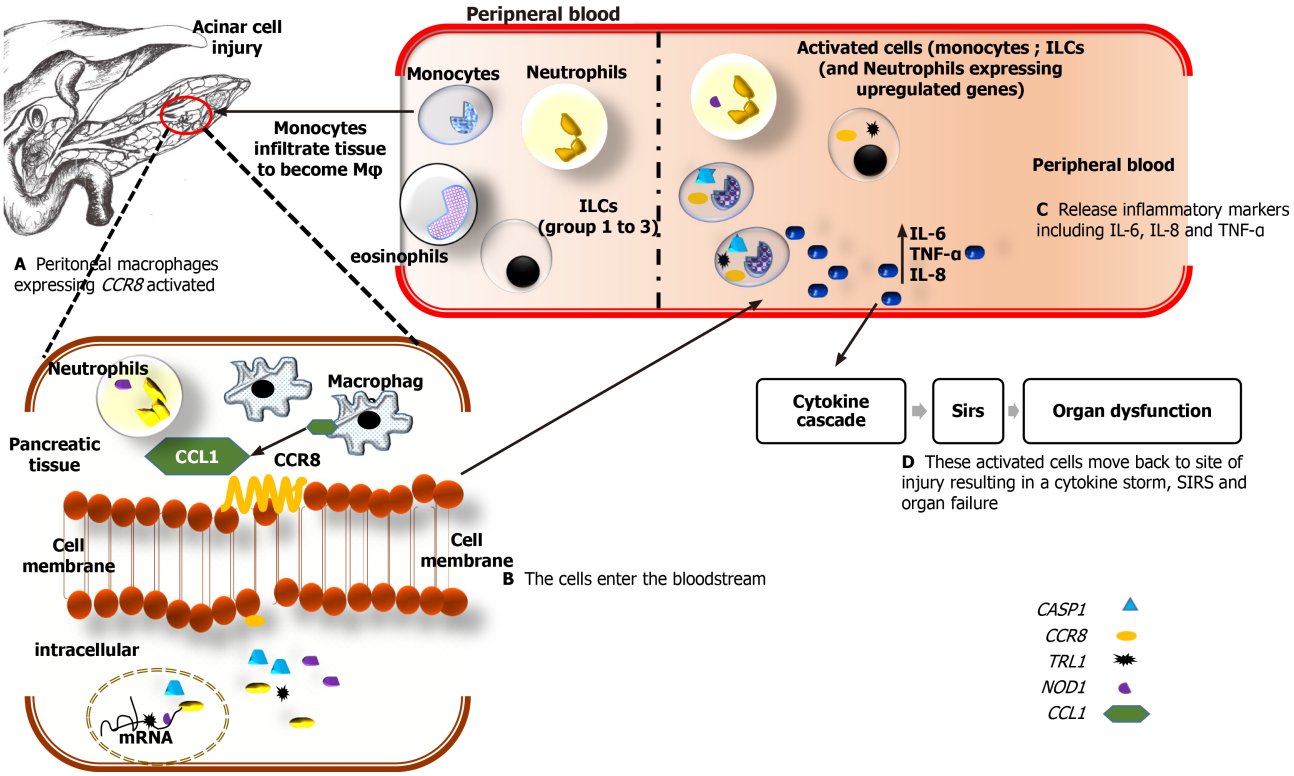

Figure 7 The schematic shows the possible mechanism by which chemokine receptor 8 is upregulated in peripheral blood. A: Upon elevation of trypsin in the pancreas due to acinar cell injury in patients with acute pancreatitis, monocytes, lymphoid cell groups (ILCs), neutrophils and eosinophils migrate to the site of injury. Once in the pancreatic tissue, the monocytes become activated to macrophages (φ) which then express the chemokine (C-C motif) ligand 1 (*CCL1*) gene. The *CCL1* binds in turn binds to the chemokine receptor 8 (CCR8) receptor on the surface of macrophages, ILC2 cells, and neutrophils. In patients with MSAP, ILC2 related cytokines, interleukin-4 (IL-4), IL-5, and IL-13 are upregulated while downregulation of these cytokines was observed in SAP patients (Supplementary Table 3). The observed increases in *CCR8* in the SAP patients may be due to excessive activation of macrophages and monocytes. The activated cells (ILCs, neutrophils, monocytes) may be releasing IL-1-β, tumor necrosis factor alpha (TNF-α), IL-6 at local sites, which send signals that activate and recruit inflammatory cells which include macrophages, neutrophils, ILC2, and ILC3; B-D: These cells migrate into the bloodstream and will express Toll-like receptor 1, Nucleotide-binding oligomerization domain, caspase 1 genes. These genes are mainly expressed on monocytes that will release proinflammatory cytokines such as IL-6, IL-8, and TNF-α and, depending on their levels in the peripheral blood, will cause dysregulation that leads to a systemic inflammatory response and consequently single or multiple organ failure. IL: Interleukin; *CASP1*: Caspase 1, apoptosis-related cysteine peptidase; *CCR8*: Chemokine receptor 8; *TRL1*: Toll-like receptor-1; *NOD1*: Nucleotide-binding oligomerization domain-containing protein 1; *CCL1*: Chemokine ligand 1; TNF-α: Tumor necrosis factor alpha.

and *IL 23A* genes[19]. This is expected since *STAT3* is responsible for differentiation in Th17 Lineage and has been implicated in autoimmune diseases[46]. Therefore, it is likely that an elevation of these Th17 cytokines may be due to the ILC3 group[22].

This preliminary study has its limitations. Like many clinical studies, obtaining the ideal sample size, which is adequate (not too small or too big) for the interpretation of the results is important in how the results are extrapolated. Here, we sampled 40 patients overall with MAP, MSAP, and SAP at three different time points (D3, 5, and 7) and tested samples from 29 as shown in Figure 1. Due to this being a time study, we noted a trend where patients dropped out after consenting or were too weak or too sick to participate, especially from the SAP group. Presentation at the hospital was also usually delayed and this could be attributed to the socio-economic state of the patients who tend to delay seeking treatment. To circumvent this challenge, where applicable (especially for the SAP group), the results presented here have been discussed with inferences to supporting literature and further work with expanded numbers is planned.

Possible concerns about treatment affecting the expression of *CCR8* and IL-6 are valid. However, the general treatment guideline for AP in the hospital unit is based on supportive care where all patients are treated according to the same protocol, none of which can influence immune responses. In mild AP, only analgesia and fluids are prescribed and nutrition is maintained with a combination of enteral and/or parenteral feeding. In the Moderate and severe group, organ support is implemented depending on the organ dysfunction. Antibiotics nor steroids are used routinely in the first phase of the inflammatory response in any of the patients and as such, we do not think that the treatment will influence the expression of IL-6 or CCR8 up to and including day 7.

CONCLUSION

This study proposes possible linkages between the upregulation of *CCR8* and IL6 elevation with AP disease severity. Simultaneously, monocytes, ILCs, and Th2 Lymphocyte frequencies, found to differentiate MAP, may differentiate MSAP and SAP groups. These findings may be beneficial as prognostic parameters in early AP stratification. Despite the limitation in sample sizes, these preliminary findings are supported by the literature. The data indicate that *CCR8*, IL-6 Levels, and associated immune molecules and cell types may be promising parameters to improve or complement existing ones for patient risk stratification in AP. The data further contributes to the scarce literature in AP from an African setting.

ARTICLE HIGHLIGHTS

Research background

Chemokine receptor 8 (*CCR8*) is a chemokine receptor that is highly expressed on monocytes and cells of T helper type-2 Lineage including innate lymphoid cells group 2 and 3 (ILC2 and 3). Upregulation in more severe cases of acute pancreatitis (AP) may be linked to elevated levels of interleukin (IL)-6 and upregulation of *CCR8*.

Research motivation

There is currently no known treatment for AP and no clear early immune markers to effectively distinguish between moderately severe AP and severe AP. The complex underlying pathophysiology further complicates this, necessitating studies to better understand the ensuing immune responses for improved stratification.

Research objectives

To identify the role of the CCR8, expressed by Th2 Lymphocytes and peritoneal macrophages, and its possible association to IL-6 as early markers to assist with AP stratification.

Research methods

A total of 40 patients were recruited from the Chris Hani Baragwanath Hospital and the Charlotte Maxeke Johannesburg Academic Hospital in Johannesburg, South Africa. Bioassays were performed on 29 patients consisting of 14 mild AP (MAP), 11 moderately severe AP (MSAP), and 4 severe AP (SAP) and 6 healthy controls as part of a preliminary study. A total of 12 mL of blood samples were collected at Day (D) 1, 3, 5, and 7 post epigastric pain. Using multiplex immunoassay panels, real-time polymerase chain reaction (RT-PCR) arrays, and multicolour flow cytometry analysis, immune response-related proteins, genes, and cells were profiled respectively. The fold change (FC) analysis was used to determine differences between the groups.

Research results

This study shows possible linkages between increasing *CCR8* expression and severity in mainly MSAP patients when compared to MAP. The concentration of IL-6 was significantly different at D3 post epigastric pain in both MAP group and MSAP group with $P = 0.001$ and $P = 0.013$ respectively, in a multiplex assay. *CCR8* was shown to increase with severity with the following FC for MAP (1.33), MSAP (38.28) to SAP (1172.45). Further verification studies using RT-PCR showed fold change increases of *CCR8* in MSAP and SAP ranging from 1000 to 1000000 times when represented as Log_{10}, compared to healthy controls respectively at Day 3 post epigastric pain.

Research conclusions

Notable increases in *CCR8* and IL-6 in severe patients were observed. Lymphocyte and monocyte cell frequencies suggest that in MAP, IL-6 was highly expressed in lymphocytes, and the severe patients (MSAP and SAP) were highly expressed by monocytes. This provides an avenue for exploring AP stratification to improve management.

Research perspectives

There is an opportunity to further investigate IL-6 producing cells such as T helper 2 lymphocytes, monocytes, and innate lymphoid cells group 2 and associated *CCR8*

increases, to determine cell-associated cytokine as a novel approach for AP risk stratification.

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ORIGINAL ARTICLE

Observational Study

Prediction of hereditary nonpolyposis colorectal cancer using mRNA *MSH2* quantitative and the correlation with nonmodifiable factor

Tjahjadi Robert Tedjasaputra, Mochammad Hatta, Muh Nasrum Massi, Rosdiana Natzir, Agussalim Bukhari, Rina Masadah, Muh Lutfi Parewangi, Prihantono Prihantono, Rinda Nariswati, Vincent Tedjasaputra

ORCID number: Tjahjadi Robert Tedjasaputra 0000-0002-6024-6200; Mochammad Hatta 0000-0002-8456-4203; Muh Nasrum Massi 0000-0002-3347-6529; Rosdiana Natzir 0000-0001-6444-7037; Agussalim Bukhari 0000-0002-6340-8615; Rina Masadah 0000-0002-1380-4759; Muh Lutfi Parewangi 0000-0001-9257-9344; Prihantono Prihantono 0000-0002-8247-0457; Rinda Nariswati 0000-0002-0758-5991; Vincent Tedjasaputra 0000-0003-3217-0285.

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Institutional review board statement: The study follows the international review ethical board with approval from the Hasanuddin University Ethics Committee (Indonesia).

Tjahjadi Robert Tedjasaputra, Department of Internal Medicine, Tarakan General Hospital, Medical Faculty University of Hasanuddin, Jakarta 10720, DKI Jakarta, Indonesia

Mochammad Hatta, Department of Immunology and Biomolecular, Hasanuddin University, Makassar 90245, South Sulawesi, Indonesia

Muh Nasrum Massi, Department of Microbiology, Faculty of Medicine, University of Hasanuddin, Makassar 90245, South Sulawesi, Indonesia

Rosdiana Natzir, Department of Biochemistry Meidcal Faculty, University of Hasanuddin, Makassar 90245, South Sulawesi, Indonesia

Agussalim Bukhari, Department of Nutrition, Faculty of Medicine, Hasanuddin University, Makassar 90245, South Sulawesi, Indonesia

Rina Masadah, Department of Pathology Anatomy, Faculty of Medicine, Hasanuddin University, Makassar 20945, South Sulawesi, Indonesia

Muh Lutfi Parewangi, Department of Internal Medicine, Faculty of Medicine, Hasanuddin University, Makassar 20945, South Sulawesi, Indonesia

Prihantono Prihantono, Department of Surgery, Faculty of Medicine, Hasanuddin University, Makassar 90245, South Sulawesi, Indonesia

Rinda Nariswati, Department of Statistic, School of Computer Science, Bina Nusantara University Jakarta, Jakarta 11530, Indonesia

Vincent Tedjasaputra, American Association for the Advancement of Science (AAAS), Science and Technology Policy Fellow, Alexandria, VA 22314, United States

Corresponding author: Tjahjadi Robert Tedjasaputra, MD, PhD, Consultant Physician-Scientist, Doctor, Senior Lecturer, Department of Internal Medicine, Tarakan General Hospital, Medical Faculty University of Hasanuddin, Jl. Gunung Sahari XI Dalam 70B RT8 RW 3, Jakarta 10720, DKI Jakarta, Indonesia. rtedjasaputra@yahoo.com

Abstract

BACKGROUND

Hereditary non-polyposis colon cancer is a dominantly inherited syndrome of

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colorectal cancer (CRC), with heightened risk for younger population. Previous studies link its susceptibility to the DNA sequence polymorphism along with Amsterdam and Bethesda criteria. However, those fail in term of applicability.

AIM

To determine a clear cut-off of *MSH2* gene expression for CRC heredity grouping factor. Further, the study also aims to examine the association of risk factors to the CRC heredity.

METHODS

The cross-sectional study observed 71 respondents from May 2018 to December 2019 in determining the CRC hereditary status through *MSH2* mRNA expression using reverse transcription-polymerase chain reaction and the disease's risk factors. Data were analyzed through Chi-Square, Fischer exact, t-test, Mann-Whitney, and multiple logistics.

RESULTS

There are significant differences of *MSH2* within CRC group among tissue and blood; yet, negative for significance between groups. Through the blood gene expression fifth percentile, the hereditary CRC cut-off is 11059 fc, dividing the 40 CRC respondents to 32.5% with hereditary CRC. Significant risk factors include age, family history, and staging. Nonetheless, after multivariate control, age is just a confounder. Further, the study develops a probability equation with area under the curve 82.2%.

CONCLUSION

Numerous factors have significant relations to heredity of CRC patients. However, true important factors are staging and family history, while age and others are confounders. The study also established a definite cut-off point for heredity CRC based on mRNA *MSH2* expression, 11059 fc. These findings shall act as concrete foundations on further risk factors and/or genetical CRC future studies.

Key Words: Colorectal cancer; *MSH2* gene; Non-modifiable factors; Risk probability

Core Tip: The study has determined a definitive cut-off for grouping colorectal cancer (CRC) with its heredity using the *MSH2* mRNA gene expression, which amounts to 11059 fc. The gene expressions may differ between blood and tissue sample of the CRC group, yet none between CRC and control group. Nevertheless, subsequent risk factors of family history and staging are found to be significant toward the heredity. The after-mentioned risk factors act as urgent reminder for highly risky people with family history of CRC and/or high CRC staging to have themselves and their immediate family members to undergo routine examinations as well as strict preventions.

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INTRODUCTION

Colorectal cancer (CRC) or also known as colorectal adenocarcinoma is a group of cancer that manifest in the colon and/or rectum. The cancer first arises as polyps that comprised of extraneous cells from the uncontrolled proliferation because of genetic

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mutations. These benign masses of flesh will then achieve even greater hyper-replication, survival, and angiogenesis, leading to a malignant carcinoma (CRC) which then can metastasize[1].

In fact, CRC holds the fourth place in the top ten of the most diagnosed global cancer with around 2000000 incidence cases, while taking third place for worldwide cancer mortality with approximately 1000000 deaths[2]. Within very high human development index countries, Hungary and Norway have the highest age-standardized rates of CRC over 100000 populations for male and female respectively (70.6 and 39.3)[3]. Meanwhile, the top age-standardized death rates are taken both for male and female by Hungary with 31.2 and 14.8 over 100000 population[3].

Specifically, CRC can be further divided according to its differentiation, epidemiology, and hereditary. Interesting potential lies dormant in CRC hereditary status, where as far as the author's known, there has been no gold standard measure to classify one's CRC into hereditary [*i.e.*, hereditary non-polyposis colon cancer (HNPCC)] or sporadic.

HNPCC or sometimes known to the general citizens as lynch syndrome (LS) is a hereditary mutation of the *MLH1*, *MSH2*, *MSH6*, *EpCAM*, and *PMS2* genes which contribute to the development of CRC yet also the passing of the autosomal dominant mutated genes and thus the heightened susceptibility to the offspring[4]. LS can be found in younger people compared to overall CRC as these mutations provide grounds for CRC rapid development. Within his or her lifetime, the risk of developing LS is around 4.1%-4.4%[5].

The incidence of LS can be said to mainly comes from the mutation of *MSH2* gene on chromosome 2. The protein translated by that subsequent gene encode MutS Homolog 2 protein which functions as a DNA mismatch repair protein. When doing its intended functions, it bonds with MSH6 or MSH3 to procure MutSα or MutSb complex according to specifications of the DNA damage, namely: Transcription repair, base excision repair, homologous recombination, *etc*[6-8].

Identification of those who carry the mutated genes of *MSH2* or the groups would benefit the patients as early detection and adequate prevention can reduce morbidity, mortality, and recurrence risk of LS[9,10]. Several studies have tried to implement the Bethesda and Amsterdam criteria to solve this dynamic screening of LS, yet the effort failed due to its complexity and arduousness especially in small family and late age of onset settings[11-15]. Consequently, when trying to address the problem from its roots of the mutated genes, establishment of the definite gene expression may act as a well cut-off point to categorize CRC into LS or non-LS with high potential of becoming a gold standard measure. The subsequent practices utilize specific enzymes that pinpoint the post-transcription mRNA strategically[16].

These techniques of separating the CRC intro groups, enable risk factor assessments toward hereditary and sporadic CRC types. Modifiable risk factors for LS and CRC are body mass index (BMI), physical activity, diet, lifestyle (*i.e.*, smoking, alcohol, and narcotics), routine medications, and diabetes mellitus (DM). Insulin resistance and the hyperglycemic state of the body can predispose a person to CRC. The excess of blood sugar trigger Warburg effect (carcinogenic glycolysis) through modulation of glucose metabolism[17,18]. Studies on DM relations to CRC prove a 1.17-1.42 hazard ratio along with 11536/559375 DM patients have CRC[19,20]. On the other hand, non-modifiable risk factors for LS and CRC involve the race, age, gender, heredity, radiations, and some diseases (*i.e.*, inflammatory bowel disease or cystic fibrosis).

Above all, the hazardous nature of CRC and LS, scarce information on CRC risk factors identifications, and the lack of gold standard for categorizing hereditary measurements, thus the present study urgently aimed to compute suitable *MSH2* gene expression for appropriate cut-off and certify the associations from the risk factors.

MATERIALS AND METHODS

Study population and subject enrollment

The present study was conducted using cross-sectional design with 71 respondents divided into 31 respondents in the control group, those who have been sequentially matched (*i.e.*, age, sex, and BMI) with the case group. The study involves tumor and malignancy sector which are a sensitive section of health as it rapidly deteriorates health while integrated to other bodily system. Henceforth, strict exclusion criteria were adapted in the current study, namely: (1) The presence or history of other cancer; (2) The presence or history of inflammatory bowel disease; (3) Chemotherapy or radiotherapy in progress or history; (4) Refusal of participation; and (5) Missing or

incomplete data.

From May 2018 to December 2019, the respondents are taken from the internal medicine outpatient and inpatient department of Tarakan General Hospital and Siloam Hospitals Lippo Village through consecutive sampling. Sample size calculated using 5% alpha and power 80%.

Research operatives

Three major steps are contained within this study. Initially, the study collects respondents and their clinical data of demographics and malignancy characteristics. Then, the respective mRNA gene expression of *MSH2* was quantified using polymerase chain reaction (PCR) and studied in assessing within groups and between groups differences as well as its hereditary significance. Whereas the last component of the study involves risk factor analysis toward hereditary of CRC and its probability model.

Sample collection and data measurement

Biopsy tissues of suspected CRC tissues and venous blood are the key samples of this study. Then, the samples are placed in a L6 buffer and have their RNA extracted. The L6 buffer are concocted earlier according to the RNA Boom extraction method of the Hasanuddin laboratory. Next, RT-PCR targeting the *MSH2* mRNA were done to measure the gene expression.

The PCR are conducted through the DNA multiplication, denaturation, primer attachment, and amplification stage. Specific Korean primers are supplied to specifically target the *MSH2* gene: CAT-CCA-GGC-ATG-CTT-GTG-TTG-A (forward) and GCA-GTC-CAC-AAT-GGA-CAC-TTC (reverse). The mechanics and PCR analysis follow the Bio-Rad protocols from Unites States of America using the power SYBR green master mix kit[21-23].

Statistical analysis

Data tabulation was done through Microsoft Excel 365, while SPSS v26 is the software of choice for the statistical analysis. Missing data is excluded from the study. The respondents' demographics are characterized using descriptive statistics; yet, Chi-Square or Fischer test are applied for categorical factors, while t-test or Mann-Whitney for numerical factors. Significance obtained if P value < 0.05.

RESULTS

The present study employs 71 respondents among the 19-mo study period, which comprised of 56.34% in the case group and 43.66% in the control group. Respondents within the control group are adequately matched according to the case group characteristics, proven with no significant difference (P > 0.05) in the demographic characteristics between the groups as depicted in Table 1.

Among the respondents on the CRC group, histopathological samples are taken and observed. Specifically, 90.00% respondents have adenocarcinoma while 7.50% have adenocarcinoma with Signet ring cell and 2.50% have neuroendocrine carcinoma. Nevertheless, specifications on the histopathological profile can also be seen from the level of differentiation. Well-differentiated biopsies are found in 26 (66.7%) respondents, intermediately differentiated in 6 (15.4%) respondents, and poorly differentiated in 7 (17.9%).

Subsequently, the study utilizes PCR analysis to measure the *MSH2* mRNA gene expression in blood and tissue between the groups. Significance is observed when comparing the gene expression within the CRC group between blood and tissue (12554.50 *vs* 7485.00). However, as pictured in Table 2, there is no significant difference of MSH2 mRNA expression between CRC and control groups (P = 0.116 and 0.465).

Moreover, the group with CRC were then subdivided based on each respondent hereditary status. One is considered having hereditary condition if his or her blood mRNA *MSH2* gene expression less than the cut-off from the fifth percentile, 11059 fc. It was established that 67.50% of the CRC group respondents have non-hereditary status, even including one-third of those with positive family history of CRC. Likewise, Table 3 portrayed the relationship of risk factors to hereditary status.

CRC has numerous substantial risk factors in theory. However, within the 40 CRC group respondents, only three factors are deemed essential hereditarily: Age, tumor staging, and family history. Uniquely, among all locations and proximity potential for CRC, hereditary does not hold any significance (P = 0.595 and 0.476). There is also no

Table 1 Baseline characteristic and *MSH2* gene expression

Characteristic	CRC group	Control group	*P* value
Age (yr)	56.8 ± 8.4	51.6 ± 13.4	> 0.05
Sex			
Male	21 (52.5)	13 (41.9)	
Female	19 (47.5)	18 (58.1)	
Body mass index (kg/m^2)	22.4 ± 3.3	23.6 ± 3.4	

CRC: Colorectal cancer.

Table 2 *MSH2* gene expression

MSH2 expression	CRC group	Control group	*P* value
Blood			
Median (range)	12554.50 (4230.00-14559.00)	12146.00 (11029.00-13633.00)	0.116
mean ± SD	11411.05 ± 2912.45	12219.87 ± 756.87	0.465
Tissue	7 485.00 (4174.00-14218.00)		

CRC: Colorectal cancer; SD: Standard deviation.

difference on biopsies differentiation between the groups (P = 0.287 and 0.999).

The study found 5.60 times increase in risk of CRC between those < 50 years old and over, in which a 9.05-year difference is found between the subsequent groups. Similarly, respondents with hereditary CRC are mostly within the C stage (54.85%) while the non-group mostly in B (37.04%). This pattern holds true even when the stages are divided into C-D and A-B clusters, where the CRC group dominate the former cluster while the latter cluster for the rest. Yet, the clustering of stages is insignificant (P = 0.116).

Family history of CRC and its hereditary follow a significant linear relationship (P = 0.008). Those who has history of CRC in his/her family majorly belong to the hereditary group (61.54%) and vice versa. There is also a notable risk increase for those who has CRC history amounting to 9.20 times than those who don't.

Bringing further to multivariate perspective, the current study applies multiple logistic analysis to find the truly significant risk factors toward CRC hereditary and its subsequent probability. Table 4 explain the regression where staging and family history are truly significant (P = 0.034 and 0.006), while age is just confounder. The unstandardized coefficients can be morphed to a LOGIT and probability functions of CRC hereditary as follows (Eq. 1 and 2).

$$\text{LOGIT} = -3.165 + 2.395 \times \text{staging} + 3.126 \times \text{history} \quad \text{(Eq. 1)}$$

$$\text{Probability} = \frac{1}{1 + e^{-\text{LOGIT}}} = \frac{1}{1 + e^{-(-3.165 + 2.395 \times \text{staging} + 3.126 \times \text{history})}} \quad \text{(Eq. 2)}$$

The variables of staging and history hold the value of either one or zero. Representatively, score of one amount to C or D in the staging component and presence of family history. Furthermore, the equations and probabilities are having good fit and not due to chance by having significant Hosmer and Lameshow statistics as well as 82.2% area under the curve (AUC) for receiving operator curve (ROC) (Figure 1).

DISCUSSION

From May 2018 to December 2019, 71 respondents were collected with 56.34% prevalence of CRC among the pre-elderly population (45-59 years old). The CRC group is predominantly male (52.5%) and classified with normal BMI. The discrepancy also found on similar studies in the Asia region (*i.e.*, Japan, China, Korea, and Hong Kong) where CRC has 20.7-64.8 incidence rate over 100 thousand populations, in which differ by 6.3-28.1 compared to the female incidence rate[24-27]. The theory on hormonal difference between gender is suspected to be the leading cause of the CRC

Table 3 Colorectal cancer hereditary risk factors

Factor	CRC hereditary, yes (n: 13)	CRC hereditary, no (n: 27)	OR (95%CI)	*P* value
Age	50.69 ± 14.99	59.74 ± 11.68		0.043
< 50	8 (61.54)	6 (22.22)	5.60 (1.33-23.62)	0.031
> 50	5 (38.46)	21 (77.78)		
Gender			0.69 (0.18-2.59)	0.826
Male	6 (46.15)	15 (55.56)		
Female	7 (53.85)	12 (44.44)		
Location			-	0.595
Caecum	1 (7.69)	1 (3.70)		
Ascending colon	2 (15.38)	4 (14.81)		
Transverse colon	2 (15.38)	2 (7.41)		
Descending colon	0 (0.00)	1 (3.70)		
Sigmoid	3 (23.08)	7 (25.93)		
Rectum	5 (38.46)	12 (44.44)		
Proximity			1.79 (0.44-7.32)	0.476
Proximal colon	5 (38.46)	7 (25.93)		
Distal colon	8 (61.54)	20 (74.07)		
Staging			-	0.020
A	0 (0.00)	7 (25.93)		
B	4 (30.77)	10 (37.04)		
C	7 (54.85)	9 (33.33)		
D	2 (15.38)	1 (3.70)		
Group staging			3.83 (0.93-15.72)	0.116
C-D	9 (69.23)	10 (37.04)		
A-B	4 (30.77)	17 (62.96)		
Family history			9.20 (1.97-42.97)	0.008
Yes	8 (61.54)	4 (14.81)		
No	5 (38.46)	23 (85.19)		
Differentiation[1]				
Poor	4 (30.77)	3 (11.54)	2.52 (0.46-13.80)	0.287
Intermediate	0 (0.00)	6 (23.08)	-	0.999
Well	9 (69.23)	17 (65.38)		

[1]Loss of differentiation data in one sample. CRC: Colorectal cancer; OR: Odds ratio; CI: Confidence interval.

dominancy in male.

CRC higher prevalence in the male gender is the result of female protectiveness from the disease. The sex hormone of estradiol and progesterone acts as protective mechanism for CRC development in the body. The hormonal clinical trial in 2019 observe that introduction of estradiol and progesterone combination treatment provide increased apoptosis of tumor cells ($P < 0.05$) while lowering tumor cell proliferations ($P < 0.01$)[28]. Aside from its function as a sexual hormone, estrogen or estradiol play an important role in cell turnover. It acts as a bridge for ion transport that regulates cells' pH, intracellular ions, and several protein activations[29]. Estrogen also exudes anti-inflammatory properties due to its ability to bond and modulate leukocytes including natural killer cell, neutrophils, dendritic cells, *etc*[30].

Table 4 Multivariate regression of colorectal cancer hereditary risk factor

Factor	B	SE	*P* value	OR (95%CI)
Staging	2.395	1.130	0.034	10.970 (1.199-100.382)
History	3.126	1.143	0.006	22.784 (2.423-214.273)
Constant	-3.165	1.094	0.004	0.042

SE: Standard error; OR: Odds ratio; CI: Confidence interval.

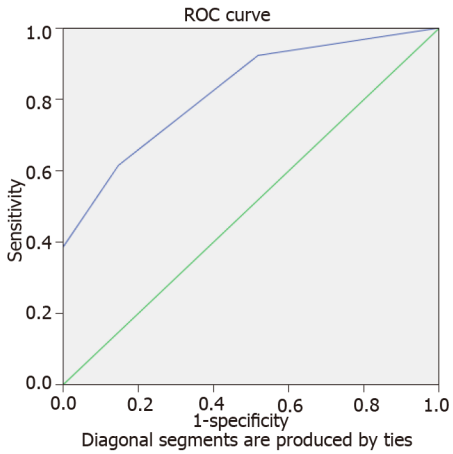

Figure 1 *MSH2* colorectal cancer hereditary probability receiving operator curve. ROC: Receiving operator curve.

Moreover, associations are observed of body anthropometry to CRC within many global and Asian studies. For instance, a Japanese prospective study finds significant difference of BMI to colon cancer respondents (P = 0.004-0.007)[31]. Akin notion is discovered in a Korean study where every five cm increment in height increase the risk for CRC in men by 1.04-1.06 and in women by 1.00-1.08[32]. Both after multivariate control with other factors. Another Korean study also view significant association of BMI to CRC especially the distal colon and rectal cancer (P < 0.001 and 0.016)[33]. These trends are not restricted only to the Asian region. A global meta-analysis obtains a relative risk of 1.25 (1.18-1.32) for CRC with the highest *vs* lowest height[34]. Likewise, a prospective systematic review corroborates the idea where CRC's risk multiply by 1.06 for every 5 kg/m^2 increase in BMI[35].

BMI has indirect relations to CRC. Higher BMI equates to higher number of cells and tissues in the body, giving rise to higher chance of genetic mutations and malignancy. People with higher stature also found to have longer intestine length than others (r: 0.827), leading to more proliferation rate and chance of cancer[36]. Further, CRC can be influenced by other diseases. Acromegaly and insulin-like growth factor abnormality for example procure modulation in body height, BMI, and thus CRC[37-39].

With the advancement of technology in healthcare and information, genetic studies for diseases' prevention, detection, and treatment have developed in a rapid pace. Specimens with DNA or RNA materials of the patients or family can be analyzed to account for the disease. This methodology has been implemented for several diseases [9]. Representatively, in assessing the hereditary status of a given CRC patient, his or her mRNA gene expression can be compared to a defined cut-off, where ≥ equates to positive status. Henceforth, the study uses 5th percentile cut-off points which are often used in M, C, D, and A statistic of circular data in a wrapped Cauchy distribution[40]. Although significant dissonance is present between blood and tissue samples in the CRC group, there are no significant difference between the first, third, and fifth percentile of *MSH2* gene expression which leads to the acceptance of utilizing 5th percentile cut-off amounting to 11059 fc. Consequently, 32.5% of CRC patients are categorized to the hereditary group. The rate supports the discovery of 22% hereditary CRC by Chang *et al*[41].

Table 3 provide risk factors assessment between the hereditary and sporadic CRC groups. Significant risk factors fall on the age, staging, and family history. The sporadic CRC has older patients than the counterpart with Δ: 9.05 years and 77.78% proportion for those > 50 years old. Generally, age of 40 years old and over has significantly higher incidence of CRC[42]. A 2018 study paints that there is a sharp increase in CRC age-specific incidence as early as 35, then the pre-elderly age of 35-64 [Δ: 60.2 (male) and ± 35 (female)], and over 65 years old [Δ: 237.5 (male) and 131.4 (female)][24]. Nonetheless, Yurgelun *et al*[43] describe that LS mutation carriers have significantly younger age at CRC diagnosis with Δ: 11.1 years ($P < 0.001$)[43]. HNPCC specifically has only ± 20% probability to develop around the age of 50 and 50% for 70 years old or above[44].

Age is an unfortunate risk for malignancy. The older a person is getting, he or she accumulate a lot of endogenous factors (*i.e.*, diet, chronic inflammation, metabolism, waning immune system, *etc.*) and exogenous factors (*i.e.*, genotoxins, mutations, medications, environmental triggers, *etc.*) which stimulate oxidative stress and reactive oxygen species that initiate DNA damage, mutations, and uncontrolled cell growth [45]. The body proinflammatory state as time passes also become a progressive breeding ground for malignancy[46].

Insignificant relations are found in gender, tumor location, and histological differentiation. The hereditary CRC are predominated by female (53.85%) while the sporadic by male (55.56%) yet the difference is negligible ($P = 0.826$). This is consistent with earlier studies where Dominguez-Valentin *et al*[47] ascertain that in earlier years of elderly age, *MSH2* carries similar risk of CRC in terms of gender[47]. Further, this ascertain that even though female is protected from overall CRC due to its hormonal effect, there is no importance to the heredity status of the CRC.

Both hereditary and sporadic CRCs tend to be in the distal colon (61.54% *vs* 74.07%), especially the rectum (38.46% *vs* 44.44%). Supremacy of CRCs in the rectum also seen in a 2020 general hospital study where rectal CRC amounts to 61.8% prevalence[48]. Yet the locations are inessential to the heredity status ($P = 0.476$). Theory upon this predicament include the intrinsic and extrinsic factors within everyone. Carethers[49] in his 2018 study disclose that the overall risk of CRC and the CRC risk of different regions of the colon are affiliated to one's physical activity, gender, height, BMI, smoking status, alcohol intake, diabetes, medications, and hormonal therapy[49]. For example, physical activity reduces the overall CRC risk and the proximal colon CRC risk; while increase in height do not affect the rectal specific location while profoundly heightening risk of overall, proximal, and distal locations.

Likewise, no significance can be seen between histopathological differentiation and CRC heredity status. Current study observes higher poorly differentiated CRC in the hereditary compared to the sporadic group (30.77% *vs* 11.54%), which is akin to the study by Sun dictating that HNPCC features prominent lymphocyte infiltrations and RER+ status, which easily translates to poor differentiation and resulting in more within the HNPCC compared to the sporadic group[50]. On the contrary, the heredity group also has more well differentiated specimens (Δ: 3.85%). The contrast may be due to amounts of proteins and cytokines within the tissue. The tissue staining with chromogranin A produce significant difference of 13.6% between hereditary and sporadic group[50]. The positive staining with after-mentioned stain has high correlation with tumor's grade and stage[51,52]. Meanwhile, the dissonance may also happen due to defects on the sample when taken by colonoscopy biopsy as opposed to a surgery.

Independent staging of the CRC produces significant results between hereditary and sporadic groups ($P = 0.020$), with the former mostly in stage C (54.85%) and the latter in stage B (37.04%). The findings contradict data by Yurgelun *et al*[43], where most LS mutation carriers are in stage II (45.5%)[43]. Difference may occur due to the health system flaw in detecting cancer and the pathophysiology of the CRC.

HNPCC or LS is very hard to be detected as a cancer diagnosis must precede the genetic diagnosis. Even in the developed country of United States, only < 1% of the Americans with LS know about the disease presence[53,54]. The rate may lessen in Indonesia where technology is not as advanced in the United States, genetic testing is not a routine test and very expensive, as well as Indonesians' tradition to not seek the healthcare center if there are no symptoms or still bearable.

Incidentally, family history has linear relationship to the hereditary *vs* sporadic type of CRC ($P = 0.008$). Hereditary CRC majorly has positive history patients (61.54%) while the sporadic group doesn't (14.81%). The conditions amount to 9.20 (1.97-42.97) times increase of risk in developing hereditary CRC when one has family history of CRC. HNPCC is an autosomal dominant disease leading to its presence in every generation of the familial generation, as a dominant trait will always be expressed

Table 5 Scenarios of colorectal cancer hereditary probability

Scenario	Family history[1]	Staging[2]	LOGIT[3]	Probability[4]
1	1	1	2.356	0.913
2	1	0	-0.039	0.490
3	0	1	-0.770	0.316
4	0	0	-3.165	0.041

[1]1: Yes, 0: No.
[2]1: C or D, 0: A or B.
[3]LOGIT: -3.165 + 2.395 × staging + 3.126 × history.
[4]Probability: $1/(1 + e^{-LOGIT})$.

according to the mendelian law of inheritance. Simultaneously, similar relationship also observed in a 2017 LS study where LS mutation carriers have $P < 0.001$ for all first- and second-degree family history of CRC[43].

Multiple regression control of the factors demonstrates that staging and family history is truly significant ($P = 0.034$ and 0.006) while age just a confounder. The analysis then developed applicative equations (Eq. 1 and 2) to predict the heredity of CRC, where examples of their usage are listed in Table 5. The analysis has a satisfactory fit criteria with significant Lemeshow and adequate AUC.

Subsequently, the probability prediction model of the current study can be one of the prospective tools to overcome the weaknesses of the Amsterdam and Bethesda criteria. Personal family history and Mendelian family genogram are important for the diagnosis of Hereditary CRC, with the Amsterdam and Bethesda criteria being the standard diagnosis tools for LS. However, those tools often face difficulties, especially for smaller families and late age of disease onset[11-15,55,56]. In addition, individual specific genotype and environmental traits assessment may be utilized to overcome the hurdle of empirical recurrence risk removal because of its impracticality in incomplete penetrance and late onset[11-15]. Meanwhile, aside from the practical probability prediction model of the present study, mRNA *MSH2* gene expression can be used through Bayesian theorem with prior pedigree risk modifications and conditional information.

Nevertheless, the limitation of the present study includes the un-generalization of the study sample**.** Participants are taken from the hospitals which indicates the possibility of selection bias and unrepresentativeness of the public. Future studies should determine whole genome sequencing to validate these findings and establish a gold standard for Hereditary CRC.

CONCLUSION

Numerous factors have significant relations to heredity of LS CRC patients. However, true important factors are staging and family history, while others (age) are confounders. The study also established a definite cut-off point for heredity LS CRC/HNPCC based on mRNA *MSH2* expression, 11059 fc. These findings shall act as concrete foundations on further risk factors and/or genetical LS CRC future studies.

ARTICLE HIGHLIGHTS

Research background

The lack of golden standard for categorizing hereditary status of colorectal cancer (CRC) poses diagnostic and management problems. Identifying proper techniques is urgent to procure the best care, prevention, risk factors management, and treatment of CRC be it hereditary or sporadic, along with judicious resource consumption.

Research motivation

The lack of golden standard leaves a gaping hole in the LS CRC healthcare system. Previous guideline of Bethesda and Amsterdam have tried yet fail in the applicability

area especially with later age onset and smaller family. These coupled with the hazardous nature of CRC or lynch syndrome and scarce information on CRC risk factors identifications motivate the authors to commence the present study.

Research objectives

To determine the gold standard cut-off of *MSH2* gene expression for hereditary cluster as well as to identify and examine the relationship of hereditary non-polyposis colon cancer (HNPCC) with its non-modifiable risk factors.

Research methods

Consecutive sampling of the hospital internal medicine patients with CRC provides the case group. Then, the control group was concocted by matching the characteristics of the case group. *MSH2* mRNA was then analyzed through blood and tissue collection and reverse transcription-polymerase chain reaction. Further, the gene expression cut-off determined using percentile technique akin to Cauchy distribution of M, C, A, and D circular data statistics. CRC groups then clustered into hereditary and sporadic according to the *MSH2* gene expression against the cut-off. Lastly, risk factors are contrasted between each cluster and developed into a prediction model.

Research results

In a group of 40 CRCs differentiated into 13 hereditary and 27 sporadic through MSH2 mRNA cut-off in 11059 fc, significant risk factors for the hereditary CRC are family history and staging with (OR: 22.784, 95%CI: 2.423-214.273, P = 0.006; OR: 10.970, 95%CI: 1.199-100.382, P = 0.034). Moreover, a prediction model is concocted with area under the curve 82.2%.

Research conclusions

The cut-off of *MSH2* mRNA 5th percentile provided rough clustering of hereditary and sporadic CRC groups. Significant risk factors toward HNPCC are family history and staging, while age is just a confounder.

Research perspectives

Future research directions include validation of the determined cut-off and reliability testing of the risk factors in a bigger sample size and/or with the general population. Further, a longitudinal study on the risk factors effects should be evaluated.

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