Evaluation of circulating host- and pathogen-derived markers of infection and inflammation in the laboratory diagnosis of sepsis.

By

Moliehi Potjo

Submitted in fulfilment of the requirements for the degree

Of

Doctor of Philosophy

In

The Department of Immunology

Faculty of Health Sciences

University of Pretoria

November 2017

DECLARATION

I, Moliehi Potjo, declare that the work contained in this dissertation is my own original work and has not been presented for a degree at any other institution. I have performed the following tests: measurement of circulating cytokines, C-reactive protein (CRP), high mobility group box protein 1 (HMGB1), soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), procalcitonin (PCT), matrix metalloproteinase-9 (MMP-9), neutrophil gelatinase-associated lipocalin (NGAL), fungitell assay for (1-3)-β-D-Glucan (BG) and a multiplex PCR for detection of pathogen-derived deoxyribonucleic acid . The sTREM-1 results were shared with Dr N. N. Sipholi from the Department of Internal Medicine, University of Pretoria who provided the patients' blood samples and clinical data for me. This dissertation is submitted in fulfilment for the PhD degree at the University of Pretoria.

Signed: _____ Date:_____

PUBLICATION

Potjo, M., Theron, A., Cockeran, R., Sipholi, N.N., Steel, H., Bale, T.V., Meyer, P., Anderson, R. & Tintinger, G.R. Interleukin-10 and interleukin-1 receptor antagonist distinguish between patients with sepsis and SIRS, has been submitted for consideration for publication in the "Journal of Infection in Developing Countries" .

ACKNOWLEDGEMENTS AND DEDICATION

This journey has not been easy, but it could not have been better. First and foremost I would like to thank **God Almighty** for guiding me through this journey, for giving me strength to carry on even when my body and mind wanted to let go. I am truly humbled.

I would like to thank everyone that has in some way been involved in my PhD journey within the Immunology Department and beyond. I would not have made it without you.

Special thanks go to:

Prof. R. Cockeran and Prof. G.R. Tintinger: My mentors thank you for taking a chance with me and making me a part of your research group. I have learned from your scientific knowledge and have become a better person under your supervision.

Prof. R. Anderson: Your door is always open to come in and ask questions, however big or small they might be. You are a good role model in the world of research and science.

Prof. A. J. Theron: Thank you for your caring personality, you are always willing to give a helping hand and do so with a smile on your face.

Dr P.W.A. Meyer: My co-supervisor thank you for all your guidance and your sense of humour, sometimes that's all one needed.

Dr N. N. Sipholi: Thank you for collecting all the clinical data and blood samples, you made it happen.

Dr H.C. Steel: Thank you for your guidance with the cytokine work.

Ms T. V. Bale: Thank you for being a friend, and a sister I never thought I had; we have gone through a lot together in the past few years, both in lab-life and private life, tears and laughter. I can share anything with you and you always manage to make me feel good about myself. Indeed I am stronger now "PPC".

My dear **team Crazzzyy:** Thanks for all the gossip and fun moments - you guys are very special in your own ways. You always support me when I am most in need, whether it is listening to my complaints or motivating and cheering me up.

My **wonderful family**: In the memory of my father **Mokhothu**, surely you will be smiling now. May his soul rest in peace. To my mother, **Matseliso**, thanks a million, if it was not for your prayers I would not be where I am today. You are a gift to be treasured. To my sister **Nthabiseng** and my brother **Tumelo**, you may not know it, but you guys are the best. Special thanks to my sister **Melita**, you are a pillar of strength and you are always there to listen to and comfort me. I know that often you became more of a dumping site for me, but hey! I am not complaining, you kept me going my dear. Last but not least, to my Husband **Paulos**, thank you for all the support and patience, it really means a lot to me.

The entire thesis is dedicated to my two precious children, **Tlhompho** and **Keorapetse**. What is this life without you? Thank you for everything that you have done for me. You often endured loneliness while mommy was working on this thesis, but never complained. Love you always.

SUMMARY

Patients with sepsis need to be distinguished from those with systemic inflammation due to non-infectious causes (SIRS) so that empiric antimicrobial therapy can be administered timeously to those with sepsis. Consequently, the identification of host- and/or pathogen-derived biomarkers of infection or inflammation is important to identify patients with sepsis. The current study was designed to evaluate the potential of clinical parameters and circulating biomarkers to distinguish sepsis from SIRS.

Patients who presented to the Emergency Unit, medical wards and ICU at the Steve Biko Academic Hospital in Pretoria, South Africa, with systemic inflammation (SIRS) were included in the study. Clinical parameters, white blood cell count and platelets were measured for each patient. C-reactive protein (CRP), procalcitonin (PCT) and cytokine concentrations were quantified using laser immunonephelometry, immunoluminescence and a Bio-Plex suspension bead array system, respectively. Fungal infections were also investigated by (1-3)-β-D-Glucan (BG) and multiplex-PCR procedures. Samples of blood, sputum, peritoneal and cerebrospinal fluid were sent for microscopy and culture.

Based on available clinical information and the results of microbiological testing, patients were classified retrospectively into 2 groups, those with sepsis or SIRS. Sixty two patients were included in the study, 37 with sepsis and 25 with SIRS. Mean body temperature was higher in patients with sepsis, while blood pressure was lower in this group. The serum concentrations of CRP, PCT, interleukin (IL)-10 and IL-1Ra were significantly higher in patients with sepsis and associated, although not significantly so, with lower levels of IL-1β and IL-8, low platelet

counts ($p<0.05$) and increased IL-6 ($p<0.05$) and to a lesser extent IL-1Ra was associated with overall mortality in the combined group of patients, and IL-6 concentrations were higher in non-survivors compared to survivors. However, there were no significant differences between the two groups of patients with respect to the other biomarkers investigated. Disappointingly, the fungal identification results did not correlate with those of the multiplex PCR as 4 patients with positive PCR results had β-D-glucan values < 80 pg/ml, while 19 patients with β-D-glucan concentrations > 80 pg/ml were negative using the PCR technique.

In conclusion, relative to patients with SIRS, those with sepsis were found to have increased levels of the immunosuppressive/anti-inflammatory cytokines, IL-Ra and IL-10, consistent with a more intense counteracting anti-inflammatory response. The apparent association of increased and decreased levels of IL-1Ra and IL-1β respectively with mortality in both groups of patients appears to underscore the role of an adequate inflammatory response in promoting survival.

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AC	Adenylate cyclase
ACCP	American College of Chest Physicians
AIDS	Acquired immune deficiency syndrome
AKI	Acute kidney injury
APC	Antigen presenting cells
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BG	(1,3)-β-D-glucan
CRP	C-reactive protein
cAMP	Cyclic adenosine 3',5' monophosphate
CD	Cluster of differentiation
CLP	Cecal ligation and puncture
CoNS	Coagulase-negative *staphylococci*
COPD	chronic obstructive pulmonary disease
DAMPS	Damage-associated molecular patterns
DAP12	DNA activation protein 12
DCs	Dendritic cells
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid

DPO	Dual primary oligonucleotide
dsRNA	Double-stranded ribonucleic acid
ECs	Endothelial cells
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
galf	Galactofuranose
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM	Galactomannan
FasL	Fas ligand
HMGB1	High mobility group box 1 protein
ICU	Intensive care unit
Ig	Immunoglobulin
IFI	Invasive fungal infections
IFN	Interferon
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
LPS	Lipopolysaccharides
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemotactic protein 1
MMPs	Matrix metalloproteinases
MIP-1	Macrophage inflammatory protein 1
MODS	Multiple organ dysfunction syndromes
MOF	Multiorgan failure
MPO	Myeloperoxidase
MRSA	Methicillin-resistant Staphylococcus aureus
NADPH	Nicotinamide adenine dinucleotide phosphate
NGAL	Neutrophil gelatinase-associated lipocalin
NF-κB	Nuclear factor κB
NO	Nitric oxide
NLR	Nucleotide-binding domain and leucine-rich repeat
NOD	Nucleotide-binding oligomerization domain
PAI-1	Plasminogen activator inhibitor-1
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PCT	Procalcitonin
PKA	Cyclic AMP-dependent protein kinase
PRRs	Pattern-recognition receptors
RIG-1	Retinoic-acid-inducible protein 1

RLRs	RIG-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RV	right ventricular
SIRS	Systemic inflammatory response syndrome
SCCM	Society of Critical Care Medicine
SPP	species
SST	Serum-separating tubes
sTREM-1	Soluble triggering receptor expressed on myeloid cells-1
TAFI	Thrombin-activatable fibrinolysis inhibitor
TGF	Transforming growth factor
Th	T-helper cells
TLRs	Toll-like receptors
TIMPs	Tissue inhibitors of matrix metalloproteinases
TNF	Tumor necrosis factor
TREM-1	Triggering receptor expressed on myeloid cells-1
USA	United States of America
WBC	White blood cells

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. INTRODUCTION

Despite progress in the diagnosis and treatment of infectious diseases, sepsis remains a leading cause of morbidity and mortality in hospitalized patients (Martin 2012). Notwithstanding the lack of specificity and sensitivity of current clinical diagnostic criteria, the major obstacles to improved outcome are: i) difficulty in distinguishing between infective and non-infective causes of the systemic inflammatory response syndrome (SIRS); ii) in many cases, failure to identify the causative pathogens; iii) because of this, the use of empiric antibiotic therapy, which may, albeit inadvertently, exacerbate harmful inflammatory responses through the release of pro-inflammatory components resulting from dying bacteria; and iv) advanced immunosuppression during the later stages of SIRS, which limits the efficacy of antimicrobial agents (Lafreniere & Lehmann 2017).

In an effort to address these issues, this study is focused primarily on the following: i) evaluation of a range of circulating, host-derived biomarkers of inflammation and infection as a rapid, non-specific strategy to distinguish between infective and non-infective causes of SIRS, as well as to establish the degree of SIRS-related immunosuppression; and ii) evaluation of the diagnostic potential of PCR-based procedure for the detection of fungal DNA, as well as an enzymatic/colorimetric-based method for the detection of fungal cell wall polysaccharides.

1.1 SEPSIS DEFINITION

Throughout the ages, mankind has suffered from diseases caused by microbial pathogens. These pathogenic microorganisms often cause severe disease and significantly reduce life expectancy (Van Amersfoort, Van Berkel &

Kuiper 2003). Under normal circumstances, many bacteria live in coexistence with humans and do not cause diseases. Pathogenic microorganisms trigger the immune response if they or their components cross the barrier between the external and internal environment. Sepsis is defined as the systemic host response to an infection (Okazaki & Matsukawa 2009, Chen, Yin & Zhang 2011, Kibe, Adams & Barlo 2011, Coelho & Martins 2012, Berger et al. 2013), and is evident with the presence of infectious microorganisms in the blood (Daniels 2011). Very often in the clinical setting, sepsis and septicaemia are used interchangeably (Daniels 2011).

It was reported by Bone et al 1989 that "sepsis syndrome is associated with specific clinical symptoms and evidence of infection". However, these symptoms were frequently manifested in the absence of identifiable bacteria in the blood, as well as in non-infectious conditions, such as acute pancreatitis, ischaemia, immune-mediated organ injury and trauma (Balk & Bone 1989). A consensus conference held by the Society of Critical Care Medicine (SCCM) and the American College of Chest Physicians (ACCP) in 1992 resolved these discrepancies and introduced the term "systemic inflammatory response syndrome" (SIRS), for which pathological infection was required (Bone et al. 1992, Khilnani & Hadda 2009, Namas et al. 2012). Additionally, the terms "severe sepsis", "septic shock" and "multiple organ dysfunction syndromes" were introduced to differentiate between the various stages of disease (Khilnani & Hadda 2009).

Until very recently sepsis has been defined as the presence of microbial infection and physiological changes collectively referred to as SIRS (Okazaki & Matsukawa 2009, Khilnani & Hadda 2009, Gogos et al. 2010, Mancini et al. 2010,

Nachimuthu & Haug 2012, Dellinger 2013, Llewelyn et al. 2013), as shown in Table 1.1 (page 6). Severe sepsis is represented by organ dysfunction and hypoperfusion or hypotension in the presence of sepsis. As a result, acute respiratory distress syndrome (ARDS) and cardiac dysfunction, confusion and disseminated intravascular coagulation (DIC) are apparent as perfusion abnormalities arise during sepsis (Ntusi et al. 2010). Septic shock was defined as severe sepsis accompanied by hypotension unresponsive to fluid resuscitation (Martensson et al. 2013). Severe sepsis and septic shock are the most common causes of mortality among intensive care unit and post-surgical patients (Coelho & Martins 2012).

Multi-organ failure is often a consequence of severe sepsis and septic shock, and refers to the presence of altered function affecting more than one organ in critically ill patients, leading to an imbalance of homeostasis, which cannot be corrected without intervention. Multiple organ dysfunction syndromes (MODS) are either primary (directly attributable to a well-defined insult) or secondary (as a consequence of the host response) (Ntusi et al. 2010).

During sepsis, the inflammatory response to the pathogen is also evident at sites other than those of the primary infection. Exaggerated host responses to microorganisms, result in a widespread release of inflammatory mediators (Marshall & Reinhart 2009, Bernard &, Bernard 2012, Balk 2014). Although a compensatory increase in heart output maintains a reasonable blood pressure and adequate organ perfusion, the consequences of these events include a decrease in blood pressure eventually leading to multiple organ failure (Offord 2002), mostly involving the lungs, liver and kidneys and possibly resulting in the death of the patient.

Early initiation of antimicrobial treatment remains a cornerstone of successful therapy in patients with sepsis (Bloos & Reinhart 2014). The reported challenges in treatment of sepsis include infections caused by antibiotic-resistant microbes, with the increasing number of these community-acquired infections rendering the choice of empirical therapy more difficult (Huttunen & Aittoniemi 2011). Accordingly, implementation of empiric broad-spectrum antimicrobial therapy should be individualized and undertaken with caution due to the rising evidence of antimicrobial resistance.

Table 1.1: Diagnostic criteria for sepsis. Reproduced with permission Dellinger et al. Surviving sepsis campaign: international guidelines for management of sever sepsis and septic shock: 2012. Crit Care Med. 2013; 14(2): 580-637. Copyright© by the Society of Crit Care Med. & Lippincott Williams & Wilkins.

Infection, documented or suspected, and some of the following:
General variables
Fever (> 38.3°C)
Hypothermia (core temperature < 36°C)
Heart rate > 90/min^{-1} or more than two SD above the normal value for age
Tachypnea
Altered mental status
Significant edema or positive fluid balance (> 20 mL/kg over 24 hr)
Hyperglycemia (plasma glucose > 140 mg/dL or 7.7 mmol/L) in the absence of diabetes
Inflammatory variables
Leukocytosis (WBC count > 12,000 μL^{-1})
Leukopenia (WBC count < 4000 μL^{-1})
Normal WBC count with greater than 10% immature forms
Plasma C-reactive protein more than two SD above the normal value
Plasma procalcitonin more than two SD above the normal value
Hemodynamic variables
Arterial hypotension (SBP < 90 mm Hg, MAP < 70 mm Hg, or an SBP decrease > 40 mm Hg in adults or less than two SD below normal for age)
Organ dysfunction variables
Arterial hypoxemia (Pao_2/Fio_2 < 300)
Acute oliguria (urine output < 0.5 mL/kg/hr for at least 2 hrs despite adequate fluid resuscitation)
Creatinine increase > 0.5 mg/dL or 44.2 μmol/L
Coagulation abnormalities (INR > 1.5 or aPTT > 60 s)
Ileus (absent bowel sounds)
Thrombocytopenia (platelet count < 100,000 μL^{-1})
Hyperbilirubinemia (plasma total bilirubin > 4 mg/dL or 70 μmol/L)
Tissue perfusion variables
Hyperlactatemia (> 1 mmol/L)
Decreased capillary refill or mottling

WBC = white blood cell; SBP = systolic blood pressure; MAP = mean arterial pressure; INR = international normalized ratio; aPTT = activated partial thromboplastin time.

Diagnostic criteria for sepsis in the pediatric population are signs and symptoms of inflammation plus infection with hyper- or hypothermia (rectal temperature > 38.5° or < 35°C), tachycardia (may be absent in hypothermic patients), and at least one of the following indications of altered organ function: altered mental status, hypoxemia, increased serum lactate level, or bounding pulses.

1.2 EPIDEMIOLOGY OF SEPSIS

Although sepsis accounts for approximately 1.8 million cases worldwide annually, this is likely to be an underestimate of the true incidence, which may be over 18 million (Slade, Tamber & Vincent 2003), equivalent to 3/1,000 of the general population. Indeed sepsis represents the most frequent cause of fatality in non-coronary intensive care units around the world accounting for mortality rates of 30% to 70%, with the elderly, the immunocompromised and critically ill being most susceptible (Ntusi et al. 2010).

To my knowledge, population-based studies of the incidence and prevalence of sepsis in South Africa have not been undertaken. Furthermore, no incidence or mortality results have been reported in the African context, with the exception of one study in Nigeria evaluating the aetiology of sepsis (Jawad, Lukšić & Rafnsson 2012). The paucity of data from different countries makes it difficult to draw clear conclusions regarding the global incidence of sepsis, and only limited results from isolated countries have been reported (Jawad, Lukšić & Rafnsson 2012).

In 2005, it was reported that "the annual incidences of sepsis and severe sepsis in several industrialized nations have been reported as 50 – 100 cases per 100,000 persons" (Danai & Martin 2005). Gender, age and race may play a role in sepsis. Males, elderly and non-whites are more likely to develop sepsis (Vogel et al. 2010, Mayr, Yende & Angus 2014).

Most of the sepsis studies are derived from hospitalized patients in academic tertiary care centers. As a result, it is difficult to generalize these observations to hospital populations at large, and a lack of knowledge about the size and demographics of the underlying populations served by these hospitals precludes estimation of the incidence rates (Jawad, Lukšić & Rafnsson 2012). Additional population-based studies are required to better delineate the incidence of sepsis in the general population, and further analysis of such data will assist in the understanding of the factors that influence the epidemiology of sepsis.

1.3 MANAGEMENT OF SEPSIS, SEVERE SEPSIS AND SEPTIC SHOCK

The Surviving Sepsis Campaign, organized by more than 11 international societies has concluded that the treatment of patients with sepsis and septic shock should include three components: i) antibiotics and source control to eliminate infection; ii) fluid resuscitation and vascular agents to stabilize hemodynamics; and iii) modulation of the sepsis response (Rangel-Frausto et al. 2005, Dellinger 2013, Dellinger et al. 2013).

In 2003, a panel of critical care and infectious disease experts developed the Surviving Sepsis Campaign Guidelines aimed at improving the awareness of difficulties related to the management of patients with severe sepsis and septic shock. An important challenge associated with sepsis is the difficulty in rapidly identifying the causative pathogen so that appropriate therapy can be instituted (Slade, Tamber & Vincent 2003). The sepsis guidelines were revised in 2016 with sepsis defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection" (Rhodes et al. 2017). These guidelines provide a template for clinicians to follow when managing patients with suspected sepsis.

1.4 CAUSATIVE MICROORGANISMS

Sepsis is defined as the systemic response to infections caused by various classes of microorganisms (bacteria, viruses and fungi may be responsible for the condition), with bacteria accounting for the majority of cases. The incidence of gram-positive sepsis has increased recently and although gram-negative micro-organisms were responsible for most cases of sepsis in the early 1980s this is no longer the case (Mayr, Yende & Angus 2014), as illustrated in Figure 1.1.

In a survey performed in 2000, involving patients in the United States of America (USA), Martins et al. (2003) reported that "gram-positive bacteria accounted for 52.1% of sepsis cases, gram-negative bacteria 37.6%, polymicrobial infections 4.7%, anaerobes 1.0%, and fungi 4.6%; the greatest relative changes were seen in the incidence rates of gram-positive bacterial and fungal infections". The increasing frequency of fungal infections is concerning due to the poor prognosis associated with this form of sepsis (van der Poll & Opal 2008). A recent large study that included 14,000 patients from intensive care units in 75 countries, found the relative proportions of culture- positive infections to be 62%, 47% and 19% for gram-positive, gram-negative and fungal sepsis, respectively (Angus and van der Poll 2013), as shown in Figure 1.2.

The increasing number of fungal nosocomial infections has been associated with a trend towards more resistant *Candida* species such as *Candida glabrata* and *Candida krusei* replacing *Candida albicans* (Martin 2012). With respect to bacterial pathogens, *Staphylococcus aureus* and *Streptococcus pneumoniae* represent the most commonly isolated gram-positive organisms, while *Escherichia*

coli, *Klebsiella* spp. and *Pseudomonas aeruginosa* account for the majority of gram-negative bacteria (Opal 2003).

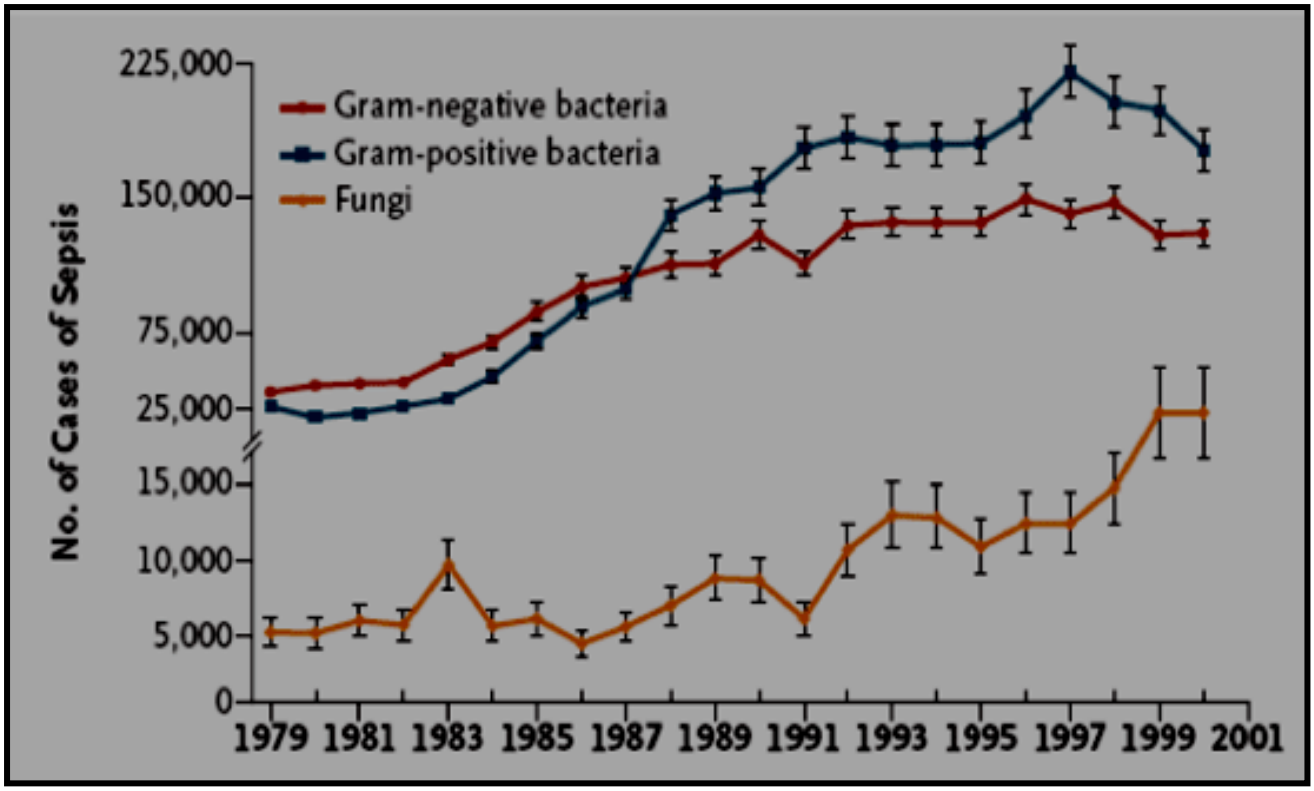

Figure 1.1: Represents the number of cases of sepsis in the United States, according to the causative organism from 1979 to 2000. Reproduced with permission from Martin et al. 2003, copyright Massachusetts Medical Society.

1.5 PATHOGENESIS OF SEPSIS

During microbial infection the most common host response is a local inflammatory response, whereby leukocytes are attracted to the area of infection by various chemoattractants in an attempt to localize the infection. If the infection is not contained, widespread activation of inflammatory cells results in the release of numerous of inflammatory mediators (Van Amersfoort, Van Berkel & Kuiper 2003).

In septic patients, normal homeostasis is impaired due to an imbalance of pro- and anti-inflammatory responses (Lacatus 2013, Wiersinga et al. 2013). The pathogenesis of sepsis involves activation of numerous inflammatory cells such as neutrophils and monocytes, as well as endothelial cells, resulting in the release of pro-inflammatory cytokines and activation of the complement cascades and a pro-coagulation state (Vincent, Martinez & Silva 2009), as shown in Figure 1.2. Septic patients often succumb to a systemic inflammatory response, of which multiple organ failure is a serious complication.

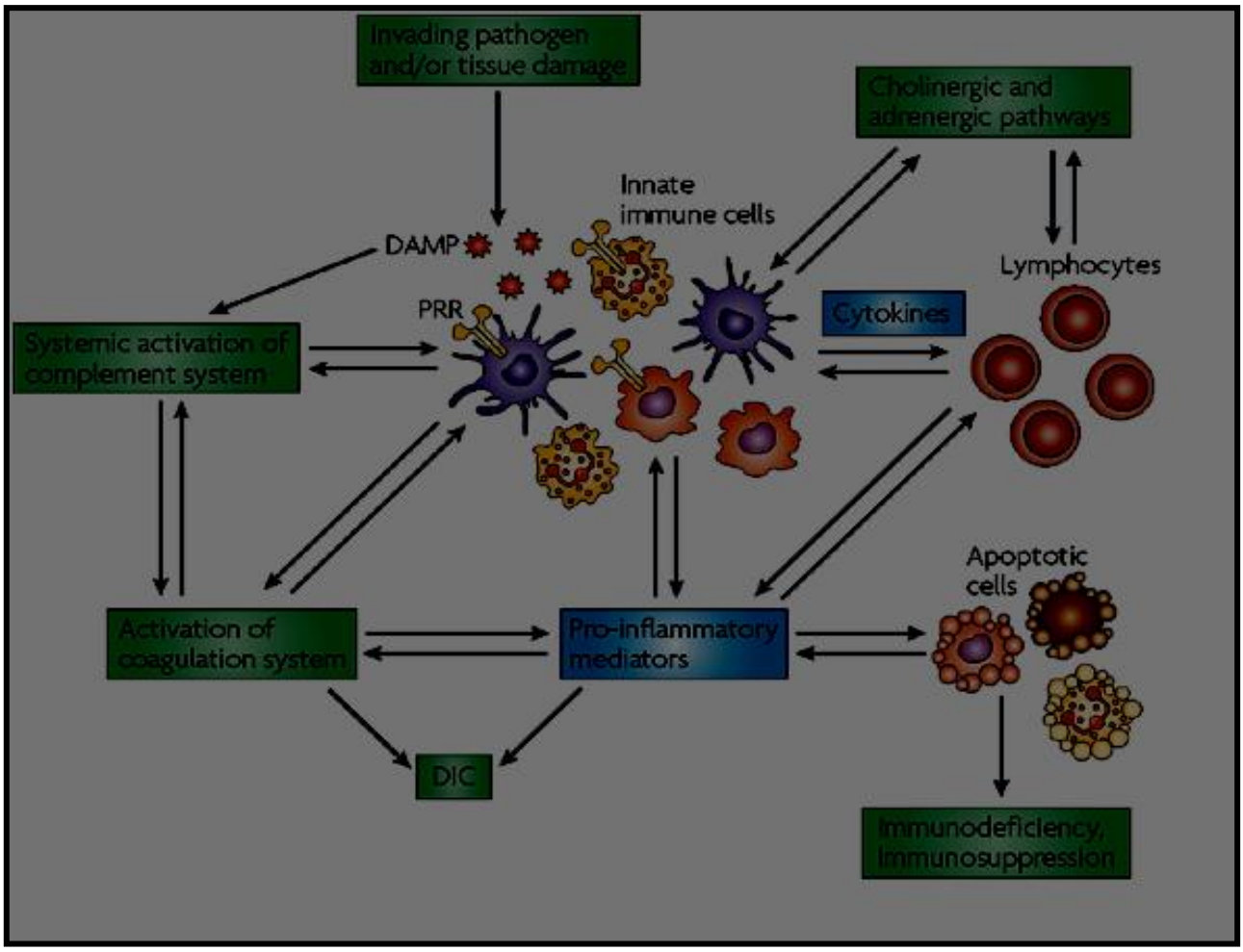

Figure 1.2: The inflammatory network in sepsis. "During sepsis, homeostasis between the various biological systems of the inflammatory network is highly imbalanced. In the initiation of sepsis, the release of a large amount of damage-associated molecular patterns (DAMPs) from invading microorganisms and/or damaged host tissue results in the overstimulation of pattern-recognition receptors (PRRs) on immune cells. Activated immune cells release excessive amounts of pro-inflammatory mediators (resulting in a 'cytokine storm'), free radicals and enzymes, which converts the normally beneficial effects of inflammation into an excessive response that damages the host. Activation of the adrenergic branch of the autonomic nervous system (ANS) and/or decreased activity of the cholinergic anti-inflammatory pathway (of the parasympathetic branch of the ANS) further amplifies the pro-inflammatory responses of

neutrophils, macrophages and dendritic cells in sepsis. The presence of invading microorganisms or their products in the blood can cause systemic activation of the complement system, which results in the excessive generation of complement anaphylatoxins, which, at high concentrations, induce numerous harmful effects. Simultaneous activation of the coagulation system and the inhibition of fibrinolysis as a result of the pro-inflammatory environment and/or damaged endothelium can result in disseminated intravascular coagulation (DIC), which is a major complication of sepsis, and in the amplification of the inflammatory response. The complement, coagulation and fibrinolysis systems are tightly connected through direct interactions of serine proteases, and imbalances in each cascade are intensified in a positive-feedback loop. Finally, the sustained pro-inflammatory environment affects the functional state of immune effector cells, eventually causing the dysfunction of neutrophils and immunoparalysis. Alterations in leukocyte apoptosis in the later stages of sepsis further account for immunosuppression, which increases the susceptibility to secondary infections." Reproduced with permission from the author, Nature Reviews Immunology, vol. 8, no. 10, pp. 776-787, Rittirsch, Flierl & Ward copyright 2008, with the legend as described by the authors indicated with the quotation marks.

1.5.1 PATHOGEN RECOGNITION

An immune response is initiated when local barriers are overwhelmed by the invading pathogens. Recognition of pathogens by cells of the immune system is a critical step, which is facilitated by activation of pattern recognition receptors (PRRs) (van der Poll & Opal 2008, Namas et al. 2012), such as Toll-like receptors (TLRs) (Wiersinga et al. 2013). TLRs play an important role as these molecules

recognize pathogen-associated molecular patterns (PAMPs) present on microbes (Okazaki & Matsukawa 2009, Wynn & Wong 2010).

Lipopolysaccharide (LPS) is an important example of a PAMP that mediates systemic inflammation and septic shock (Rittirsch, Flierl & Ward 2008) by activating cell surface receptors (TLR4) and cell surface adaptor proteins (CD14 and MD2) (Kawai & Akira 2009, Namas et al. 2012). Lipoteichoic acid present on gram-positive bacteria signals through TLR2, while TLR3 is involved in the recognition of viral double stranded RNA (Wynn & Wong 2010).

Damaged or dying cells release damage associated molecular patterns (DAMPs) (Denk, Perl & Huber-Lang 2012), such as high mobility group box protein 1 (HMGB-1), which is recognized by TLRs and may contribute to the progression of sepsis and septic shock (Rittirsch, Flierl & Ward 2008, Wiersinga et al. 2013)

In addition to TLRs, other intracellular non-TLR PRRs such as NOD-like receptors (NLRs) and RIG-like receptors (RLRs) are important for pathogen recognition (Cinel & Opal 2009, Wiersinga et al. 2013, Abderrazak et al. 2015, Rabes, Suttorp & Opitz 2016). Nucleotide-binding oligomerization domain (NLR) detects peptidoglycan of gram-positive bacteria, and retinoic-acid-inducible protein 1 (RIG-1) detects viral RNA and induces production of type 1 interferon (Wiersinga et al. 2013). Following engagement of pathogens by PRRs, an immune response is initiated via activation of mitogen activated protein kinase (MAPK) and the transcription factor, nuclear factor κB (NF-κB) (Cinel & Opal 2009).

Cytokines and chemokines released following activation of PRRs, amplify the innate response that is directed against invading pathogens. Among the many cytokines implicated in septic shock, tumor necrosis factor-α (TNF-α) and interleukins (IL), particularly IL-1 and IL-6, play key roles in the pathogenesis of sepsis (Wynn & Wong 2010, Boomer, Green & Hotchkiss 2014). TNF-α and IL-1 help to keep the infection localized, but once the infection becomes systemic, the pro-inflammatory effects of these cytokines can also be detrimental. Interleukin-8, an important regulator of neutrophil function, also contributes to acute lung injury and multi-organ dysfunction. Other cytokines that probably play a role in sepsis are IL-10, interferon-γ (IFN-γ), IL-12, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Boomer, Green & Hotchkiss 2014).

Cytokines have been classified on the basis of their biological properties into pro-inflammatory (Th1-cell response), for example, IFN-γ and IL-2, as well as macrophage-derived IL-1 (α and β), TNF-α, IL-8, IL-12, and IL-6, or anti-inflammatory (Th2-cell response), for example, IL-10, IL-4, transforming growth factor-β1 (TGF-β1) and IL-13, which can result in profound immunosuppression (Rittirsch, Flierl & Ward 2008, Chen, Yin & Zhang 2011, Boomer, Green & Hotchkiss 2014). Recent reports indicate that an IL-23-dependent Th-cell population produces IL-17, but not the Th1 and Th2 cytokines, IFN-γ and IL-4, respectively (Weaver et al. 2007).

These Th17 cells may play an important role in protecting the host against a range of extracellular bacterial and fungal pathogens, primarily through mobilization and activation of neutrophils and macrophages (Webster & Galley 2009). Despite the beneficial effects of pro-inflammatory cytokines in the activation of host defenses, overwhelming production of these mediators can lead to vasodilation, increased vascular permeability, hypotension, multiple organ failure and ultimately shock and death (Bozza et al. 2007, Jaffer, Wade & Gourlay 2010, Pierrakos &, Vincent 2010, Lvovschi et al. 2011, Tamayo et al. 2011). In addition, a variety of chemokines are released during sepsis, including IP-10, CCL5 (RANTES), MCP-1, MIP-1, and IL-8. Increased concentrations of chemoattractants induce complement proteins C3 and C5, as well as host defense proteins and peptides, such as cathelicidins and defensins (Wynn & Wong 2010) that may also play an important role during sepsis.

To counteract this intense, pro-inflammatory response, the host, in turn, mounts an anti-inflammatory response characterized by the production of anti-inflammatory cytokines, cytokine antagonists, and acute phase reactants. Although potentially protective, the anti-inflammatory response may result in a prolonged state of SIRS-related, acquired immunosuppression (Aziz et al. 2013), which, in turn, compromises the effectiveness of antimicrobial chemotherapy (Hotchkiss, Monneret & Payen 2013). Insight into the status of the host with respect to immune activation or immunosuppression has important implications for antimicrobial chemotherapy. During the phase of intensive immune activation, bactericidal agents are potentially harmful because they may exacerbate inflammatory responses by causing the release of pro-inflammatory toxins and cell wall components from disintegrating bacteria. On the other hand, bactericidal

antibiotics may be essential during the phase of advanced immunosuppression (Anderson et al. 2010, Aziz et al. 2013).

1.5.2 IMPORTANT CYTOKINES IN SEPSIS

Two of the most important cytokines implicated in sepsis are TNF-α and IL-1. These cytokines stimulate target cells to produce an array of inflammatory mediators (van der Poll &, Opal 2008, Schulte, Bernhagen & Bucala 2013, Wiersinga et al. 2013). Interleukin-17 produced by Th17 cells, as mentioned above, may play a novel role in sepsis by amplifying pro-inflammatory responses consequent to enhanced production of IL-1β, IL-6, and TNF-γ (Weaver et al. 2007, Wiersinga et al. 2013).

1.5.3 ROLE OF COMPLEMENT IN SEPSIS

The complement system has traditionally been considered to be a central part of the host defense against invading pathogens; however, complement activation may also contribute to an adverse outcome in patients with sepsis (Anas et al. 2010). There is strong evidence that both TLRs and the complement system optimize clearance of antigen-antibody complexes in plasma, while playing an important role in opsonization of invading pathogens followed by phagocytosis and cell lysis, thereby promoting inflammation, resulting in the recruitment of immune cells (Sarma & Ward 2012). Unfortunately, in some patients homeostasis is not restored by these inflammatory response mechanisms. The inflammatory response continues and becomes greatly exaggerated, resulting in septic shock and a potentially lethal outcome (Ward & Gao 2009, Ward 2010, Sarma & Ward 2012, Wiersinga et al. 2013).

In patients with sepsis there is a vigorous activation of the complement system, as signified by the loss of the haemolytic activity of complement (CH50) and by the appearance of the complement anaphylatoxins, C3a and C5a, in plasma (Ward 2010). It is well recognized that complement activation products are generated in sepsis and this pathway is presented in Figure 1.3. Activation products of complement, including C5a anaphylatoxin and the membrane attack complex [MAC] C5b-9, have been reported to be generated following infusion of endotoxin, or in response to caecal ligation and puncture (CLP) in a murine model of polymicrobial sepsis. C5a then interacts with its receptors C5aR and C5L2 inducing a "cytokine storm" that may result in multi-organ failure (MOF) (Ward 2010), as shown in Figure 1.4.

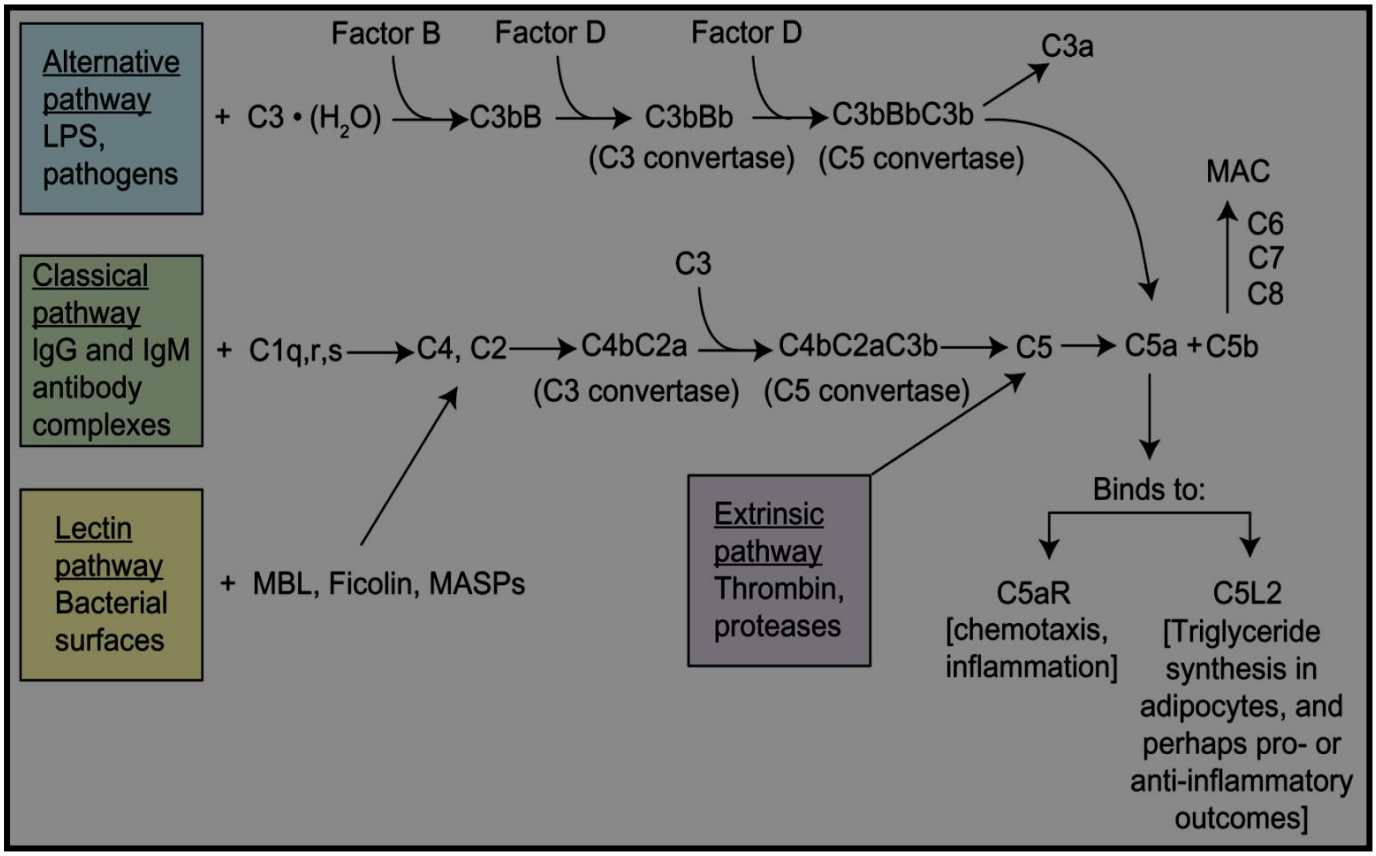

Figure 1.3: Complement-activating pathways leading to production of C5a. "Reprinted from Cell Health and Cytoskeleton, Vol 4, Sarma & Ward 2012, 73-82, copyright (2012), with permission from Dove Medical Press Ltd."

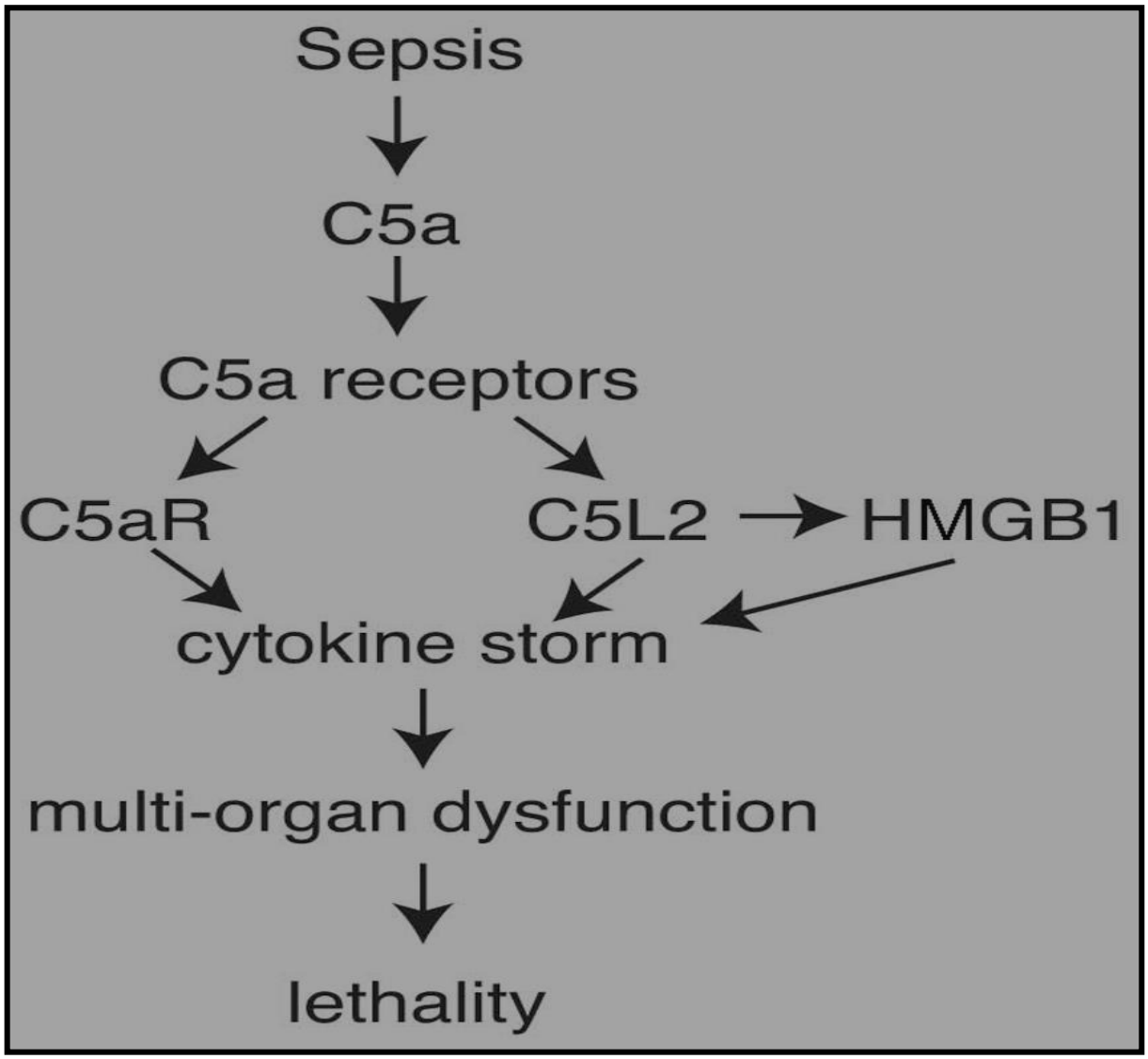

Figure 1.4: Interaction of complement component 5a with its receptor. After the Complement component C5a interacts with its receptor augmenting the onset of sepsis induced by CLP in rodents. The downstream effects are the "cytokine storm" together with production of HMGB1 from macrophages, which accentuates the cytokine storm. Ultimately, these events lead to multiple organ dysfunctions (MODs) and lethality. Reprinted with the permission from the author, Journal of innate immunity, vol. 2, no. 5, pp. 439-445, Ward 2010.

1.5.4 COAGULOPATHY AND SEPSIS

Severe sepsis is characterized by the dysregulation of host-derived mediators of inflammation (Schouten et al. 2008), which, in turn, are associated with hypotension, coagulopathy and multi-organ dysfunction. Importantly, sepsis may be associated with activation of the coagulation system (Sriskandan & Altmann 2008). The link between sepsis and activation of the coagulation system may manifest as subclinical hypercoaguability, as well as localised venous thromboembolism or acute disseminated intravascular coagulation (DIC) (Schouten et al. 2008). Disseminated intravascular coagulation is characterized by massive thrombin formation and widespread microvascular thrombosis, with consumption of platelets and coagulation factors. Disseminated intravascular coagulation contributes to multi-organ dysfunction or failure and may result in significant bleeding (Saracco et al. 2011).

Mechanistically, over-expression of inflammatory mediators in sepsis, together with the pathogenic microorganisms, produce DIC by: i) up-regulation of tissue factor (TF) expression, a procoagulant molecule released by activated macrophages and other cell types; ii) impairment of endogenous anticoagulant pathways including antithrombin, protein C and tissue factor pathway inhibitor; and iii) decreased fibrinolysis due to increased activity of plasminogen activator inhibitor-1 (PAI-1) and probably to thrombin-mediated activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (Lolis &, Bucala 2003, Semeraro et al. 2010, Wiersinga et al. 2013). Inflammatory mediators, either directly or indirectly, also induce apoptosis or necrosis, leading to the release of cell-derived nucleic acid, nuclear proteins and other macromolecules and enzymes that may, in turn, amplify inflammation, coagulation, tissue injury and MODS.

1.5.5 APOPTOSIS IN SEPSIS

Apoptosis is programmed cell death without damage to the cell membrane, release of cell contents or the development of an inflammatory response (Jedynak, Siemiątkowski & Rygasiewicz 2012). Apoptosis may be induced by several factors including glucocorticoids, cytokines (such as TNF-α, IL-1β, and IL-6), Fas ligand (FasL), heat shock, reactive oxygen species (ROS), nitric oxide, and products of FasL-expressing cytotoxic T lymphocytes (Hattori et al. 2010, Jedynak, Siemiątkowski & Rygasiewicz 2012). There is increasing evidence that accelerated apoptosis, particularly of lymphocytes, may be actively involved in the immunopathogenesis of sepsis. In this context, it is noteworthy that apoptosis associated production of immunosuppressive cytokines may contribute to a transient state of immunological tolerance associated with impaired maturation of dendritic cells (Jedynak, Siemiątkowski & Rygasiewicz 2012, Condotta et al. 2013)

1.6 DETECTION OF SEPSIS PATHOGENS

As mentioned above, despite the early optimization of hemodynamics and implementation of empirical antibiotic therapy, sepsis remains the most common cause of death in severely ill, hospitalized patients. Antibiotic therapy may be reviewed on acquisition of results of microbiological pathogen and sensitivity testing (Ferrer et al. 2014, Amaro et al. 2017). Although isolation of pathogens from blood cultures remains central to the diagnosis of sepsis, this microbiological procedure is limited by low sensitivity, length of time taken to acquiring results, and the risk of contamination (Chang et al. 2013), as well as prior empirical antibiotic therapy.

Although the use of instrumentation which continuously monitors bacterial growth may expedite the detection of causative pathogens, even this may be constrained in the setting of infections caused by slow-growing, fastidious organisms such as *Bartonella, Mycoplasma* and *Nocardia* spp, as well as *Francisella tularensis* and several molds. Further delays in acquisition of an accurate diagnosis may occur in the setting of infections caused by obligate anaerobes (Mancini 2010). Delayed acquisition of results has therefore provided the impetus not only for development of molecular diagnostic procedures, but also for the identification of both host- and pathogen-derived diagnostic/prognostic systemic biomarkers (Dierkes et al. 2009)

Molecular microbiological procedures are based on the amplification of pathogen-derived nucleic acid in the blood, cerebrospinal fluid and other bodily fluids. Tests based on these procedures may improve clinical care by shortening the time to diagnosis of microbial positivity and are less likely to be affected by prior antibiotic treatment. Polymerase chain reaction (PCR)-based procedures have been introduced in an attempt to expedite pathogen detection (Cinel & Opal 2009). Detection of bacterial DNA fragments by PCR in blood samples, or 16S rRNA fragments of gram-positive and gram-negative bacteria and *Candida* spp. is most promising in facilitating early detection of sepsis, due to a high degree of sensitivity and specificity. The main disadvantages of these techniques are high costs, the lack of standardization, and the need for skilled personnel (Coelho & Martins 2012).

Individual single-plex PCR assays have been developed and approved for the identification of individual, specific pathogens. However, to be clinically useful,

this procedure necessitates the performance of multiple single-plex PCR assays for each patient, which is costly and inefficient. The use of multiplex-PCR, results in the simultaneous amplification of more than one genetic locus using multiple primer pairs, which can differentiate between various aetiological agents responsible for sepsis (Laakso & Mäki 2013) based on the molecular weight of the fragments amplified.

Drawbacks which hinder PCR-based pathogen detection in patients with sepsis are largely associated with nucleic acid extraction procedures and include: i) microbial contamination of PCR reagents; ii) the presence of PCR inhibitors in the biological fluid; iii) preventing carryover contamination in the successive rounds of PCR; and iv) interference by high concentrations of human DNA (Handschur et al. 2009, Mancini et al. 2010). With a few exceptions, most PCR-based diagnostic procedures do not determine the antibiotic sensitivity of the causative pathogen, representing an additional limitation (Mancini et al. 2010).

In addition, the rapid accurate diagnosis of invasive fungal infections (IFI) is often particularly difficult due, most commonly, to the slow appearance of symptoms and radiological changes during the course of infection. In this context, classic mycological laboratory procedures including histology, microscopy and culture often have limited diagnostic utility. Alternative diagnostic approaches include the detection of fungus-specific cell wall components using serologic and enzymatic procedures. Examples of these include: galactomannan (GM, *Aspergillus* spp.), mannan (*Candida* spp.), and (1,3)-β-D-glucan (BG, both *Aspergillus* and *Candida* spp.) with the latter (BG) consisting of (1,3)-β-D-glucopyranosyl polymers with randomly dispersed single β-D-glucopyranosyl units joined by (1-6)–β linkages

giving a comb-like structure (Kedzierska et al. 2007, Persat et al. 2008, Mancini et al. 2010, De Vlieger et al. 2011, Del Bono et al. 2011, Montagna et al. 2011, Fontana et al. 2012, Mikulska, Furfaro & Viscoli 2012, Delaloye & Calandra 2013).

Galactomannan (GM) is a polysaccharide present in the cell wall of *Aspergillus* spp that is released during the growth of the fungus at the tips of the hyphae (Cray, Watson & Arheart 2009). It is not a single molecule, but rather a family of molecules which are more appropriately referred to as galactofuranose (galf)-antigens (Mennink-Kersten et al. 2006). β-D-Glucan is present in the cell wall of a wide variety of fungi and detection is based on its ability to activate factor G of the horseshoe crab coagulation cascade via activation of its serine protease zymogen β subunit (Persat et al. 2008, Posteraro et al. 2011, Delaloye & Calandra 2013). Detection of these antigens in biological fluids, including serum, has useful applications in the diagnosis of invasive disease due to *Aspergillus* and *Candida* spp. (Hachem et al. 2009).

Although the presence of circulating GM may precede the presence of clinical signs of invasive aspergillosis, a major disadvantage of this assay is the high potential for false-positive results (Mancini et al. 2010, Millon et al. 2010, Mikulska et al. 2011, Ng et al. 2013). Several possible causes of false-positive reactions have been reported, including the intake of certain foods such as ice cream, processed meat products, canned vegetables, and some oral nutritional supplements (Millon et al. 2010, Ng et al. 2013). Cross-reaction with other fungal species such as *Blastomyces dermatitidis*, *Penicillium* spp., and *Fusarium* spp. has been described (Mikulska et al. 2012, Tortorano et al. 2012), while false-positive

results have also been reported in patients treated with amoxicillin-clavulanate (Ng et al. 2013).

Circulating biomarkers also have the potential to contribute to the diagnosis and prognosis of sepsis because of their ability to: i) indicate disease severity; ii) differentiate bacterial from viral and fungal infections; and iii) differentiate systemic sepsis from localized infections. Other potential uses of biomarkers include evaluating the response to therapy and recovery from sepsis, as well as predicting the complications that lead to the development of organ dysfunction (Pierrakos & Vincent 2010, Delaloye & Calandra 2013, Faix 2013, Henriquez-Camacho & Losa 2014, Biron, Ayala & Lomas-Niera 2015).

1.7 Circulating host-derived markers of inflammation and infection

The selection of the biomarkers described in this thesis was based on the following:

- Potential to distinguish between infective and non-infective causes of SIRS: soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and procalcitonin (PCT).
- Utility as markers of the severity of SIRS: IL-1β, IL-6, IL-8, IL-17, TNF-α, G-CSF, GM-CSF, and CRP, neutrophil gelatinase-associated lipocalin (NGAL) and matrix metalloproteinase-9 (MMP-9).
- Utility as markers of advanced immunosuppression: IL-Ra, IL-4, IL-10, TGF- β1, HMGB1 and CRP.

1.7.1 Host-derived markers of infection

Two circulating, host-derived markers of microbial infection which have attracted considerable recent attention with respect to their abilities to function as non-specific markers of bacterial infection are sTREM-1 and PCT.

1.7.1.1 Triggering receptor expressed on myeloid cells-1

The triggering receptor expressed on myeloid cells is described as causing activation of receptors on PMNs, monocytes/macrophages, microglia, osteoclasts, dendritic cells, as well as on megakaryocytes and platelets (Ford & McVicar 2009, Poukoulidou et al. 2011, Tao et al. 2013) and appears to play an important role in the pro-inflammatory response in sepsis and septic shock (Tao et al. 2013, Coa, Gu & Zhang 2017). TREM-1 is a member of the immunoglobulin (Ig)-like superfamily of receptors (Ormsby et al. 2011). It is a type 1 transmembrane protein possessing a single extracellular immunoglobulin-like domain, a transmembrane region, and a short cytoplasmic tail (Molloy 2009, Lin et al. 2011, Cao, Gu & Zhang 2017).

Like other types of membrane receptor, TREM-1 has a short intracellular domain and is shed from the membrane of activated phagocytes without the transmembrane and intracellular domains and is detected as soluble TREM (sTREM)-1 in body fluids (Gibot 2005, Ho et al. 2008, Kwofie et al. 2012). Following binding to its undefined counter-receptor on target cells, sTREM-1 induces transduction of DNA activation protein 12 (DAP12), triggering the release of pro-inflammatory cytokines, thereby augmenting the host response to bacterial stimuli (Gibot 2005, Tessarz & Cerwenka 2008, Barati et al. 2010, How et al. 2011, Ormsby et al. 2011).

Extracellular bacteria, specifically their cell wall components, such as LPS or lipoteichoic acids, are potent inducers of upregulation of expression of TREM-1 on phagocytic cells (Molloy 2009, Wu et al. 2012), which is accompanied by the release of pro-inflammatory cytokines (Dower et al. 2008). Indeed, TREM-1 appears to act in concert with agonists of TLRs such as LPS, amplifying potentially harmful responses during severe bacterial infection (Gibot 2005, Klesney-Tait & Colonna 2007, Dower et al. 2008, Molloy 2009).

1.7.1.2 Procalcitonin

Procalcitonin (PCT), the precursor of the polypeptide hormone, calcitonin, is a 116 amino acid protein, encoded by the *CALC-1* gene on chromosome 11 (Beqja-Lika et al. 2013, Boysen et al. 2013), which undergoes cleavage in the neuroendocrine cells of the thyroid, lung and pancreas, resulting in the formation of three distinct molecules viz. calcitonin, katacalcin and an N-terminal fragment, aminoprocalcitonin (López et al. 2011). PCT has been reported to be elevated in patients with severe bacterial and fungal infections and in sepsis (Fazili et al. 2012, Perrakis et al. 2012, Vijayan 2017). During viral infections, high levels of IFN-γ are present, which suppress the production of PCT, resulting in only moderate levels of this biomarker (Fazili et al. 2012).

Production of PCT is initiated following exposure of target cells to endotoxin and several pro-inflammatory mediators (in particular TNF-α) and its levels of production appear to correlate with the degree of insult (Simon, Milbrandt & Emlet 2008). Although PCT can be elevated in non-infectious conditions such as transplant rejection during immunotherapy with monoclonal antibodies to CD3, elevated levels of PCT are a reliable and specific marker of invasive infection, and

the adequate treatment of such infection results in a reduction in the levels of circulating PCT (Lee 2013, Vijayan et al. 2017).

1.7.2 Host-derived markers of inflammation

The predominant host-derived markers of inflammation are the pro-inflammatory neutrophil and monocyte/macrophage–mobilizing cytokines/chemokines briefly discussed in section 1. These include: i) the bone-marrow-targeted growth factors G-CSF and GM-CSF, produced mainly by monocytes/macrophages/fibroblasts/endothelial cells and epithelial cells, as well as Th1 cells/macrophages; ii) the neutrophil mobilizing/activating cytokines/chemokines, IL-17 and IL-8, produced by Th17 cells and monocytes/macrophages, respectively; iii) predominantly pro-inflammatory cytokines, particularly IL-1β, IL-6 and TNF-α produced by cells of the innate (monocytes/macrophages, mast cells) and adaptive (T cells) immune systems, as well as by structural cells (fibroblasts, epithelial cells, and others); and iv) the acute phase reactant, CRP, which possesses both pro- and anti-inflammatory properties, which are operative early and late in the inflammatory response respectively.

1.7.2.1 C-reactive protein

C-reactive protein (CRP) is of particular importance during the early innate immune response to infection and appears to link innate and acquired immune responses. This acute-phase protein, which is produced predominantly by hepatocytes and alveolar macrophages (Mancini et al. 2010, Bloos & Reinhart 2014), binds to microbial surface polysaccharides and peptidoglycan via a calcium-dependent mechanism, apparently promoting opsonization via interaction of bound

CRP with surface immune adherence-promoting receptors on phagocytic cells, such as Fcγ receptors (Casey et al. 2008). A similar mechanism appears to promote the clearance of apoptotic cells and cellular debris (Casey et al. 2008). In addition, the formation of complexes between CRP and micro-organisms, as well as CRP with dead and dying host cells and tier products, has also been reported to initiate activation of the classical complement pathway, augmenting clearance of pathogen and cellular debris via phagocytosis (Faix 2013).

The concentration of CRP in serum shows a rapid rise following severe trauma and/or bacterial infection (Hofer et al. 2012). In 1982, Morley and Kushner demonstrated that, within six hours of an inflammatory stimulus, the serum CRP concentration rises dramatically and may increase a hundredfold over normal values of less than 1.0 mg/dl. The CRP concentration decreases rapidly if the stimulus for increased synthesis is removed. Consequently, changes in the serum CRP level may indicate changes in the inflammatory process or tissue damage (Morley, Kushner 1982, Hofer et al. 2012).

1.7.2.2 Matrix metalloproteinase-9

Proteolytic enzymes, released predominantly by activated phagocytes and damaged connective tissue, have also been implicated in harmful inflammatory host responses (Bourboulia, Stetler-Stevenson 2010, Halade, Jin & Lindsey 2013). Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, which facilitate the extravasation and movement of inflammatory cells across the extracellular matrix (ECM), as well as promoting tissue remodeling (Lorente et al. 2009, Halade, Jin & Lindsey 2013). They are classified according to substrate specificity into collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), elastases (MMP-7 and -12), and membrane–

types (MT-MMPs, MMP-14, -15, -16, and -17) (Lorente et al. 2009). Along with other metalloproteinases, MMP-9 plays a key role in physiological processes such as inflammation, tumor invasion and metastasis (Renckens et al. 2006).

The proteolytic activities of these various MMPs are usually highly regulated by counteracting inhibitors of proteolysis, particularly tissue inhibitors of matrix metalloproteinases (TIMPs) and alpha-2 macroglobulin, which play a key role in maintaining the integrity of tissue architecture, although this balance may be compromised during severe infection (Maitra et al. 2010). MMP-9 is stored in both the secondary and tertiary granules of phagocytic cells and may be released to an excessive extent during acute inflammatory disorders, including sepsis, with elevated concentrations of MMP-9 having been reported in sepsis patients, which were found to correlate with disease severity (Renckens et al. 2006, Lorente et al. 2009, Maitra et al. 2010).

1.7.2.3 Neutrophil gelatinase-associated lipocalin

Human neutrophil gelatinase-associated lipocalin (NGAL), which has a molecular weight of 25kDa, is an iron-binding antimicrobial protein expressed predominantly by neutrophils and various other eukaryotic cell types in which it is covalently bound to gelatinase. In the case of neutrophils, NGAL is detected in the secondary granules of these cells during the early-myelocyte stage of granulopoiesis (Devarajan 2010). In addition to bone marrow, mRNA encoding NGAL has also been detected in kidney, liver, lung, colon, uterus, prostate and stomach tissue (Parravicini 2010, Soni et al. 2010).

NGAL plays a role in host defense against bacterial pathogens by competing with iron-binding bacterial siderophores to restrict bacterial growth via an essentially bacteriostatic mechanism. This protective role of NGAL has been demonstrated in a murine model of experimental infection in which NGAL-gene-knockout mice manifested significantly increased susceptibility for development of gram-negative bacterial infections and death from sepsis (Devarajan 2010, Soni et al. 2010). In addition to its role in host defense, NGAL also facilitates iron shuttling in eukaryotic cells, contributing to various cellular responses including proliferation and differentiation.

Production of NGAL undergoes considerable upregulation during exposure of epithelial organs to various stressors, including inflammation-associated damage during sepsis (Parravicini 2010) and is also produced by human cancers, being predictive of an unfavourable clinical course (Devarajan 2010).

1.7.3 Host-derived markers of advanced immunosuppression

The host utilizes several interacting strategies to counter and suppress the systemic inflammatory response. These include: i) the production of neutrophil- and monocyte/macrophage-targeted anti-inflammatory cytokines, especially IL-4/IL-10 produced predominantly by Th2 cells and type 2 macrophages, as well as TGF-β1, produced by regulatory T cells/ macrophages and structural cells; ii) cytokine antagonists, such as circulating IL-1, IL-6 and TNF receptors, with the IL-1 receptor antagonist (IL-1Ra), being produced by many different types of immune/inflammatory/structural cells; and iv) CRP, which also possesses anti-inflammatory properties.

1.7.3.1 High mobility group box 1 protein

Although thought to be predominantly pro-inflammatory in nature as described below, HMGB1 is produced relatively late in the inflammatory response. Interestingly, anti-inflammatory activities have recently been attributed to HMGB1, including inhibition of IL-6 and TNF-a production by activated macrophages (Gong et al. 2010). This combination of properties (production late in the course of the inflammatory response together with anti-inflammatory activity) suggests that measurement of circulating HMGB1 may be a useful marker of advanced immunosuppression in SIRS.

HMGB1, which is highly conserved, is a non-histone nuclear protein with multiple functions, which as mentioned above, appear to be operative late in the course of systemic inflammation (Gong et al. 2010, Walle, Kanneganti & Lamkanfi 2011, Deng et al. 2013). Originally described as an intracellular transcription factor, it has become clear that HMGB1 is released from endotoxin-stimulated macrophages after a significant delay (Wang et al. 1999, Andersson & Tracey 2003, Wang, Yang & Tracey 2004). HMGB1 binds to DNA and participates in gene transcription in target cells, and also functions as a cytokine in the extracellular milieu (Deng et al. 2013).

HMGB1 is actively secreted by various immune cells (e.g. macrophages, monocytes and neutrophils) via a regulated process involving phosphorylation, acetylation, packaging into secretory lysosomes, and exocytosis (Xu et al. 2010). In addition, damaged or necrotic immune cells, but not apoptotic cells, passively release HMGB1 (Walle, Kanneganti & Lamkanfi 2011). Extracellular HMGB1 has

been reported to interact with TLRs, as well as with receptors for advanced glycation end products (RAGE), as shown in Figure 1.5. The interaction between HMGB1 and TLR2 or TLR4 in particular has been proposed to underlie the pro-inflammatory, as opposed to the anti-inflammatory, actions of HMGB1 (Qin et al. 2009).

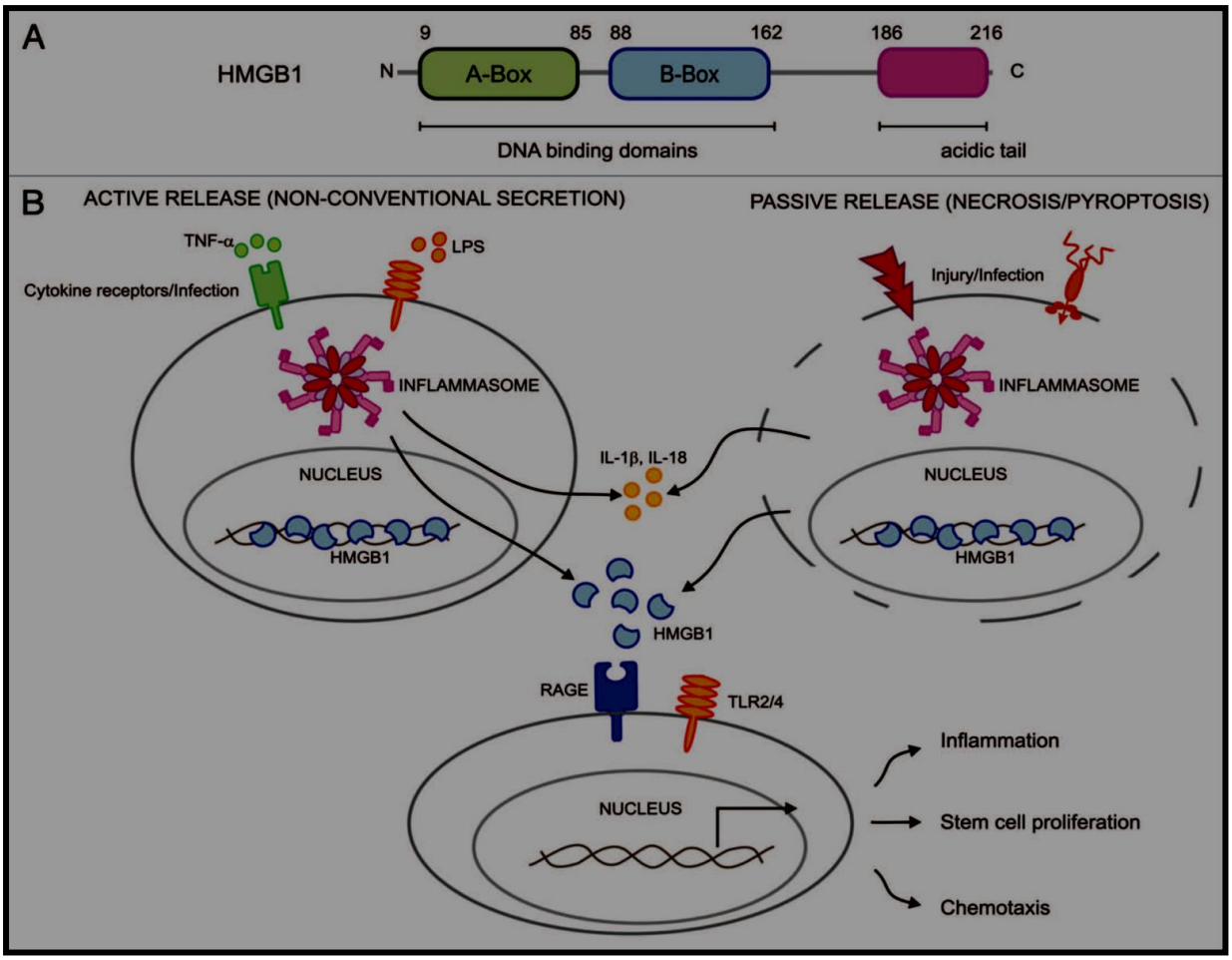

Figure 1.5: "Structure of HMGB1 and its functions in the extracellular environment. (A) Human HMGB1 is a protein of 216 amino acids, that is composed of two homologous DNA-binding domains called 'A and B boxes' that are followed by a negatively charged acidic tail in the carboxyl-terminus. (B) HMGB1 can be secreted through non-canonical mechanisms along with pro-inflammatory cytokines such as IL-1β and IL-18 following inflammasome activation in stimulated and/or infected immune cells, or it may be passively released from damaged and infected cells undergoing necrotic cell or pyroptotic cell death. Extracellular HMGB1 can bind to its receptors RAGE and TLR2/4 on effector cells in order to induce inflammation, chemotaxis and repair responses". Reproduced with permission from: Virulence, vol. 2, no. 2, pp. 162-165, Walle, Kanneganti & Lamkanfi 2011, with the legend as discussed by the author indicated with quotation marks.

1.8 Hypothesis

Measurement of circulating pro-inflammatory and anti-inflammatory cytokines, PCT, CRP, sTREM-1, HMGB1, MMP-9 and NGAL when used in combination with detection of sepsis pathogens by conventional molecular and serological procedures, may identify a profile of host- and pathogen-derived biomarkers which can be used to enable distinction between infective and non-infective causes of SIRS, as well as identifying the status of the patient with respect to the phase of sepsis and/or extent of SIRS-related immunosuppression. Alternatively, these procedures may confer little or no benefit over existing procedures.

1.9 Objectives

- To measure the circulating cytokine profiles (both pro-inflammatory and anti-inflammatory) in patients with SIRS and sepsis using suspension bead protein array technology (Bioplex®).

- To categorize patients as having infective or non-infective causes of SIRS using clinical criteria, alterations in circulating biomarkers, together with conventional, molecular and serological antigen detection procedures, including detection of fungal nucleic acid and galactomannan.

- To determine the diagnostic and prognostic utility of measurement of the concentrations of circulating, host-derived, systemic markers of inflammation and infection (cytokines, HMGB1, TREM-1, PCT, MMP-9, NGAL and CRP), or

alternatively as adjuncts to clinical and/or other laboratory diagnostic parameters.

CHAPTER 2

THE ROLE OF CIRCULATING HOST- AND PATHOGEN-DERIVED MARKERS OF INFECTION AND INFLAMMATION IN THE LABORATORY DIAGNOSIS OF SEPSIS

2.1 Introduction

The systemic inflammatory response syndrome (SIRS) defines criteria used to identify patients with systemic inflammation that may be due to infectious or non-infectious causes. If a source of infection is identified, sepsis is diagnosed and antimicrobial therapy should be initiated. However, establishing a definite source of sepsis is often delayed as the results of pathogen identification investigations may only be available after 48 – 72 hours, and even then, despite advances in molecular diagnostics, detection may only be achieved in <40% of patients (Jain et al. 2015). Importantly, antimicrobial therapy needs to be initiated early in order to reduce the mortality associated with sepsis (Ferrer et al. 2014; Amaro et al. 2017), while on the other hand inappropriate antimicrobial therapy administered to patients with SIRS without a source of infection has been shown to adversely affect the survival of these patients.

Therefore, the indiscriminate use of antimicrobial therapy for all patients with SIRS is not advocated, but should not be withheld from patients with sepsis. This creates a dilemma for treating physicians, especially when the source of systemic inflammation, either of infectious or non-infectious origin, is not immediately apparent. In this context, the identification of host-and/or pathogen-derived systemic biomarkers of inflammation/infection, preferably with both discriminatory and prognostic potential, remains a research priority with considerable translational potential.

Accordingly, the current study, undertaken in a southern African hospital setting, was designed to evaluate the potential of clinical parameters and circulating biomarkers of systemic inflammation in differentiating patients with sepsis from those with SIRS, as well as in predicting survival. This may be of

particular relevance given that patients of African-American origin are reported to be at increased risk for development of post-traumatic sepsis (Kisat et al. 2013).

2.2 Patients and Methods

The study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria and Steve Biko Academic Hospital, Pretoria, South Africa.

Patients admitted to the Emergency Unit or Medical wards and Medical Intensive Care Unit (ICU) at the Steve Biko Academic Hospital, University of Pretoria, Pretoria, South Africa, during a 6 month period, with a provisional diagnosis of the systemic inflammatory response syndrome (SIRS), were considered eligible for inclusion in the current study. Patients were excluded if they did not meet 2 or more of the SIRS criteria shown below or had received antimicrobial therapy within the preceding 12 hours.

SIRS was diagnosed if 2 or more of the following criteria were met (Schulte, Bernhagen & Bucala 2013):

1. Body temperature > 38°C or < 36°C.
2. Heart rate > 90 beats/minute.
3. Respiratory rate > 20 breaths/minute.
4. White blood cell count > 12000 or < 4000 cells/mm^3.

As the current study was initiated prior to 2016, the recently revised definition of sepsis based on the degree of organ dysfunction (SOFA score)

(Singer et al. 2016; Gül et al. 2017, Verdonk, Blet & Mebazaa 2017) was not used to classify patients.

Following informed consent, patients who met the SIRS criteria were included in the study. Clinical parameters including blood pressure, respiratory rate, body temperature and heart rate were recorded for each patient and blood or serum used to determine the white blood cell (WBC) and platelet counts, as well as concentrations of the prototype acute phase reactant, CRP and PCT, the latter a biomarker of bacterial infection (Riedel et al. 2011, Vijayan et al. 2017).

The circulating concentrations of an array of pro-inflammatory and anti-inflammatory cytokines were also determined as described below, as well as those of neutrophil gelatinase-associated lipocalin (NGAL), the soluble triggering receptor expressed on myeloid cells 1 (s-TREM-1), matrix metalloproteinase 9 (MMP-9), and the high mobility group box 1 protein (HMGB1). These biomarkers were chosen as being generally representative of activation of cells of the innate immune system, particularly neutrophils and monocytes/macrophages.

In addition, appropriate samples such as blood, sputum, urine, pus, peritoneal fluid or cerebrospinal fluid (CSF) were collected for microbiological investigation, which included microscopy, culture and sensitivity at the discretion of the treating physician. When indicated, samples were also sent for microscopy and culture to detect *Mycobacterium tuberculosis*. A chest radiograph was also performed when indicated. Patients with presumed sepsis were treated empirically with antimicrobial therapy, which was adjusted according to culture results.

Patients were monitored until discharge from hospital and the records of all patients, including those who did not survive, were retained and reviewed. Based on a detailed review of the available clinical information and the results of microbiological testing, patients were classified retrospectively into 2 groups, namely those with sepsis and those with SIRS. The differentiation into these 2 groups was based on clinical findings, microscopy and culture results of blood, sputum, urine, peritoneal fluid, CSF and bronchial aspirates, response to antimicrobial therapy and radiographic abnormalities such as signs of lobar consolidation on chest X-ray.

2.3 Laboratory Methods

To prepare serum, 5 milliliter (ml) of venous blood was collected in endotoxin-free, silicone-coated vacutainers containing a gel separator. The blood samples were allowed to stand at room temperature to coagulate (30 to 60 minutes) followed by centrifugation (3000 rpm for 10 minutes) after which the serum was removed, aliquoted, and stored at minus 20 ^{0}C until performance of the various assays described below with the exception of cytokine/chemokine analyses for which plasma was used. To prepare plasma, venous blood (10 ml) was collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes and the plasma separated by centrifugation, aliquoted and stored as described above.

2.3.1 C-reactive protein (CRP) and procalcitonin (PCT)

CRP was assayed by laser immunonephelometry (Siemens Healthcare Diagnostics, BN Prospec Nephelometer, Newark, USA), with results expressed as micrograms (μg)/ml serum, while PCT was measured by means of an immunoluminescence procedure using a chemiluminometer and compatible

reagents according to the manufacturer's protocol (Lumi Test, Brahm's Diagnostika, Berlin, Germany) with values of <0.5 nanograms (ng)/ml serum considered to be in the normal range.

2.3.2 Cytokines and chemokines

Measurement of these was performed using the Bio-Plex suspension bead array system (Bio-Rad Laboratories Inc, Hercules, CA, USA) which utilizes Luminex xMAP multiplex technology to enable simultaneous detection and quantitation of multiple different analytes in a single sample. The system uses an array of microspheres in liquid suspension, conjugated with a monoclonal antibody specific for a target protein. The beads contain different ratios of two spectrally distinct fluorophores, thereby assigning a unique spectral identity. These antibody-coupled, color-coded beads were then incubated with the plasma sample (1/4 dilution), washed, followed by addition of a biotinylated detection antibody, washed again, and finally incubated with streptavidin-phycoerythrin. A wide range of standards (0.38 - 91756.00 picograms (pg)/ml) was used to enable quantitation of individual cytokines using a Bio-Plex array reader with a dual laser detector and real-time digital signal processing. The following analytes were measured simultaneously using a 9-plex test kit: human interleukin (IL)-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-8, IL-10, IL-17A, tumor necrosis factor (TNF), granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). An additional cytokine, human transforming growth factor-β1 (TGF-β1) was measured by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) procedure (R&D Systems Inc. Minneapolis, MN, USA) following brief acidification of the plasma specimens to activate latent cytokine to the immunoreactive form and results expressed as

ng/ml plasma (Meyer et al. 2010). The upper limit of normal for these analytes was calculated as the mean ± 1 SD for 10 healthy control subjects (5 female, 5 male, average age 44.4 ± 15.0 years ranging from 26 - 65 years of age) and these values, as well as for the corresponding values for the investigations mentioned below, are shown as supplementary data in table 2.6.

2.3.3 Human neutrophil gelatinase-associated lipocalin (NGAL)

NGAL is considered to be a sensitive biomarker of sepsis severity and early renal injury (Hang et al. 2017, Kim et al. 2017), and in the current study serum levels of this biomarker were measured using a quantitative capture ELISA procedure (BioVendor Research and Diagnostic Products, Asheville, NC, USA) and the results expressed as ng/ml serum.

2.3.4 Human soluble triggering receptor expressed on myeloid cells (sTREM-1)

Following up-regulation of expression of TREM-1 on cells of the innate immune system, most commonly during bacterial and fungal infections, soluble TREM-1 (sTREM-1) is released from the cell membrane and can be detected in the circulation as a biomarker of infection and inflammation (Cao, Gu & Zhang 2017) and may predict survival in patients with neutropenic sepsis (Kwofie et al. 2012). In the current study, serum concentrations of sTREM-1 were measured using a quantitative sandwich ELISA procedure (R&D Systems Inc. Minneapolis, MN, USA) and the results expressed as pg/ml serum.

2.3.5 Human matrix metalloproteinase-9 (MMP-9)

The granule proteinase, MMP-9, which is released by activated neutrophils and monocytes/macrophages, as well as structural cells, also possesses pro-inflammatory activities which include activation of vascular endothelium (Florence et al. 2017) was measured using a quantitative solid phase ELISA procedure (R&D Systems, Biotechne, North America) with results expressed as ng/ml serum.

2.3.6 Human high mobility group box 1 protein (HMGB1)

This pro-inflammatory cytokine is produced, among other cell types, by monocytes/macrophages, dendritic cells and endothelial cells and amplifies inflammatory responses via interactions with several different receptors, particularly Toll-like receptor 4 expressed on neutrophils and monocytes/macrophages (Klune et al. 2008). In the current study, serum concentrations of HMGB1 were measured using a quantitative sandwich ELISA procedure (IBL International, Hamburg, Germany) and the results expressed as ng/ml serum.

2.4 Statistical analysis

Statistical Analysis was performed using WinStat statistical software. Results are expressed as the mean ± standard deviation, and levels of statistical significance were calculated using the Mann-Whitney U-test for comparison of non-parametric data. A p value <0.05 was considered as significant.

2.5 Results

2.5.1 Patients

Using the SIRS criteria, 68 patients were included in the study. Of these, 37 patients were classified into the sepsis group and 25 were identified as SIRS patients without an apparent source of infection. Six patients could not be classified into either group and were excluded from analysis. Demographic data for both groups of patients are shown in Table 2.1, while clinical diagnoses and micro-organisms identified from those patients with sepsis and the clinical diagnoses of those patients with SIRS are shown in Tables 2.2 and 2.3 respectively. Respiratory tract infections, particularly community-acquired pneumonia (CAP), were the most commonly encountered in patients with sepsis (29.7%). Other frequent sites of infection were the urogenital tract (18.9%), gastrointestinal system (8.1%) and central nervous system (5.4%). The most common precipitants of systemic inflammation in the SIRS group of patients included disorders of the cardiovascular system (36%) and the respiratory system (28%).

2.5.2 Clinical parameters

The clinical parameters for patients classified as either sepsis or SIRS are shown in Table 2.4. The mean body temperature of the sepsis group was significantly higher than that of the SIRS group, while systolic and diastolic blood pressure was lower in patients with sepsis. None of the other clinical parameters differed significantly between patients with sepsis or SIRS.

2.5.3 Mortality

The total number of patients who did not survive was 22; 15 and 7 in the sepsis and SIRS groups respectively. These patients had significantly lower platelet counts relative to those who survived (p <0.05).

2.5.4 Biomarkers of inflammation

The concentrations of circulating plasma cytokines and other biomarkers of infection and/or inflammation, and the total leukocyte and platelet counts of patients with sepsis or SIRS, as well as those for the survivors and non-survivors in the combined and individual groups are shown in Tables 2.4 and 2.5 respectively. With respect to comparison between the entire sepsis and SIRS groups of patients, the serum concentrations of CRP, PCT, IL-10, and IL-1Ra were significantly higher (p <0.006 – p <0.044), while leukocyte counts, IL-6, IL-17A and G-CSF tended to be higher, and platelet counts, IL-1β, IL-8 lower, albeit not significantly so, in the sepsis group. However, the concentrations of the other cytokines and biomarkers of inflammation, specifically TNF-α, GM-CSF, TGF-β1, NGAL, sTREM-1, MMP-9 and HMGB1 were comparable in both groups (Table 2.4).

With respect to comparison between the survivors (n=40) and non-survivors (n=22) for the combined sepsis (n=37) and SIRS (n=25) groups, platelet counts were significantly lower (p <0.02) and IL-6 elevated (p <0.015) in the group of non-survivors. Additionally, circulating concentrations of IL-1Ra and G-CSF appeared to be higher, while IL-1β, IL-17A, IL-8, and GM-CSF were lower in patients who did not survive, albeit not significantly so. These differences essentially mirror those detected when comparing the entire groups of sepsis and SIRS patients and may reflect the higher numbers of patients who succumbed to their infections in the sepsis group. This contention is supported by a comparison

of the corresponding data for the sub-groups of non-survivors shown in Table 2.5 which demonstrate an apparent association of mortality with elevated levels of IL-1Ra and decreased levels of IL-1β and IL-8.

Table 2.1: Demographic data of patients with sepsis and SIRS

	Total (n = 62)	**Sepsis (n = 37)**	**SIRS (n = 25)**
Male	39	21	19
Female	23	16	7
Age (mean ± SD)	43.7 ± 17.3	39.9 ± 15.4	48.2 ± 18.6

Table 2.2: The clinical diagnosis, systems affected and the micro-organisms identified from the patients with sepsis. The number of patients diagnosed with each condition and the type of specimen cultured is indicated in brackets.

System	Clinical diagnosis	Micro-organisms Identified
Respiratory	Community-acquired pneumonia (5)	*H. parainfluenzae (sputum)* *S. pneumoniae (blood)*
	Ventilator-associated pneumonia (2)	*S. aureus, K. pneumoniae (tracheal aspirate)*
	Pulmonary tuberculosis (4)	*M. tuberculosis (sputum)*
Gastrointestinal	Spontaneous bacterial peritonitis (2)	*K. pneumoniae, Enterobacter (ascitic fluid)*
	Liver abscess (1)	*E. histolytica (ascitic fluid)*
Musculoskeletal	Septic arthritis (1)	*P. mirabilis and S. aureus (pus)*
	Soft tissue infection (1)	*P. aeruginosa, S. aureus (pus)*
Urogenital	Urinary tract infection (3)	*Enterococcus, K. pneumoniae (urine)*
	Pyelonephritis (4)	*Enterobacter* spp. *(blood, urine)* *E. coli (blood)* *K. pneumoniae (blood, urine)*
Haematological	Neutropenic sepsis (1)	*S. epidermidis (blood)*
Neurological	Meningitis (2)	*S. pneumoniae, C. neoformans (CSF)*
Cardiovascular	Subacute bacterial endocarditis (1)	*E. faecalis (blood)*
Other	Septicaemia (9)	*S. aureus, E. faecalis, Enterococcus* spp., *K. pneumoniae, S. pneumoniae, E. coli, S. milleri (blood)*
	Malaria (1)	*P. falciparum (blood)*

Key: *H. parainfluenza = Haemophilus parainfluenzae, S. pneumoniae = Streptococcus pneumoniae, S. aureus = Staphylococcus aureus, K. pneumoniae = Klebsiella pneumoniae, M. tuberculosis = Mycobacterium tuberculosis, E. histolytica = Entamoeba histolytica, P. mirabilis = Proteus mirabilis, P. aeruginosa = Pseudomonas aeruginosa, E. coli = Escherichia coli, S. epidermidis = Staphylococcus epidermidis, C. neoformans = Cryptococcus neoformans, E. faecalis = Enterococcus faecalis, S. milleri = Streptococcus milleri, P. falciparum = Plasmodium falciparum,* spp. *= species.*

Table 2.3. The clinical diagnosis and systems affected for the patients with SIRS. The number of patients diagnosed with each condition is indicated in brackets.

System	Diagnosis
Respiratory	Bronchiectasis (1), COPD exacerbation (1), lung mass (1), pleural effusion (1), acute asthma (1), aspiration (1), lung nodules (1)
Gastrointestinal	Hepatic encephalopathy (1)
Trauma	Major trauma (2)
Urogenital	Chronic renal failure (1)
Haematological	Aplastic anemia (1), pancytopenia (1)
Neurological	Stroke (1)
Cardiovascular	Congestive heart failure (4), RV thrombus (1), hypertensive crisis (1), pericardial effusion (1), mitral valve disease (1), myocardial infarction (1)
Endocrine	Diabetic ketoacidosis (1)

Key: COPD = chronic obstructive pulmonary disease, RV = right ventricular

Table 2.4: The clinical parameters and biomarkers of patients with sepsis and SIRS, as well as survivors and non-survivors.

Clinical Parameters	**Sepsis (n = 37)**	**SIRS (n = 25)**	**Survivors (n = 40)**	**Non-survivors (n = 22)**
Temperature (^0C)	37.5 ± 1.28	36.9 ± 1.2*	37.2 ± 1.4	37.4 ± 1.2
Respiratory Rate (breaths/min)	24.6 ± 6.4	23.8 ± 4.8	24 ± 4.8	24 ± 7.7
Pulse Rate (beats/min)	111 ± 123	107 ± 18.4	106 ± 18.6	114 ± 165
SBP(mmHg)	117 ± 24.8	134 ± 38.5*	126 ± 35	118 ± 25
DBP(mmHg)	70.4 ± 122	84 ± 24. 4*	78 ± 20.4	12 ± 15.5
Biomarkers				
WBC	14.3 ± 10.8	11.3 ± 7.3	13.2 ± 9.9	13 ± 8.8
Platelets	228.4 ± 151	271 ± 180	278 ±169	180 ± 126*
CRP	229 ± 152	124 ± 135*	178 ± 160	208 ± 136
PCT	26.2 ± 47.8	5.0 ± 8.3*	14 ± 28.8	24 ± 52
TGF-β1	541 ± 511.5	757 ± 597	696 ± 566	474 ± 485
IL-1β	3.7 ± 9.9	10.4 ± 40.6	8.7 ± 32	1.5 ± 1.8
IL-17A	6.6 ± 14	2.4 ± 6	6.3 ± 13.5	3.0 ± 6.5
IL-6	472 ± 1133	245 ± 402	354 ± 998	426 ± 690*
IL-8	434 ± 1495	752 ± 2640	830 ± 2556	269 ± 314
IL-10	81.2 ± 293	9.8 ± 15*	54 ± 265	48 ± 112
IL-1Ra	1499 ± 2806	525 ±1222*	802 ± 1617	1720 ± 3347
GM-CSF	4.1 ± 9.9	5.1 ± 11.6	5.3 ± 11.4	2.6 ± 8
G-CSF	356 ± 1495	153 ± 434	128 ± 350	580 ± 2122
TNF-α	12.7 ± 14.7	11.1 ± 14.8	13 ± 12.9	11.7 ± 18
S-TREM	416.4 ± 360	408 ± 311	430 ± 369	366 ± 258
HMGB1	25.1 ± 24	21 ± 20.3	25.3 ± 23.4	20 ± 19.7
MMP-9	15.1 ± 5.9	13.8 ± 7.3	15.2 ± 6.4	13.6 ± 6.6
NGAL	199.4 ± 98.7	184 ± 114	191 ± 110	205 ± 93

Key: SBP = systolic blood pressure, DBP = diastolic blood pressure, WBC = white blood cell count, CRP = C-reactive protein, PCT = procalcitonin, TGF-β1 = transforming growth factor β1, IL = interleukin, IL-1Ra = interleukin 1 receptor antagonist, GM-CSF = granulocyte macrophage colony-stimulating factor, G-CSF = granulocyte colony-stimulating factor, TNF-α = tumor necrosis factor-α, s-TREM = soluble triggering receptor expressed on myeloid cells, HMGB1 = high mobility group box 1 protein, MMP-9 = matrix metalloprotein-9, NGAL = neutrophil gelatinase associated lipocalin, * $p < 0.05$.

Table 2.5: The clinical parameters and biomarkers of survivors and non-survivors in patients with sepsis and SIRS expressed as the mean ± SD.

	Sepsis		**SIRS**	
Clinical Parameters	**Survivors (n = 22)**	**Non-survivors (n = 15)**	**Survivors (n = 20)**	**Non-survivors (n = 7)**
Temperature (^0C)	37.6 ± 1.4	37.4 ± 1.2	36.7 ± 1.2	37.5 ± 1.3
Respiratory Rate (breaths/min)	25 ± 5	24 ± 8.7	24 ± 4.6	24 ± 6
Pulse Rate (beats/min)	110 ± 18	112 ± 16	102 ± 16.5	120 ± 18
SBP(MM Hg)	120 ± 27	111 ± 21	136 ± 43	132 ± 26
DBP(mm Hg)	73.5 ± 12.7	65 ± 9	84 ± 27	84 ± 18
Biomarkers				
WBC	16.1 ± 11.7	12.8 ± 9	10.4 ± 6.5	13.6 ± 9
Platelets	264 ± 162	164 ± 103	299 ± 185	207 ± 163
CRP	226 ± 169	234 ± 120	109 ± 126	159 ± 160
PCT	24 ± 35.4	30 ± 64	1.4 ± 1.9	13 ± 11
TGF-β1	632 ± 557	375 ± 380	787 ± 598	657 ± 629
IL-1β	4.9 ± 12.1	1.4 ± 1.6	13.8 ± 48	1.7 ± 2.2
IL-17A	8.4 ± 16.5	3.5 ± 7	2.5 ± 6.2	2.2 ± 5.8
IL-6	473 ± 1289	470 ± 818	205 ± 408	347 ± 397
IL-8	548 ± 378	225 ± 245	908 ± 3112	351 ± 425
IL-10	87 ± 349	70 ± 137	10 ± 17.7	9.3 ± 6.2
IL-1Ra	1266 ± 2055	1928 ± 3901	210 ± 234	1334 ± 2180
GM-CSF	4.2 ± 10.2	4.0 ± 9.8	7 ± 13.3	0
G-CSF	113 ± 217	805 ± 2506	149 ± 487	162 ± 286
TNF-α	11.8 ± 9.3	14.4 ± 22	12.8 ± 16.7	6.7 ± 7.6
S-TREM	442 ± 424	368 ± 198	426 ± 299	362 ± 363
HMGB1	26 ± 25.6	23 ± 21	24 ± 22	14 ± 16
MMP-9	16.2 ± 5.4	13 ± 6.4	13.6 ± 7.5	14.4 ± 7.3
NGAL	210 ± 106	182 ± 86	143 ± 107	252 ± 97

Key: SBP = systolic blood pressure, DBP = diastolic blood pressure, WBC = white blood cell count, CRP = C-reactive protein, PCT = procalcitonin, TGF-β1 = transforming growth factor β1, IL = interleukin, IL-1Ra = interleukin 1 receptor antagonist, GM-CSF = granulocyte macrophage colony-stimulating factor, G-CSF = granulocyte colony-stimulating factor, TNF-α = tumour necrosis factor-α, s-TREM = soluble triggering receptor expressed on myeloid cells, HMGB1 = high mobility group box 1 protein, MMP-9 = matrix metalloprotein-9, NGAL = neutrophil gelatinase associated lipocalin.

Table 2.6. Supplementary data: Serum cytokine and C-reactive protein (CRP) concentrations for 5 - 10 normal controls expressed as the mean ± SD.

IL-1β (pg/ml)	4.86 ± 3.0
IL-6 (pg/ml)	3.76 ± 4.46
IL-8 (pg/ml)	4.79 ± 6.8
IL-10 (pg/ml)	0.16 ± 0.36
IL-17 (pg/ml)	2.38 ± 5.32
IL-Ra (pg/ml)	78.9 ± 281.3
TNF-α (pg/ml)	15.9 ± 8.8
GM-CSF (pg/ml)	3.13 ± 8.43
G-CSF (pg/ml)	25.1 ± 19.7
MMP-9 (ng/ml)	2.8 ± 1.05
HMGB1 (ng/ml)	1.28 ± 0.96
CRP (μg/ml)	3.6 ± 4.1

Key: IL = interleukin, IL-1Ra = interleukin 1 receptor antagonist, GM-CSF = granulocyte macrophage colony-stimulating factor, G-CSF = granulocyte colony-stimulating factor, TNF-α = tumor necrosis factor-α, HMGB1 = high mobility group box 1 protein, MMP-9 = matrix metalloprotein-9, CRP = C-reactive protein.

2.6 Discussion

Patients with sepsis need to receive early, appropriate antimicrobial therapy in order to decrease the mortality associated with this condition (Gauer 2013; Taneja et al. 2017). However, differentiation of sepsis from SIRS in the clinical setting is often difficult. Although clinical parameters form an integral component of the clinical evaluation of all patients with SIRS (Kenzaka et al. 2012), the results of the current study suggest that only 2 clinical parameters, namely body temperature and blood pressure, may differentiate sepsis from SIRS, while heart rate and respiratory rate were similar in both groups of patients.

This limitation in respect of availability of reliable discriminating clinical parameters has led to a search for systemic biomarkers that may be used to differentiate SIRS from sepsis, with a wide range of these having been investigated in clinical trials (Faix 2013, Samraj, Zingarelli & Wong 2013). In the current study, a range of circulating cytokines and other inflammatory biomarkers was measured in patients with sepsis and SIRS. The patients with sepsis had significantly higher concentrations of CRP and PCT which may be indicative of bacterial infection. Of these biomarkers, increases in CRP are reported to parallel those of IL-6 and G-CSF with the cytokine appearing to function as a physiological component of the acute phase response (Noursadeghi et al. 2005). CRP, however, is a non-specific indicator of systemic inflammation and patients with non-infectious causes of SIRS may also have markedly increased serum concentrations of this acute phase reactant (Pepys & Hirschfield 2003). PCT has, on the other hand, been used to differentiate sepsis from SIRS (Biron, Ayala & Lomas-Neira 2015), but some patients with non-infectious causes of systemic inflammation may have increased levels of circulating PCT (Henriquez-

Camacho & Losa 2014). Therefore, in the clinical setting, CRP and PCT concentrations should be considered in conjunction with additional clinical information.

The host response to sepsis and non-infectious causes of systemic inflammation is usually characterized by a pro-inflammatory response that is counteracted by a compensatory anti-inflammatory response (CARS) (Mathias et al. 2015). In this context, IL-10 is a pluripotent anti-inflammatory cytokine that antagonizes the actions of potent pro-inflammatory cytokines such as TNF-alpha and IL-1 (Oberholzer, Oberholzer & Moldawer 2002). Interestingly, in the current study the concentrations of circulating IL-10, as well as those of another anti-inflammatory cytokine, IL-1Ra, were significantly greater in patients with sepsis compared to those with SIRS, a finding which is in keeping with the CARS response that accompanies sepsis. Both of these anti-inflammatory cytokines are released predominantly by macrophages during sepsis (Janson, Hance & Arend 1991; Arend & Guthridge 2000, Duque & Descoteaux 2014).

The profile of cytokines released during systemic inflammation may depend, however, on the nature of the stimulus (infectious or non-infectious) (Cavaillon 2006) and may differ when sepsis is caused by Gram-negative versus Gram-positive bacteria (Legrand et al. 2010). Furthermore, it has been found that higher IL-10 concentrations may correlate with increased levels of IL-6 released during the pro-inflammatory response (Friedman et al. 1997; Glynn et al. 1999), which also appeared to be the case in the current study in the sepsis group.

The magnitude of the increase of anti-inflammatory cytokines, including IL-10 and IL-1Ra, suggests that the CARS response in sepsis patients exceeds

that observed in patients with SIRS. Similar findings have been reported in previous studies of patients with sepsis or severe sepsis (Marie et al. 2000; Rodriquez-Gaspar et al. 2001), while others have found no differences (Torre et al. 2000; Tamayo et al. 2011). An additional noteworthy finding of the current study is the apparent inverse relationships between circulating concentrations of IL-Ra and those of IL-1β and IL-8 in the group of sepsis patients. This association was most pronounced in the groups of sepsis and SIRS non-survivors.

Although requiring confirmation in larger groups of patients, the consistency of this association between increased IL-1Ra with decreased IL-1β and IL-8 suggests that suppression of the host inflammatory response is a key contributor to mortality in both sepsis and SIRS, possibly reflecting the protective role of the nucleotide-binding domain and leucine-rich repeat containing protein (NLRP3) inflammasome against bacterial infection (Abderrazak et al. 2015; Rabes, Suttorp & Opitz 2016). This contention is, however, at variance with recent reports which advocate targeting of IL-1β and IL-18 as a therapeutic strategy (Shakoory et al. 2016) suggesting that timing of IL-1β–directed strategies may be critical (Osuchowski et al. 2006).

A significantly lower platelet count was also observed in the group of non-survivors, with similar, albeit insignificant trends observed in the 2 subgroups of non-survivors. In this context, it is noteworthy that platelet counts of less than $99x10^9/L$ have been reported to be predictive of mortality in sepsis patients admitted to intensive care units (Claushuis et al. 2016).

Limitations of the current study include the limited number of patients, mixed types of infection in patients with sepsis, as well as the variety of conditions causing SIRS in patients without sepsis. However, this broad spectrum of

diagnoses in both groups does mimic the clinical reality faced by many physicians. In additional the biomarkers' performance in identifying sepsis and SIRS were only assessed at presentation. Meaning, the conclusions cannot be drawn about the predictive value of the markers for the development of sepsis at a later stage or assess the impact of serial measurements. HIV tests were performed at the discretion of the clinician and not routinely. The HIV status of the patients could influence the results and need to be evaluated in future studies.

2.7 Conclusion

The findings of the current study have identified clinical parameters and biomarkers of inflammation that may be useful not only in differentiating sepsis from SIRS, but when used in combination may also identify those patients in both categories at highest risk of mortality. In this context a biomarker score based on IL-10, IL-1Ra, IL-1β, IL-8 and platelet count appears to be most promising based on the findings of the current study.

CHAPTER 3

Evaluation of the multiplex PCR and Fungitell assays for the diagnosis of fungal infections

3.1 Introduction

Fungal infection and sepsis are common problems in critically ill patients (Perrakis et al. 2012) and may also occur during the phase of immunoparalysis that develops in some patients with sepsis following the acute pro-inflammatory response to microbial pathogens (Sundar & Sires 2013). It is widely accepted that early antimicrobial therapy decreases morbidity and mortality in septic patients. However, fungal infections may be difficult to diagnose and are associated with a high mortality (Blot & Charles 2013, Posteraro et al. 2016). Microbial culture is still a preferred way of diagnosing invasive fungal infections, however this procedure has limited value due to the delay in acquisition of results and a high occurrence of either false-positive or false-negative results (Yeo et al. 2002, Chen et al. 2002, Morrell, Fraser & Kollef 2005, Garey at al 2006, Avni, Leibovici & Paul 2011, Riedel 2012, Clancy & Nguyen 2013, Arvanitis et al. 2014, Pfaller 2015). These issues necessitate the use of alternative diagnostic approaches, such as detection of the fungal-specific cell wall components, galactomannan, mannan and β-D-glucan, using serologic and enzymatic procedures.

It is therefore not surprising that there has been considerable interest in the implementation of these methods to achieve this goal (Kibe, Adams & Barlow 2011). The ultimate marker of infection would not only allow rapid identification of the microorganism in question, but also guide a target specific therapy (Faix 2013). This chapter focuses on the ability of different diagnostic procedures, namely culture, multiplex PCR and (1,3)-β-D-glucan in identifying fungal causes of sepsis (Kedzierska et al. 2007, Budhavari 2009, Theel & Doern 2013).

3.2 Methods

3.2.1 Fungitell assay for (1-3)-β-D-Glucan (BG)

The Fungitell assay (Associates of Cape Cod® international Inc., Knowsley, Liverpool, UK) is a protease zymogen-based colorimetric test, which qualitatively detects the presence of BG in the patient sera as a pathogen-derived biomarker of invasive fungal infection. Briefly, this assay is based upon specific Limulus amoebocyte lysate modification and employs the use of chromogenic peptide substrate. The presence of BG in the test sample (serum) causes activation of serine proteases in the lysate, which, in turn cleave p-nitroaniline from an added peptide substrate. During the series of experiments described in this chapter, the colored reaction product was measured spectrophotometrically with the magnitude of color intensity being directly proportional to the concentration of BG in the serum. The manufacturer's predefined value of 80 pg/ml was used as a cut-off.

3.2.2 Multiplex-PCR

Extraction, purification and PCR amplification of DNA are critical techniques used to determine genetic modification of an organism, or to identify microorganisms present in a single sample. In the current study the panel of fungal pathogens included: *Candida krusei, Candida glabrata, Candida albicans, Candida parapsilosis, Candida tropicalis and Aspergillus fumigatus.* By simultaneously amplifying multiple loci in the same reaction, multiplex PCR is gaining recognition both in research and clinical laboratories as a rapid and convenient screening assay. In the current study, the detection of causative fungal pathogens was performed using the Seeplex® sepsis DNA test from

Seegene (Bangyi-Dong, Songpa-Gu, Seoul, Korea) following the manufacturer's instructions.

This technique is a novel "dual primary oligonucleotide" (DPO) system, which has been designed to prevent non-specific priming. Unlike conventional priming systems, which utilize a single priming event, the DPO system utilizes two separate primer segments of unequal length joined by a temperature-labile polydeoxyinosine linker. Each primer segment has a preferential annealing temperature, with the longer 5' prime segment binding to the template DNA, conferring stable annealing, while the shorter 3' end binds selectively to its target site, preventing non-specific annealing. Accordingly, "only target-specific extension will result from the successive priming of the 5'-segment and the 3'-segment of the DPO" (Kommedal et al. 2012).

The assay incorporates three steps: i) preparation of sample by mechanical lysis and purification of fungal DNA, in which the target sequences are located between the 18S and 5.8S ribosomal sequences of fungi; ii) throughout the PCR reaction, specific products are determined using fluorescent dye-labelled hybridization probes designed to distinguish between the different species; and iii) separation of the amplified products by agarose gel electrophoresis and detection using UV light.

3.2.5 Microbial Cultures

Microbial and fungal cultures were performed by the Department of Microbiology at the Tshwane Academic Division of the National Health Laboratory Service (NHLS) as part of the standard laboratory evaluation of patients with SIRS. In addition to blood culture results, other positive culture results from samples such as urine, sputum, bronchial aspirate and CSF were analyzed. These results were used to group patients into sepsis and/or SIRS as described in chapter 2 *section 2.2.*

3.3 Statistical analysis

The statistical analysis was performed as described in chapter 2, section 2.4

3.4 RESULTS AND DISCUSSION

The multiplex-PCR assay detected the presence of fungal DNA in 18 of 62 patients tested. Thirteen of these patients were from the sepsis group and 5 from the SIRS group. Fungal DNA was detected from 11 patients who survived and 7 who did not survive.

The fungal species detected using the multiplex PCR assay were as follows: *Aspergillus fumigatus* (9 patients), *Candida parapsilosis* (6 patients), *Candida glabrata* (3 patients) and *Candida kruseii* (3 patients). Multiple fungal species were identified from one patient in the SIRS group.

Serum concentrations of β-D-glucan measured using the Fungitell assay were elevated (> 80 pg/ml = positive result) in 30 of the 62 patients tested. Of these, 18 were in the sepsis group and 12 from the patients with SIRS. These results did not correlate with those of the multiplex PCR as 4 patients with positive PCR results had β-D-glucan values < 80 pg/ml, while 19 patients with β-D-glucan concentrations > 80 pg/ml were negative using the PCR technique.

In addition, the results of both the multiplex PCR and the Fungitell assays did not correlate with either the clinical findings or microbiological cultures. Importantly, the β-glucan test presented with a greater number of false-positive results, and is not considered suitable for screening purposes and thus for the selection of patients that need pre-emptive antifungal therapy (Racil et al. 2010, Arvanitis et al. 2014). Likewise, interpretation of PCR fungal detection results is not always clear-cut given that fungi are frequent colonizers of humans such that the presence of fungal DNA may represent either an active infection or colonization (*Stevens 2002,* Arvanitis et al. 2014). This suggests that results

obtained when using these techniques to diagnose fungal infections should be interpreted cautiously in the context of clinical and histological findings. Clearly, intensive investigation is necessary to clarify the role of both PCR and serological techniques in the diagnosis fungal infections in patients with sepsis.

Although not shown, there was lack of correlation between microbial culture and multiplex PCR results. However, it holds the truth that molecular assays can assist in shortening the time to results, while shortcomings include an incomplete coverage of pathogens, the inability of the test to be applied directly to a biological sample, and restricted information regarding antimicrobial susceptibility. This has led to the recommendation that multiplex PCR assay can be used to supplement but not replace the traditional microbial culture.

3.5 Conclusion

The utility of the Fungitell and multiplex PCR assays for diagnosing fungal infections in patients with sepsis could not be confirmed in the current study.

CHAPTER 4

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