Investigating the biomarker potential of circulating small extracellular vesicles in patients presenting with sepsis.

by

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PREFACE

The experimental and research work described in this thesis was conducted at the University of KwaZulu-Natal (Durban, South Africa) and Nuffield Department of Women's and Reproductive Health, University of Oxford (Oxford, United Kingdom) from March 2021 to December 2024, under the supervision of Professor Irene Mackraj.

This work has not been submitted in any form for any degree to any tertiary institution, and where use has been made of the work of others, it is duly acknowledged in the text. The results reported are from investigations conducted by the candidate.

[redacted]

11 December 2024

R. Bhagwan-Valjee **Date**

As the candidate's supervisor, I agree to the submission of this thesis.

[redacted]

11 December 2024

Professor I. Mackraj **Date**

DECLARATION

I, Roushka Bhagwan-Valjee, declare that:

i. The research reported in this thesis, except where otherwise indicated, is my original work.
ii. This thesis has not been submitted in part or full for any degree or examination at any other university.
iii. This thesis does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.
iv. This dissertation does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced.
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v. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source detailed in the thesis and the references sections.
vi. My contribution to the project was as follows:

Identification of research topics, experimental design, execution, data analysis and interpretation, manuscript and thesis write-up.

The PhD candidate performed experimental work described in this thesis, and where others have contributed, they were duly acknowledged in the text. The candidate drafted this publication in full, and it has been reviewed by co-authors.

11 December 2024

R. Bhagwan-Valjee **Date**

PUBLICATIONS AND CONFERENCE OUTPUT

Peer-reviewed publications contributing to this thesis:

Bhagwan Valjee, R., Ibrahim, U. H., Xulu, K., Mahomed, S., & Mackraj, I. (2023). Circulating exosomes in sepsis: A potential role as diagnostic biomarkers, therapeutic and drug delivery carriers. Smart Materials in Medicine, 4, Pages 639-647. DOI: https://doi.org/10.1016/j.smaim.2023.06.007 **(Chapter Two of this thesis)**

Bhagwan Valjee, R., Mackraj, I., Moodley, R., & Ibrahim, U. H. (2024). Investigation of exosomal tetraspanin profile in sepsis patients as a promising diagnostic biomarker. Biomarkers, 29(2), 78–89. DOI: https://doi.org/10.1080/1354750X.2024.2319296 **(Chapter Three of this thesis)**

Conference Output:

Bhagwan Valjee, R., Mackraj, I., Moodley, R., & Ibrahim, U. H. Investigation of exosomal tetraspanin profile in sepsis patients as a promising diagnostic biomarker.

Presented at the UKEV Forum 2022, 1st and 2nd December 2022, Royal College of Physicians of Edinburgh, United Kingdom. (Invite listed in **Appendix 7.6**)

Abstract published in the UKEV Forum 2022, Programme and Abstract Book**. (Appendix 7.7)**

STATEMENT

The following publications/manuscripts have been included as chapters in this thesis

Chapter Two: Circulating exosomes in sepsis: A potential role as diagnostic biomarkers, therapeutic and drug delivery carriers. (**Published**: Smart Materials in Medicine, 2023, pages 639-647)

Chapter Three: Investigation of Exosomal Tetraspanin Profile in Sepsis Patients as a Promising Diagnostic Biomarker. (**Published**: Biomarkers, 2024, pages 78–89)

Chapter Four: Characterizing the surface marker profiles of circulating small extracellular vesicles in sepsis patients. (**Submitted**: Smart Materials in Medicine)

For the preparation of the above manuscripts, the candidate performed all the experimental and research work and interpreted the data. The co-authors contributed to editing and verifying the scientific content as well as editing the manuscripts.

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LIST OF ABBREVIATIONS

APACHE- Acute Physiology and Chronic Health Evaluation

APCs- Antigen-presenting cells

BP- Blood pressure

CDC- Centres for Disease Control and Prevention

CRP- C-reactive protein

DAMPs- Damage−associated molecular patterns

DBP- Diastolic blood pressure

DNA- Deoxyribonucleic acid

DNMT- DNA methyltransferase

EDTA- Ethylenediaminetetraacetic acid

ELISA- Enzyme-linked immunosorbent assay

ESCRT- Endosomal sorting complex required for transport

EVS- Extracellular vesicles

HIV- Human Immunodeficiency Virus

HMGB-1- High-mobility group box-1

ICU- Intensive Care Unit

IFNs- Interferons

IL- Interleukins

ILVs- Intraluminal vesicles

IR- Inflammatory response

ISEV- International Society for Extracellular Vesicles

KEGG- Kyoto Encyclopedia of Genes and Genomes

LC-MS/MS- Liquid chromatography coupled with tandem mass spectrometry

LPS- Lipopolysaccharide

MFI- Median Fluorescent Intensity

miRNA- Micro-Ribonucleic acid

MISEV- Minimal Information for Studies of Extracellular Vesicle

MOD- Multiple-organ dysfunction

mRNA- Messenger Ribonucleic acid

MVBs- Multicellular bodies

MVs- Microvesicle

NETs- Neutrophil extracellular traps

NF-κB- Nuclear factor-κB

NK- Natural Killer cell

NLR- Neutrophil-lymphocyte ratio

NODs- Nod-like receptors

NTA- Nanoparticle Tracking Analysis

P2X7- Purinoreceptor

PAMPs- Pathogen−associated molecular patterns

PBS- Phosphate Buffered Saline

PCT- Procalcitonin

PD- Programmed cell death

PRRs- Pattern recognition receptors

PTEN- Phosphatase and tensin homolog protein

RAAS- Renin-angiotensin-aldosterone system

ROS- Reactive oxygen species

SBP- Systolic blood pressure

Sepsis 3- Third International Consensus for Sepsis and Septic Shock

sEVs- Small extracellular vesicles

SIRS- Systemic inflammatory response syndrome

SNS- Sympathetic nervous system

SOFA- Sequential Organ Failure Assessment

SP-IRIS- Single-particle interferometric reflectance imaging sensor

TB- Tuberculosis

TEM- Transmission Electron Microscopy

Th- T helper

TLRs- Toll-like receptors

TNF-α- Tumor necrosis factor-alpha

Treg- Regulatory T cell

VCAM-1- Vascular cell adhesion molecule-1

WHO- World Health Organization

ABSTRACT

Sepsis is defined as an inflammatory disorder caused by a dysregulated immune response to infection, affecting approximately 48.9 million people globally. The complex and multifaceted pathophysiology of sepsis complicates clinical diagnosis, management and treatment. Without a validated gold standard for diagnosis, definitive biomarkers are critical. Recently, small extracellular vesicles (sEVs) and exosomes[1] have emerged as promising biomarkers of disease, owing to their role in intercellular communication. Therefore, the present study has evaluated the use of sEVs as possible biomarkers for sepsis. The approach included providing a theoretical framework surrounding sEVs, their role in sepsis pathophysiology and the potential these sEVs have as diagnostic, prognostic and therapeutic tools for sepsis. This led to the evaluation of commonly used surface markers for identifying extracellular vesicles using a multi-level, automated and precise platform. We identified an abundance of CD63, CD81 and CD9 positive particles in sepsis patients. Interestingly, the data provided distinct colocalization patterns which are abundant in sepsis patients. Our study highlighted that the CD63/CD9 colocalization may be of increasing interest in exploring the potential of these vesicles as biomarkers for sepsis. Furthermore, developing biomarkers for sepsis has become increasingly difficult due to the presence of co-morbidities. These co-morbidities further complicate the pathophysiology of sepsis, increasing the mortality rate and the risk of developing organ failure. Additionally, previous studies confirm a vast array of surface markers on the membrane of sEVs. These markers have roles in protein-protein interactions and target cell signalling. We then, further investigated the surface marker profiles in 4 cohorts (viz. healthy controls, hypertension, sepsis patients with no pre-existing co-morbidities and sepsis patients with previously diagnosed hypertension). In doing so, we identified specific surface marker profiles of sepsis and hypertension when compared to healthy controls or the other pathological states. These surface marker profiles could aid in the further understanding of sepsis pathophysiology and the role sEVs play therein. This study highlights that sEVs and the identified surface marker profiles could be exploited in the interest of biomarker development for sepsis.

[1] The terms “small extracellular vesicles” and “exosomes” are used in this thesis as the International Society for Extracellular Vesicles (ISEV) has updated the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines regarding appropriate nomenclature- new guidelines published as of 2024

1. CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Sepsis is the cause of more than 50% of deaths that occur in the Intensive Care Unit (ICU) and is characterized by hyper-inflammation as a result of a dysregulated immune response to infection [1, 2]. The heterogeneity of the disease has led to many aspects of the pathophysiology not being fully understood. Briefly, it is divided into two phases, the hyper-inflammatory phase and the prolonged immunosuppressive phase, ultimately leading to end-organ dysfunction and/or failure and death [1]. Sepsis remains one of the leading causes of mortality globally due to the lack of biomarkers for early diagnosis [3]. Timeous initiation of treatments is crucial in lowering the risk of mortality for patients with sepsis, hence researchers are investigating several biomarkers, although efforts have failed to validate these markers for application within clinical settings.

In recent years extracellular vesicles, specifically small extracellular vesicles (sEVs) or exosomes, have become increasingly popular as possible biomarkers for various diseases [4]. These small (30-200nm) vesicles are composed of specific cargo which reflect their cells of origin [5]. During normal physiological and pathological states, sEVs act as intercellular communicators through the cargo they carry. These vesicles can influence the phenotype of their target cells and are reflective of various cellular responses, making them ideal candidates for biomarkers [6].

Recent research has shown that the abundance of sEVs in sepsis patients is significantly higher than in normal healthy individuals, therefore associating the number of extracellular vesicles with disease progression [7]. Furthermore, it has been suggested that sEVs are implicated in the pathophysiology of sepsis by inducing inflammatory pathways and exacerbating immunosuppression. However, research on the involvement of sEVs in sepsis is limited.

The biological properties of sEVs indicate that they are strong contenders as biomarkers for sepsis. The molecular patterns within and on sEVs can aid in the diagnosis of sepsis as well as monitoring disease progression in these patients [8]. The implication of sEVs in the pathophysiology of sepsis could assist in stratifying patients into more heterogenous groups for proper treatment decisions. Therefore, this necessitates research into elucidating the role and characteristics of sEVs in the pathogenesis of sepsis to ultimately aid in their clinical application as biomarkers.

1.1 Sepsis definition and epidemiology

In 2015, The Third International Consensus for Sepsis and Septic Shock (Sepsis 3) redefined sepsis as "a life-threatening organ dysfunction caused by a dysregulated host response to infection." [9-11]. Sepsis was previously defined as a medical condition whereby the dysregulated immunological response to infection causes systemic inflammation, leading to end organ failure and death [1, 7]. Hippocrates, a Greek physician gave this medical condition its name as he considered it "the process by which flesh rots and wounds fester." [2, 10]. Over the past three decades, the definition of sepsis has evolved substantially. In 1991 sepsis was defined as "The systemic response to infection, manifested by two or more of the Systemic inflammatory response syndrome (SIRS) criteria as a result of infection." [10]. Organ dysfunction is identified as an acute increase of two or more points in the Sequential Organ Failure Assessment (SOFA) score [12]. The SOFA score is usually calculated on admission into the ICU and every 24 hours thereafter. The SOFA score accurately reflects the functions of the organ systems, by incorporating six criteria (respiratory, neurological, cardiovascular, hepatic, renal and haematological) for the calculation, by allocating a score of 0-4 per criterion [13, 14]. Affecting approximately 30 million people globally and causing an estimated 6 million deaths annually, sepsis is considered to be one of the key causes of mortality in severely ill patients [1, 7].

According to the 2020 Centres for Disease Control and Prevention (CDC) updates, a minimum of 1.7 million American adults develop sepsis annually. Sepsis is the cause of approximately 270 000 deaths among Americans and an alarming one in three deaths in hospitals (: https://www.cdc.gov/sepsis/datareports/index.html). There is an alarming rate of >50% of the total mortality in ICUs that is caused by sepsis [11, 15]. Sepsis patients have hospital stays eight times longer and a higher risk of death during hospitalization, therefore sepsis remains a major public health challenge worldwide [11]. In a recent investigation performed by Rudd et al, there were an estimated 48.9 million incident cases of sepsis recorded worldwide with 11 million sepsis-related deaths in 2017. This represents 19.7% of the total global deaths [16]. Rudd and colleagues showed that the incidence and mortality rate decreased from 1990-2017, however, it importantly stated that the incidence and mortality varied markedly between different regions with the highest burden in Sub-Saharan Africa, Oceania, South Asia, East Asia, and Southeast Asia, with 85% of global cases occurring in low and middle-income countries [16, 17]. According to the previously mentioned study, the World Health Organization

(WHO) stated that in 2017 approximately 50% of the global sepsis cases were attributed to children, with an estimated 20 million cases and 2.9 million global deaths in children under 5 years of age. A review published in 2016, stated that person/s affected by an infection, injuries of severe complications or serious non-communicable disease are at risk of developing sepsis, however, there are vulnerable populations also at a high and increasing risk including but not limited to: the elderly, pregnant or newly pregnant women, neonates, hospitalized patients (especially those admitted into ICUs), those with weakened immune systems and/or chronic illnesses [18]. Previous studies indicate that sepsis predominantly affects the elderly, stating that elderly patients account for 64.5% of sepsis patients and that the majority of these infections identified in sepsis patients are caused by gram-negative bacteria [19, 20]. These studies show that in developed nations, the common causes of infections are pneumonia, urinary tract infections and intra-abdominal infections [19, 20]. Co-morbidities such as diabetes and hypertension significantly increase the rate of mortality [20]. In contrast to this, the epidemiological data of sepsis patients in developing countries have not been extensively studied and documented [21]. A study conducted by Martin-Loechez et al., in Europe shows that age represents an independent mortality-associated risk factor in the elderly, as they show that mortality rates in patients 80 years and older are significantly higher than those aged 60-75 years as well as the younger cohort of patients [22]. However, before this study, studies conducted in 2012 and 2017, as well as a survey in 2018, stated that age is not a critical basis risk factor for survival in elderly patients [23-25]. A retrospective chart review conducted in South Africa (Durban, KwaZulu-Natal), by Ndadane et al., specified that the majority of the sepsis cases identified were below the age of 60. In Africa, there is a high prevalence of infectious diseases such as Human Immunodeficiency Virus (HIV) and Tuberculosis (TB) which could be a possible cause of the increased rates of sepsis in the younger population (<60 years). Ndadane et al., found that lower respiratory tract infections were the primary cause of sepsis, in their cohort, as well as meningitis, which the authors concluded may be attributed to HIV co-infection with central nervous system infections [21]. Data on the demographic profile and epidemiology of sepsis in South Africa is severely limited, with studies only reporting on the prevalence of sepsis in a specific study site (i.e. a particular hospital). An example of this is the previously mentioned study by Ndadane et al., who reported that 40.3% of patients admitted, with the diagnosis of infection, to the emergency centre of a particular district hospital in Durban, South Africa, met the criteria for severe sepsis [21]. Understanding the epidemiological patterns of sepsis in South Africa will aid in improved disease management and contribute to a more accurate global representation of sepsis prevalence.

1.2 Sepsis pathophysiology

Sepsis is considered a heterogeneous and multifaceted disease, with multiple physiological systems being affected [11, 26, 27]. Even now, the actual mechanisms underpinning the pathophysiology of sepsis are still not fully understood. In recent years, the pathophysiology of sepsis and/or septic shock has been considered biphasic [1]. The death distribution of sepsis has been considered to peak in the initial phase due to the overwhelming inflammatory response, also known as the "cytokine storm" [11, 28, 29]. This phase of sepsis is attributed to an overwhelming inflammatory response (cytokine storm) whereby there is a drastic increase in the release of pro- and anti-inflammatory cytokines [2]. It is known as the "hyperinflammatory phase" which results in several signs and symptoms used to diagnose sepsis. The cytokine storm usually results in fever, early cardiac and pulmonary failure, endothelial dysfunction, refractory shock, and difficulty breathing [30]. If left uncontrolled, the high levels of pro-inflammatory cytokines result in excessive tissue damage leading to septic shock and/or multiple-organ dysfunction (MOD) [31]. Septic shock is defined as the most severe complication of sepsis. Septic shock is a result of a drastic drop in blood pressure that can cause damage to various vital organs [32]. The second phase of sepsis is known as the "immunosuppressive phase". During this state, the body is less effective at fighting off infections as the immune system has deteriorated, which is characterized by dysfunctional immune cells (reduced antigen presentation and cytokine production), increased immune cell death, reduced immune responses, and the excessive release of anti-inflammatory cytokines [33, 34]. The death distribution of sepsis shows that there is also a late peak in mortality during this phase, occurring several weeks to months later, caused by prolonged immunosuppression leading to organ injury and sometimes multi-organ failure [28].

1.2.1 Hyperinflammatory phase

Sepsis is considered a heterogeneous disease as multiple physiological systems are affected during the progression of sepsis [35]. It is defined as a dysregulated immune response to infection. The host response is initiated by both pathogen-associated molecular patterns (PAMPs), such as endotoxin and lipoteichoic acid, and damage-associated molecular patterns (DAMPs), such as heat shock proteins, high-mobility group box-1, nucleotides, and mitochondria released from injured host cells [1, 12]. PAMPs and DAMPs bind to specific pattern recognition receptors (PRRs). Immune cells express PRRs on the extracellular surfaces [of antigen-presenting cells (APCs) and monocytes], including some Toll-like receptors (TLRs)

and in the cytosol called Nod-like receptors (NODs) [30, 36]. The activation of TLRs by PAMPs and DAMPs stimulates the activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factor pathways to release inflammatory cytokines [29, 30, 37]. In the coronary endothelium, adhesion molecule expression (such as vascular cell adhesion molecule-1 (VCAM-1)), stimulated by pro-inflammatory cytokines, contributes to inflammation and neutrophil infiltration in the myocardium, thereby reducing cardiomyocyte contractility [37]. Inflammatory markers include pro-inflammatory interleukins (IL), e.g., IL-1, IL-12, IL-18, tumor necrosis factor-alpha (TNF-α), and interferons (IFNs), which subsequently initiate the activation of additional cytokines (e.g., IFN-y, IL-6, IL-8), complement and coagulation pathways, and, by negative feedback, downregulation of components of the adaptive immune system [38, 39]. The activation of the adaptive immune system occurs by antigen-presenting cells (APCs), dendritic cells, and B and T lymphocytes [40]. Pathogen-specific antibodies with immunological memory are produced by the slower initiation of the adaptive immune system [41]. Therefore, the pathophysiology of sepsis and the progression of sepsis is influenced by both pro- and anti-inflammatory pathways [30].

1.2.2 Immunosuppression phase

The term "immune exhaustion" describes the immunosuppression induced by sepsis and this is attributed to the depletion of immune cells via apoptosis [30, 42]. Apoptosis plays a vital role in determining immune cell populations and, therefore, maintaining the immune response [42]. The regulated cell death of immune cells by apoptosis is advantageous to the host as this diminishes the inflammatory response in sepsis [43]. Conversely, a vast depletion in immune cells could prevent the host response from eliminating the invading pathogen [11]. The types of immune cells which are affected by apoptotic cell depletion are lymphocytes (T and B lymphocytes), T helper (Th)1/Th2 ratio, circulating Th17 and regulatory T (Treg), neutrophils, macrophages, and dendritic cells [44, 45]. The depletion of lymphocytes in sepsis correlates with the severity of sepsis in the patient. Therefore, studies show that sepsis survivors' lymphocyte populations were higher when compared to the non-survivors [46]. Apoptotic depletion occurs with cells representing a specific inflammatory response (CD4+ T and B cells) and those representing a non-specific inflammatory response (macrophages/monocytes, dendritic cells, NK cells, γδ T cells) [47]. Immune cells such as neutrophils and platelets are involved in coagulation dysfunction in sepsis. Coagulation dysfunction is attributed to the activation of the coagulation cascade by tissue factors released from damaged endothelial cells [48]. Hyperinflammation causes damage to the endothelium causing dysregulated vascular

tone and impairing vascular permeability [45]. Platelet aggregation is stimulated by neutrophil extracellular traps (NETs), which are released by activated neutrophils which have adhered to damaged endothelial cells [18]. The successive activation of the coagulation systems causes increases in the formation of fibrin-rich microvascular clots in sepsis. This then leads to loss of smooth muscle tone reactivity and peripheral vasodilation, with ensuing organ hypoperfusion, impaired oxygen delivery to the tissues, and anaerobic glycolysis lactic acidosis [30]. Therefore, sepsis is described as a multifaceted disease triggering cascades of various systems.

The complex, multifaceted pathophysiology of sepsis restricts the effective diagnosis and treatment of all manifestations of the disease. To date, sepsis treatment includes antibiotic therapies, ventilator management, blood glucose maintenance, and resuscitative strategies, as well as supportive therapies that are recommended by the Surviving Sepsis Campaign [49, 50]. However, persistent infections are responsible for 70–80 percent of sepsis deaths, revealing widespread antimicrobial resistance and the lack of effective treatment choices [51]. Antibiotic treatment within the first hour following sepsis diagnosis is crucial in reducing the mortality rate of sepsis patients. There is also a subset of patients diagnosed with sepsis who have non-infectious causes, and with these patients rapid antibiotic administration increases the risk of antibiotic resistance [32]. It is known that antibiotic resistance adds to the burden and jeopardizes sepsis management efforts. With these restricted treatment options, there is an urgent need for novel biomarkers that can readily predict the possibility of a person developing sepsis and track its progression to efficiently avoid treatment failure due to unintended effects on interacting processes or improper timing of treatment initiation [37].

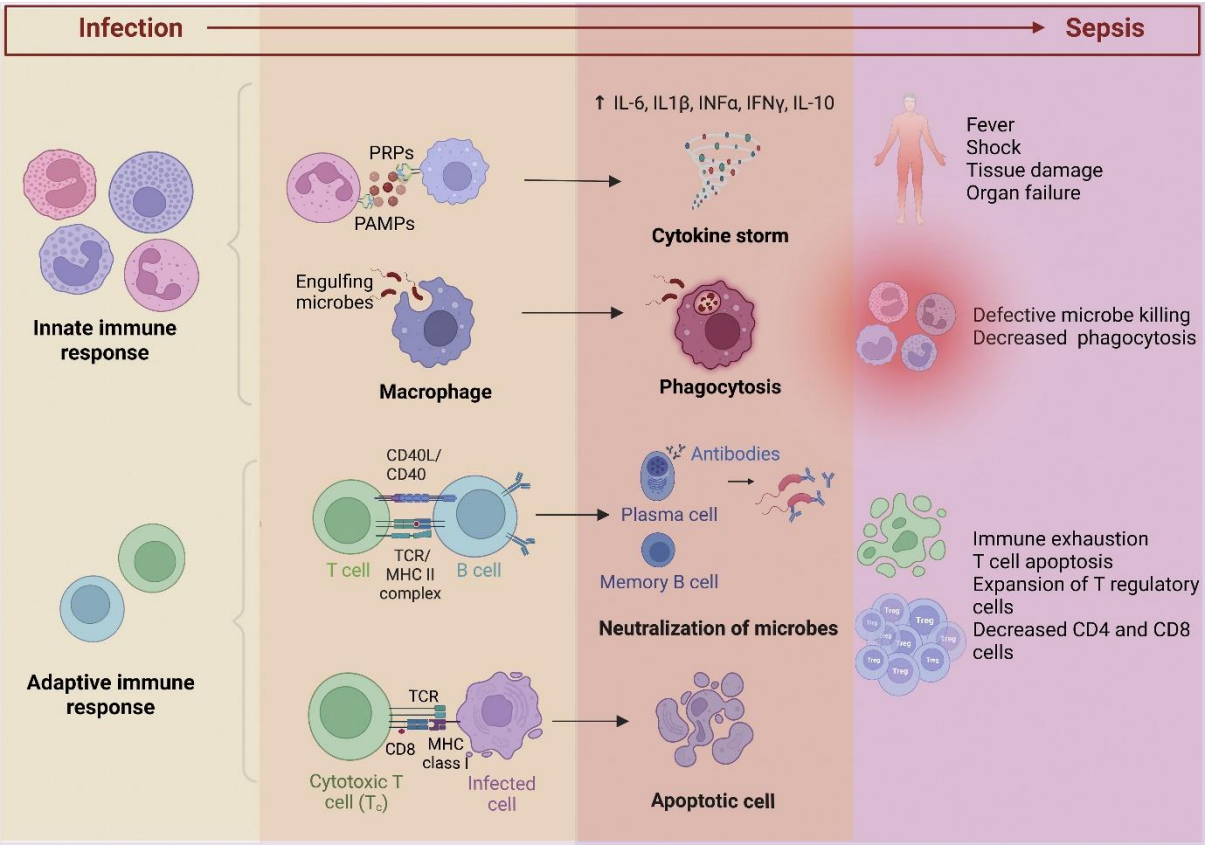

Figure 1 Activation of the innate and adaptive immune responses during sepsis. The inflammatory response is caused by the systemic activation of the immune system and is characterized by the rapid increase of pro-inflammatory cytokines also known as the cytokine storm. The cytokine storm is associated with fever, shock and tissue/organ damage. The adaptive immune response causes the production of pathogen-specific antibodies which have immunological memory. The apoptotic depletion of immune cells and immune exhaustion is caused by sepsis-induced immunosuppression [30].

1.3 Biomarkers of sepsis: The current state

Early sepsis detection is critical to initiate appropriate treatment methods timeously [3]. Several markers have been proposed for the diagnosis of sepsis, but are still not ready to be deployed widely in various clinical settings, due to limited accuracy and none of these markers are specific to sepsis and may be elevated in other diseases [1, 52]. Based on a sound understanding of the pathophysiology of sepsis, novel diagnostic and therapeutic interventions are possible for a heterogenous patient population [47]. Biomarkers that include expression patterns such as in sEVs/exosomes (as discussed below) may aid in stratifying patients into

more homogeneous subgroups or in developing targeted therapeutic interventions within the realms of personalized medicine.

The outcome of sepsis for patients depends on the timely initiation of effective treatments. Regarding the case of patients admitted to general wards and those in post-surgery recovery, this has proven to be significantly challenging and may also contribute to high mortality rates in this population. Since sepsis symptoms are ambiguous, a significant cause for concern is a delayed diagnosis. Novel biomarkers have been thoroughly investigated in recent years. However, there is still no gold standard for diagnosis and no particular biomarker that is employed in a clinical context for specific diagnosis. The definition of biomarkers has evolved since the term was first used in 1973. Currently, the term biomarker stands as *"a defining characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention"* [53].

Biomarkers that have been studied for sepsis vary from the action of particular cells to the state/phase of the septic patient i.e., hyperinflammatory phase and/or immunosuppressive phase. Lactate, procalcitonin (PCT) and C-reactive protein (CRP) are among the most popular markers used in the clinical setting.

1.3.1 Lactate

Blood lactate concentrations show the difference between lactate production and clearance [54]. Hyper-inflammatory states or disorders such as sepsis cause an increase in the production of lactate by accelerated glycolysis [54, 55]. During cell hypoxia, lactate is produced from pyruvate, a metabolite of glucose. Glucose is converted to pyruvate via the aerobic pathway; therefore, when under stress (cell hypoxia), this causes a switch from aerobic to anaerobic metabolism, leading to the overproduction of lactate [56]. Typically, the liver removes the majority of the lactate. Therefore, liver diseases cause a diminished removal of lactate. This leads to speculation that lactate may be more of a biomarker of organ dysfunction. Elevated levels of lactate have been used as a marker of sepsis-induced hypoperfusion and are highly associated with high mortality rates [57]. The SEPSIS-3 definition states that sepsis patients can be identified by a serum lactate level of >2 mmol/L in the absence of hypovolemia [9]. The systemic metabolism of lactate can be influenced by various factors and therefore suffers from a lack of specificity as a biomarker of sepsis [58]. A study by Singer et al., reported that the sensitivity of lactate measurements as low as 2mmol/L has a low sensitivity for identifying sepsis at all stages of the disease, whereas elevated lactate measurements at 4mmol/L or more

have a high to very high sensitivity [59]. Although monitoring the lactate levels may be helpful with patients' response to therapy, lactate levels have not been able to provide therapeutic endpoint details for patients.

1.3.2 PCT and CRP

Procalcitonin (PCT) and C-reactive protein (CRP) are produced in response to infections and/or inflammation. Under normal circumstances, PCT is produced in the parathyroid glands, but during infections, PCT is produced by the neuroendocrine cells of the lungs and intestines [60]. The release of PCT is facilitated by pro-inflammatory cytokines such as TNF-α and IL-6 [55]. Studies in animal models show a significant rise in PCT during early sepsis. With PCT being a reliable biomarker for infections, studies have shown that the rise of PCT in sepsis does not correlate with the severity of the disorder or the mortality rates [61, 62]. A systematic review investigating the diagnostic accuracy of PCT, reported that the overall diagnostic performance was low with sensitivity and specificity being 71% [63]. PCT, although marginally more specific than CRP, may be elevated in patients following major surgery or in conditions such as pancreatitis [52]. CRP is produced in the liver, and its release is mediated by IL-6. CRP has been widely studied as a biomarker of sepsis as it is a promising biomarker of inflammation [61, 62]. Normal CRP measurements are usually <1.0mg/dL. However, studies show that while there is a significant rise in the CRP levels in patients with sepsis, severe sepsis, and septic shock, the overall specificity of CRP is debatable, as elevated CRP levels are characteristic of other inflammatory disorders such as autoimmune diseases and even severe trauma, and are influenced by sex, age, and co-morbidities [61, 64]. CRP sensitivity and specificity vary with cutoff values. A study showed that using 50 mg/L as a cutoff value, the assessment of CRP was sensitive to sepsis patients but not specific enough, indicating the use of a high cutoff value still introduced diagnostic inaccuracy [61]

1.3.3 Other biomarkers

Pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, initiate the first response of the innate immune system to injury and/or infection and are considered to be biomarkers of the hyperinflammatory phase of sepsis [55]. It has been shown that TNF-α and IL-1β are not specific to sepsis. IL-6 has been the most popularly studied pro-inflammatory cytokine as a sepsis biomarker, as studies show that elevated IL-6 levels correspond with the severity of sepsis and mortality rates [65, 66]. On the other hand, it has been suggested that the use of IL-6 as a biomarker is more prognostic than it is diagnostic, as it may help detect which patients with sepsis are at risk of severe sepsis [67, 68]. C5a, a pro-inflammatory peptide, causes the

activation of the complement cascade. The complement cascade has been proven to stimulate the inflammatory state during sepsis [69]. Activation of the complement cascade enhances phagocytotic properties by complement protein 3 (C3) [55]. Although C5 has been considered to be a biomarker of complement proteins, its use as a biomarker of sepsis is not widely used as C5a has both pro-inflammatory and anti-inflammatory roles during sepsis. Biomarkers of activated monocytes and neutrophils and biomarkers of the immunosuppressive state of sepsis have also been studied. Each marker lacks specific biomarker qualities to be used in the clinical setting as a diagnostic tool for sepsis as none of these markers are 100% specific to sepsis and the dynamic nature of cytokines over time [52, 70]. TNF-α has been shown to correlate with sepsis-related mortality but not with the microbiological cause of sepsis [70], whereas IL-6 may be elevated in other diseases such as cancer and cardiovascular disease [71, 72]

1.4 Extracellular vesicles

Understanding intercellular communication in multicellular organisms, such as humans, is essential in unravelling pathophysiological mechanisms that underpin disorders such as sepsis. Extracellular vesicles (EVs) are lipid-bound vesicles secreted by eukaryotic cells into the extracellular space to conduct functions such as cell-to-cell communication [73-75]. EVs originate through two primary pathways: the endosomal system and plasma membrane budding [76]. EVs contain biomolecules from parent cells which are delivered to target cells which leads to alterations in the target cells' function and morphology [75]. Recently EVs have been recognized to be vital in the role of intercellular communication [77]. EVs can be classified into three main subtypes, namely, ectosomes/microvesicles (MVs), exosomes and apoptotic bodies [74, 78, 79]. The sub-classification of EVs is based on the origin, biogenesis, release pathways, size, physiochemical properties and functions specific to each [74]. These physiochemical properties play critical roles in pathological conditions such as disease states and infections. Biochemically, the composition of these EVs differs based on the source of the numerous cells they are secreted from [73].

1.4.1 Small extracellular vesicles

1.4.1.1 Biogenesis

Small extracellular vesicles (sEVs) are small EVs which include exosomes and microvesicles. The primary distinction between the two is the mechanism of biogenesis. Exosomes are

intraluminal vesicles (ILVs) that are secreted from multivesicular bodies (MVBs) upon fusion to the plasma membrane. Intraluminal vesicles are formed by the inward budding of MVB membranes [74, 80]. Early endosomes are formed by endocytosis of the plasma membrane and mature into late endosomes. Following the maturation of the late endosome, invagination of the membrane occurs forming ILVs in larger MVBs [81]. During this process, encapsulation of extracellular soluble proteins within the ILVs occurs with surface proteins present on the invaginated membrane [82]. Thereafter, MVBs fuse with the plasma membrane, releasing enclosed ILVs into the extracellular space via exocytosis. These enclosed ILVs are referred to as exosomes [83]. The synthesis and secretion of exosomes require the endosomal sorting complex required for transport (ESCRT) function. The ESCRT mechanism comprises four separate ESCRT proteins (ESCRT 0, I, II, III) and associated proteins (VPS4, TSG101, and ALIX). The ESCRT mechanism recognises ubiquitinated proteins and is responsible for the deformation of the membrane to promote budding. The cargo loading of exosomes involves ESCRT-independent pathways which utilise lipids and related proteins such as cholesterol, lipid rafts, sphingolipids, ceramide and tetraspanins. The ESCRT-independent pathways are pivotal for the sorting of cargo into exosomes as well as the variation in size and quantity. Microvesicles are formed through direct outward budding and fission of the plasma membrane [84, 85]. Micro-domains are formed within the plasma membrane via asymmetric protein and phospholipid distributions, which are regulated by aminophospholipid translocases [86]. Aminophospholipid translocase transfers phospholipids from the outer leaflet to the inner leaflet of the cell membrane. The translocation of phosphatidylserine from the inner leaflet to the outer leaflet, induces membrane budding and vesicle formation [87]. Actin-myosin interactions cause the contraction of cytoskeletal structures, completing the budding process [85, 88]. The biogenesis of microvesicles requires flippases and floppases. Flippases move particular phospholipids from the outer to the inner leaflet, while floppases move them in the opposite direction [89].

1.4.1.2 Composition

The sEVs (exosomes and microvesicles) are spherical bilayered particles with a diameter of 30-150nm. They are secreted by all cell types [5] and contain a specialized cargo and surface markers reflective of the cell/organ from which they emanate. These vesicles have unique and complex compositions of bioactive materials including proteins, lipids, and nucleic acids (mRNAs, and miRNAs) [90]. They were initially described as miniature versions of the host

cells from which they are formed, which essentially means that their structure and composition are highly dependent on the physiological or pathological state of the parent cell/organ [73]. A cohort of proteins is present on the lipid bilayer of sEVs called tetraspanins. Tetraspanins are a protein family that is involved in cell invasion, fusion and penetration. Typically, the presence of tetraspanins such as CD63, CD9, CD81, CD82 and CD37 is generally conserved among sEVs [83, 90]. Studies show that CD63 and CD81 are the most abundant tetraspanins among a broad range of sEVs [73]. Therefore, tetraspanins have been considered markers for sEV detection. Small heat shock proteins such as HSP27, HSP60, HSP70 and HSP90 are found in sEVs with functions such as cellular response and antigen presentation. MVB formation proteins (responsible for sEV formation and release) are also found within sEVs.

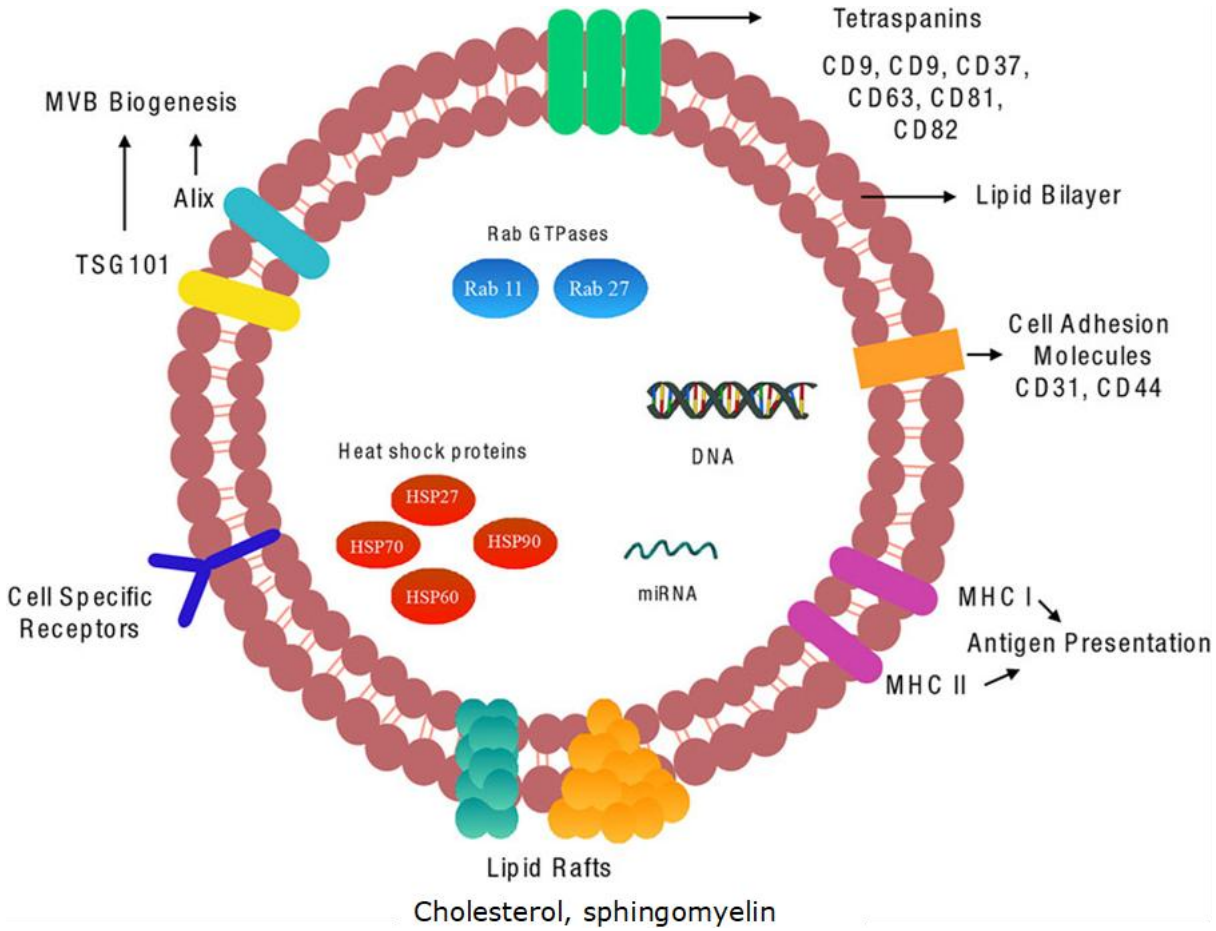

Figure 2 Structure and composition of sEVs. They are composed of a lipid bilayer encapsulating biomolecules specific to the cell of origin. Various components such as proteins, miRNAs and lipids are found within sEVs, with tetraspanins and other protein markers on the surface [90].

1.4.1.3 Biomarker potential

The sEVs were initially thought to be the cellular mechanism responsible for eliminating unwanted cellular compounds and obsolete and toxic materials [5, 91]. However, using sEVs in clinical trials for diagnostic and therapeutic purposes has become increasingly popular in recent years. The sEVs play a pivotal role in intercellular communication in health and disease by transporting their cargo (functional proteins, metabolites and nucleic acids) to recipient cells [5]. As mediators of intercellular communication, sEVs act in a paracrine and endocrine manner with the ability to influence the phenotype of the target cell, modulating immune responses, cellular migration, cancer metastasis or the spreading of neurotoxic protein aggregates in neurodegenerative diseases [91]. Therefore, sEVs reflect cellular processes which could be beneficial in the diagnosis of various diseases.

The sEVs are found in most bodily fluids including blood, urine, cerebrospinal fluid, semen, saliva and sweat. They are to some extent stable in these biofluids and can be isolated in clinical settings for evaluation in the early stages of disease [90]. Within the past decade, the association of elevated levels of sEVs and disease progression, such as cancer, has been extensively studied. These sEVs are novel and exciting biomarker candidates for personalized medicine since they are secreted by most cell types and are easily accessed in the form of liquid biopsies [92]. Liquid biopsy-based sEV profiling affords the use of these vesicles as biomarkers with great potential in terms of aiding in clinical diagnosis and assessing the severity of diseases more accurately. The membrane composition and the size of sEVs allow them to cross significant biological barriers, including the blood-brain barrier [93]. These factors and their molecular fingerprint make sEVs excellent candidate biomarkers for disease diagnosis, prognosis and therapeutic interventions.

1.5 The role and biomarker potential of sEVs in sepsis

Within the past decade, the role of EVs in the pathophysiology of various diseases such as cancer [94], liver diseases, neurodegenerative diseases [95, 96], infectious diseases [6] and cardiovascular diseases [97, 98] has been extensively reviewed. Studies conducted within the past decade have reported that DNA mutations in plasma sEVs were sensitive tumour prognostic indicators for various types of cancer, including melanoma and pancreatic cancer [99, 100]. Additionally, studies on breast cancer and ovarian cancer reported that RNAs and

proteins from tumour-derived sEVs have diagnostic value. Interestingly, the first sEV-based test was launched in 2017 which was approved by the US Food and Drug Administration (FDA) and uses sEV gene transcripts to identify high-grade prostate cancer among men, subsequently reducing the number of biopsies performed by 27% [101]. Importantly, within the research of neurodegenerative diseases, circulating EVs can cross the blood-brain barrier and the lipid bilayer of EVs protects the contents against enzymatic degradation, making EVs more stable than DNA and RNA [102]. A study has highlighted the potential of plasma EVs, enriched with proteins related to Alzheimer's disease, as a tool for distinguishing patients with mild cognitive impairments and patients with Alzheimer's disease [103]. This provides evidence that sEVs have emerged as significant contenders for biomarker development strategies, which will impact not only diagnosis but also prognosis and possibly treatment [104]. The transport of bioactive cargo between donor and recipient cells through sEVs is recognized to provide pleiotropic activities in intercellular communication. They are also thought to have a significant role in immunosuppression and tumor development [4]. The ability of sEVs to modulate immunity is dependent on several variables, including the illness type, cellular origins, and the cargo that is delivered to the recipient cells [105]. There is considerable intricacy and pleiotropy in the immunological control of sEVs in sepsis. The sEVs with varying cargos from the same source or separate biological sources can stimulate or inhibit immune cells. Therefore, endogenous sEVs have both advantageous (intercellular communication, enhancing the immune response, tissue repair) and detrimental (disease progression and inflammation) roles [106, 107].

In vivo studies show that there was an increase in the number of sEVs in the sera of LPS-induced mice while the blockage of sEV generation diminishes sepsis-induced inflammation, suggesting an increase in the release of sEVs during sepsis [7, 108, 109]. The sEVs play pivotal roles in inducing inflammation and exaggerating immunosuppression and organ dysfunction in sepsis. In vivo studies suggest that sEVs in the plasma of sepsis-induced mice, promote the release of pro-inflammatory cytokines during the early and late phases of sepsis [7, 110]. Apart from directly increasing the production and release of cytokines, sEVs induce inflammation by disrupting immune cells involved in the production of pro-inflammatory cytokines and inflammation [111], via the activation of various PRRs and signalling pathways to induce pro-inflammatory responses [112]. As a result, sEVs have been shown to play crucial roles in the development of sepsis and the immunosuppressive state, as studies have shown that EVs

derived from septic rats reproduced oxidative stress and inflammatory patterns associated with sepsis [113-115].

At present, there is no single test that can be said to predict mortality with a high degree of sensitivity and specificity. Based on their biological properties, sEVs could fulfil the role of possible contenders, in conjunction with other tests [116]. The sEVs are easily accessible and include expression patterns that could aid in the diagnosis and prognosis of sepsis as well as stratifying patients into more homogenous subsets for quick decision-making [117]. Nucleic acids found within sEVs are promising biomarkers in terms of monitoring the progression of sepsis [117]. Exosome-derived miR-125b-5p was upregulated in sepsis patients when compared to healthy individuals, suggesting the significance of nucleic acids within sEVs as tools for survival prediction in sepsis [118]. Furthermore, proteomic analyses of sEVs proteins and genes are crucial for understanding the role they play in biological processes and disease pathology [8, 119, 120], which is explained in detail in the upcoming chapter of this thesis. A study reported that the protein composition of sEVs allowed for the differentiation of pulmonary diseases in children [121]. Therefore, the observations made from this study will contribute to the possible association between sEV surface markers and their role in sepsis and as possible biomarkers. The role of sEVs in sepsis as well as their biomarker potential has been extensively reviewed and discussed in Chapter Two.

1.6 Purpose of the study

Modern biotechnological applications have transformed how we view, identify, and treat illnesses, resulting in ground-breaking medical discoveries. EV diagnostics is an emerging and rapidly expanding field in medical biotechnology. Research surrounding EVs and sEVs offers an effective means of identifying promising biomarkers and therapeutic agents for diseases due to their unique molecular cargo, which includes proteins, lipids, nucleic acids and the markers present on the surface membrane. These molecules carry precise biological codes related to their cellular origin and can alter target cells' normal and pathological functions.

To date, sepsis treatment includes antibiotic therapies, ventilator management, blood glucose maintenance, and resuscitative strategies, as well as supportive therapies that are recommended by the Surviving Sepsis Campaign. Given the limitations pertaining to treatments, the need for novel biomarkers that can readily predict the possibility of a person developing sepsis and

thereafter track its progression is rapidly increasing. Early detection of sepsis is critical to initiate appropriate treatment methods timeously. Whilst several markers have been proposed for the diagnosis of sepsis, they are still not ready to be deployed widely in various clinical settings.

It has therefore become clear that orthodox approaches with regard to diagnosing and treating sepsis need to be re-examined. Advanced molecular biotechnology and other new technologies are critically required for the further understanding of sepsis, its progression, treatment and diagnosis. The discovery of an effective and appropriate biomarker for sepsis is still an ongoing challenge. With the rapid advancements surrounding the availability and significance of sEV isolation methods and analytics, the search for sepsis biomarkers has become increasingly important. The gap in research has therefore prompted the design of this project using novel technologies to help mitigate challenges faced in sEV research and unique South African cohorts to further understand the role of these vesicles in disease. This project aims to apply novel technologies and identify unique surface marker signatures within the field of sEV research for prognostic, diagnostic, and possible future therapeutic interventions for sepsis within the framework of acceptable healthcare guidelines.

The investigations presented contribute to the foundational framework in support of the clinical application of sEVs as biomarkers of sepsis. These were categorised into three studies as follows:

Therefore, our aims and objectives are as follows.

1. **Aim 1: To determine the theoretical framework surrounding the potential of exosomes as biomarkers in sepsis. (Chapter Two)**
   Objectives
   - To evaluate the current role of exosomes in the pathophysiology of sepsis
   - To evaluate the application of exosomes in diagnostic, prognostic and possible therapeutic interventions in sepsis

2. **Aim 2: To characterise and identify specific colocalization patterns in South African sepsis patients using a single-particle interferometric reflectance imaging sensor (SP-IRIS)-based platform (Chapter Three)**

Objectives

- To quantify and characterise circulating exosomes in South African sepsis patients and identify exosomal tetraspanin biomarkers using the cutting-edge technique provided by NanoView Biosciences, Boston, MA.

3. **Aim 3: To identify specific surface marker profiles in the pathophysiology of sepsis as possible biomarkers using a Bead-Based Multiplex Flow Cytometry Assay. (Chapter Four)**

   Objectives

   - To isolate and quantify sEVs in South African sepsis patients
   - To evaluate the expression of sEV surface markers in various cohorts (viz, sepsis with no pre-existing co-morbidities, sepsis with previously diagnosed hypertension, hypertension and healthy controls) in order to identify specific profiles using the MACSPlex exosome kit, human (Miltenyi Biotec).

The outcomes of this study are novel and will hopefully contribute to the understanding of the pathophysiology of sepsis, which may lead to the subsequent identification of biomarkers for the diagnosis and prognosis of sepsis. The basis of this study will significantly contribute to the biomarker research in this field.

1.7 Methodology Overview

This study and methodology were reviewed and approved by the regulatory and institutional ethics committee, the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004587/2022) and the South African Department of Health (NHRD: KZ_202107_008). The participants involved in this study were recruited from Inkosi Albert Luthuli Central Hospital and King Edward VIII Hospital in Durban, South Africa. According to the declaration of Helsinki, all participants were provided with one of two methods of consent (viz. deferred consent or next-of-kin proxy consent). Blood samples from all recruited participants were taken by the attending physician where he had drawn approximately 8mL of blood per patient via an arterial vein. Blood samples were taken in tubes containing

ethylenediaminetetraacetic acid (EDTA), and plasma was obtained by centrifugation for 15 minutes at 3500 x g. Blood samples were centrifuged within an hour following collection. The plasma was stored at -80°C until the date of use. The plasma was used for analysis within 3 months of sample collection. The control group used in this study consisted of age, gender and race-matched healthy volunteers with no chronic illnesses. A statistical overview regarding sample size was conducted by a statistician at the University of KwaZulu-Natal, concluding that a sample size of n=12 was to be used. However, due to limitations in a part of the study (Chapter Three), this sample size could not be met. All statistical analyses in this project were done using GraphPad Prism 8.0 (C.A., La Jolla).

The aim of Chapter Three was to characterize and identify specific colocalization patterns in South African sepsis patients. This was carried out using a cohort of patients presenting with sepsis with the following criteria: black South Africans admitted into the ICU of the respective hospital with a SOFA score greater than two (n=6). A cohort of healthy participants (volunteers), consisting of age-matched black South Africans, was also used in this study (n=6). sEVs were isolated using the total exosome isolation kit from plasma (Invitrogen by life technologies) as per the manufacturer's instructions. The isolated sEVs were then characterized using Transmission Electron Microscopy (TEM) for morphology and imaging and a CD63 Exo-ELISA for the quantification of isolated sEVs. Thereafter, sEV analyses were performed using the ExoView R100 platform (NanoView Biosciences, USA). The ExoView R100 platform is a single-particle interferometric reflectance imaging sensor (SP-IRIS)-based platform which uses antibodies (anti-CD81, anti-CD63 and anti-CD9) to fluorescently analyse the presence of these markers on the surface membrane of the isolated sEVs.

The aim of Chapter Four was to identify specific surface marker profiles in the pathophysiology of sepsis as possible biomarkers. The presence of chronic illness (such as hypertension) in patients with sepsis has severe effects on the prognosis and outcomes of patients and still requires further elucidation. Hypertension is a global health problem, with these patients being at a greater risk of developing sepsis due to shared physiological pathways between the conditions. This chapter investigated surface marker profiles of sepsis in the presence of a previously diagnosed co-morbidity (i.e. hypertension). This was done using four cohorts of patients, i.e., healthy controls, hypertension, sepsis with no pre-existing chronic illnesses, and sepsis with previously diagnosed hypertension (n=12 per group). The healthy controls and hypertension group was age, sex and ethnicity matched for the sepsis and sepsis with hypertension group. For the purpose of this study, sEVs were isolated using the total exosome

isolation kit from plasma (Invitrogen by Life Technologies, CA, USA) as per the manufacturer's instructions. Thereafter, characterization of the isolated sEVs was done using Nanoparticle Tracking Analysis (NTA) and a CD63 Exo-ELISA for the quantification and TEM for the morphological analysis. Isolated sEVs were subjected to bead-based multiplex exosome flow cytometry assay using the MACSPlex exosome kit, human (Miltenyi Biotec) using the short protocol as per the manufacturer's instructions. This assay uses capture beads which identifies 37 different antibody-coated bead subsets. The Spectroflo® (Cytek Biosciences B.V., Amsterdam, Netherlands) software was used to read and generate data from each sample.

1.8 Thesis Overview

Chapter One: Methodology Overview

This section provides a brief summary of the methodology used throughout this thesis and specifies the approvals that were obtained for the purpose of this study. All kits and reagents, as well as the manufacturers, are stated.

Chapter Two: Manuscript One

This publication provides an in-depth theoretical framework surrounding the role of circulating sEVs as possible biomarkers of sepsis. This review emphasizes the use of novel techniques for the purpose of sepsis diagnosis, prognosis and possible therapeutic interventions.

Chapter Three: Manuscript Two

This publication consists of an experimental paper using a novel and automated technique for the identification of commonly used surface markers found on the surface membrane of sEVs as possible biomarkers of sepsis. This publication highlights the disadvantages of other sEV isolation techniques and shows how the ExoView R100 platform has overcome them. The results obtained from this technique provide evidence that CD63/CD9 positive sEVs are abundant in sepsis patients. The results also show colocalization patterns specific to sepsis patients, which could serve as biomarkers for sepsis patients.

Chapter Four: Manuscript Three

This manuscript elaborates on the heterogeneity of the surface markers present on sEVs and disease-specific subsets. This manuscript also incorporates the presence of co-morbidities with sepsis into the experimental design. Herein, a Bead-Based Multiplex Flow Cytometry Assay was used to identify 37 different surface markers found on the isolated sEVs of healthy controls, sepsis, hypertension and sepsis with previously diagnosed hypertension. The results provide foundational evidence of disease-specific sEV subsets which could aid in the stratification of patients and towards the development of sepsis biomarkers.

Chapter Five: Synthesis

This chapter provides an overview of the study, identifying the gap in research and how the above manuscripts help to fill that gap. This chapter also highlights the findings of each manuscript and provides evidence to support the use of sEVs as possible biomarkers for sepsis diagnosis and prognosis. Also highlighted are major conclusions, limitations and recommendations of this study.

NB. The term "exosomes" is used in Chapters Two and Three as the manuscripts have been published; however, for the purpose of this thesis, the term "small extracellular vesicles (sEVs) will be used in all written texts. This was done as the International Society for Extracellular Vesicles (ISEV) has updated the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines regarding appropriate nomenclature- new guidelines published as of 2024. MISEV states that unless the isolation method used utilizes size as a way to derive EVs specifically from the endosomal pathway, the term "exosome" should not be used.

2 CHAPTER TWO: MANUSCRIPT ONE

Circulating exosomes in sepsis: A potential role as diagnostic biomarkers, therapeutic and drug delivery carriers. (Published: Smart Materials in Medicine, 2023, pages 639-647)

Bridging Text

This publication critically reviews the role of circulating sEVs in the pathophysiology of sepsis, highlighting the significant advances of sEVs in sepsis diagnosis and management. Herein, the future prospects, current challenges and recommendations are also highlighted to provide further understanding of applying novel techniques within the field of sEV research for prognostic, diagnostic and possible future therapeutic interventions in sepsis within the framework of acceptable healthcare guidelines.

NB. Formatting, tables/figures and referencing was done in accordance with the journal requirements.

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Circulating exosomes in sepsis: A potential role as diagnostic biomarkers, therapeutic and drug delivery carriers

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ABSTRACT

Sepsis and sepsis-related organ dysfunction have been identified as significant global life-threatening health threats, with a high mortality rate despite ongoing research in the area. Timely diagnosis is essential such that treatment could be initiated as early as possible to ensure the best outcome, since delayed intervention is associated with a higher mortality. Patient stratification and disease monitoring, present significant challenges in sepsis treatment and management strategies, largely due to the heterogenicity of sepsis signs and symptoms. Hence a focus on potential biomarkers to overcome these challenges is needed. Recently, extracellular vesicles (EVs), mainly the exosome subtype, have been investigated regarding their potential role in sepsis diagnostics, therapeutics and as drug delivery vehicles. Herein, we present an up-to-date review covering the role of circulating exosomes in the diagnosis and monitoring of the progression of sepsis and in therapeutics and drug delivery for sepsis. To provide context, sepsis pathophysiology and the role of circulating exosomes in sepsis have been highlighted. Future prospects, current challenges and recommendations regarding the role of exosomes in sepsis are also identified.

1. Introduction

Sepsis, one of the leading causes of death globally [1,2], is a complex multisystem disorder whereby the dysregulated immunological response to infection causes systemic inflammation, which may lead to end-organ failure and death [3,4]. Although infections are a commonly encountered health issue in individuals of various ages and the response and treatment are minor and appropriate, in a percentage of patients, these infections may be severe, causing the treatment to be ineffective [5]. Consequently, this may lead to organ dysfunction or failure. Recent literature showed that about 48.9 million sepsis cases and 11 million sepsis-related death were reported in 2017, representing a staggering 20% of all global deaths. Septic shock and multiple organ dysfunction are among them, affecting approximately 30 million people and causing an estimated 6 million deaths annually [3,4], with mortality rates as high as 50% [6]. Even though the management of critically ill sepsis patients has improved over the last decade, sepsis-related mortality remains high [7].

The complex pathophysiology of sepsis has been separated into 2 phases, the first being the hyperinflammatory phase, followed by an immunosuppressive phase ultimately leading to organ dysfunction and/or failure [8–10]. Recent studies show that exosomes play crucial roles in both phases of sepsis pathophysiology. Exosomes have been shown to mediate an increase in the release of pro-inflammatory cytokines during the hyperinflammatory phase [11]. Moreover, they have the ability to impact upon the immune response during the disease state, therefore aiding in the immunosuppressive state and the progression of sepsis [12]. Finally, sepsis leads to the dysfunction of various organ systems, exosomes have been found to exacerbate this dysfunction and accelerate organ failure [13].

Furthermore, the prognosis of sepsis is dire, challenged by diagnostics that lacks a gold standard and heterogenous clinical presentation. Moreover, the delay in the diagnosis and the initiation of appropriate treatment methods were shown to increase the mortality in critically ill patients. Furthermore, a total of 30% of survivors may experience long-term dysfunction and cognitive impairment [14], posing a significant burden on society. The outcome of sepsis for patients depends on the

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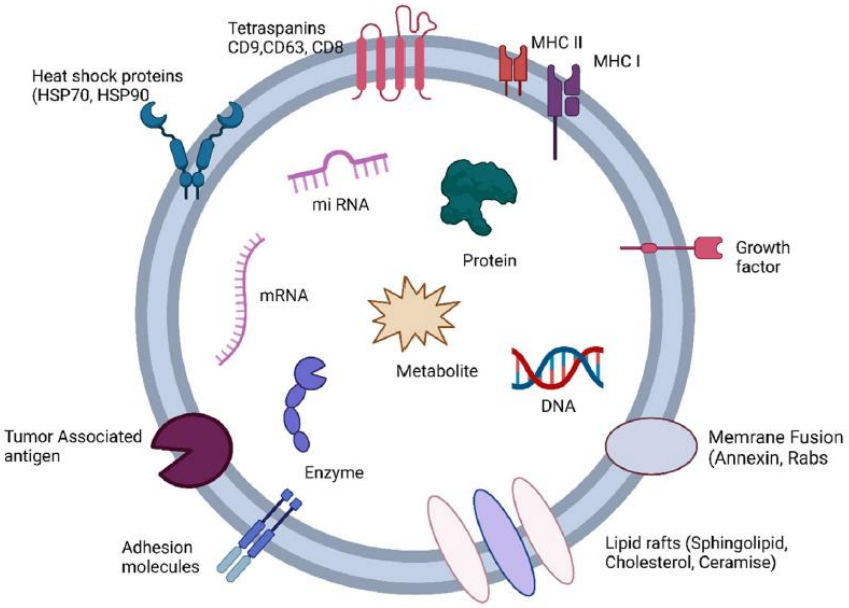

Figure (1). Diagrammatic representation of exosomal cargo and biomolecules present on the membrane of exosomes. Exosomes consist of a large variety of elements, including proteins (tetraspanins and heat shock proteins), deoxyribonucleic acid, messenger ribonucleic acid, micro-ribonucleic acid (DNA, mRNA, miRNA), cell adhesion molecules, enzymes and lipids. The components of exosomes are highly dependent on the cell type from which they originate (Created using Biorender.com).

timely initiation of effective treatment. In the case of patients admitted to the general ward and those in post-surgery recovery, it has proven to be significantly challenging and may also contribute to high mortality rates. In recent years, despite the extensive reports on novel biomarkers, there is still no gold standard for diagnosis, and no specific biomarkers in the clinical setting for a definitive diagnosis.

Current sepsis treatment strategies include antibiotic therapies, ventilator management, blood glucose maintenance, resuscitative strategies, and supportive therapies recommended by the updated Surviving Sepsis Campaign [15,16]. However, persistent infections are responsible for 70–80% of sepsis deaths, attributed to widespread antimicrobial resistance and the lack of effective treatment choices [17]. Antibiotic resistance adds to the burden and jeopardizes sepsis management efforts. With restricted treatment options, there is an urgent need for novel biomarkers that can readily predict the possibility of a person developing sepsis, and track its progression to avoid therapeutic failure due to unintended interactions or improper timing of the intervention [18]. Therefore, the quest for reliable biomarkers for diagnostics, and novel therapeutics for sepsis has become critical in the clinical setting. Current research shows that exosomes play a crucial role in sepsis

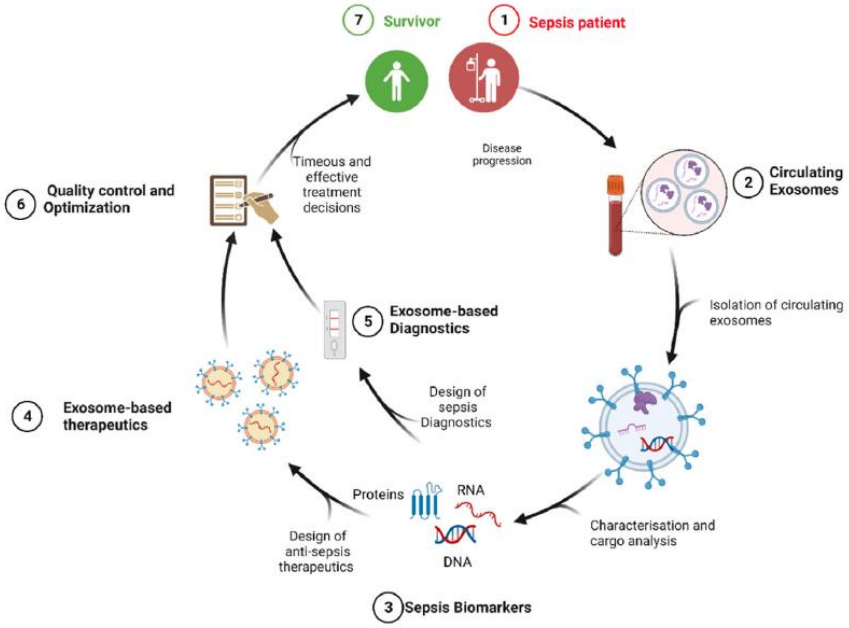

Figure (2). Schematic diagram representing the role circulating exosomes in sepsis diagnosis and treatment.

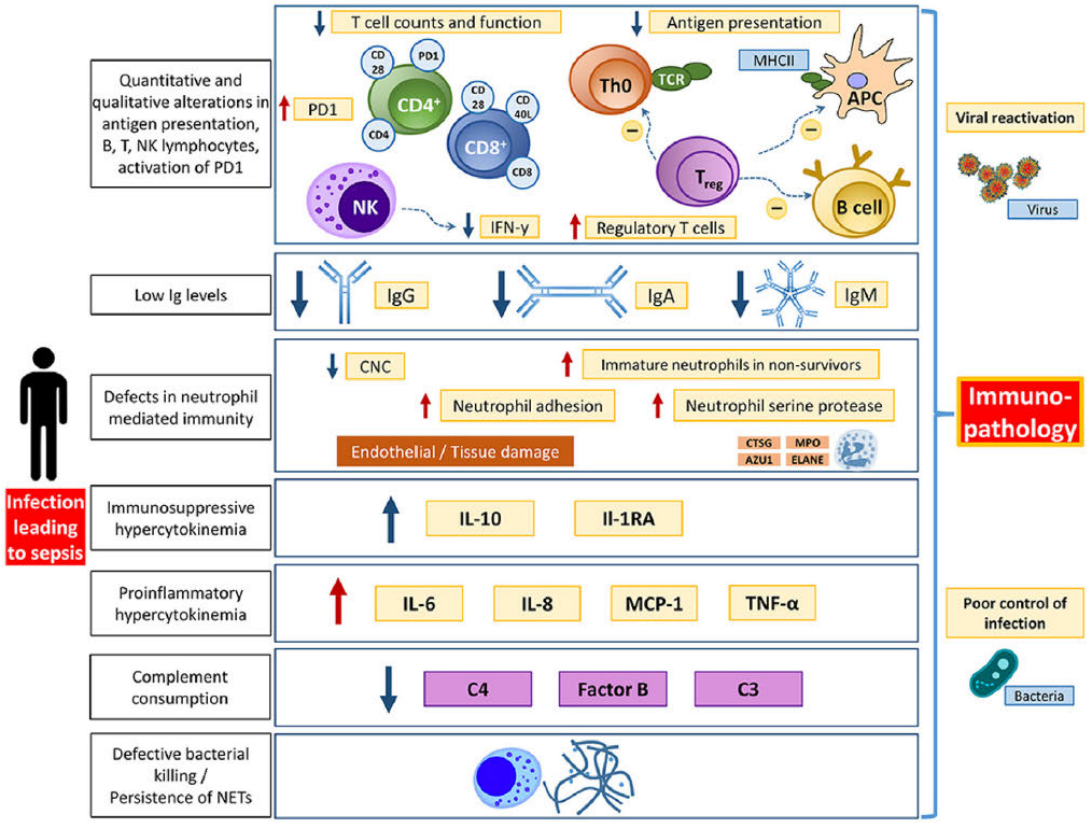

Figure (3). Diversity of immunological dysfunction and essential components of immunosuppression state in sepsis through various immunopathological pathways that lead to virus reactivation and poor infection control. Adapted from Jarczak et al. (2021) [45].

pathophysiology through inflammatory pathways, thus displaying significant potential to be used as diagnostic biomarkers and therapeutic agents for sepsis [4,19].

Circulating exosomes possess great potential as diagnostic and prognostic agents because of their various intercellular communication methods and the specificity of the molecules they carry under different pathological conditions. Moreover, circulating exosomes have been extensively studied as a novel drug delivery platform and therapeutics that confer targeted delivery with low immunogenicity, and less toxicity for various disease conditions, including cancer, cardiovascular diseases and inflammation [20–24]. Circulating exosomes are now acknowledged to play a crucial part in normal biology and pathophysiology of various health conditions (including sepsis) due to their role as communicators, and their ability to elicit a response from target cells [25].

Circulating exosomes that have been discharged into the bloodstream [26], may travel via the blood circulation to reach distant regions, directly interacting with target cells and swiftly controlling intracellular signalling. Under both healthy and pathological conditions, they carry different cargo represented by proteins, lipids, and miRNA, which are easily detectable in the circulation [27–29]. Exosomes, a subset of extracellular vesicles (EVs), are intercellular communication vehicles involved in many physiological processes [30] that are found in blood, saliva, urine, semen, breast milk, and cerebrospinal fluid [31]. Biogenesis and size are used to classify extracellular vesicles (EVs) as exosomes, micro-vesicles, and apoptotic bodies [31], which have gained interest due to their significant participation in several processes (invariably intercellular communication) [32]. In particular, exosomes are nanosized lipid-bound vesicles, generated by eukaryotic cells into the extracellular space to perform cell-to-cell communication [33–35]. Exosomes are released from the cell surface and originate from the endosomal system through several molecular pathways and possess a variety of proteins, nucleic acids such as various subtypes of RNAs, and lipids (see Figure (1) [36,37]. Importantly, exosomes carry biomolecules from parent cells and transfer them to target cells, causing changes in the function and shape of the target cells [35].

In this review, we demonstrate the significance, recent advances and contribution of circulating exosomes in sepsis diagnosis and treatment and their potential as sepsis-targeted drug delivery systems as presented in Fig. 2. The role of circulating exosomes in the pathophysiology of sepsis has been discussed to provide a background that could illustrate the importance of exosomes in sepsis generally. Moreover, we identify the current challenges and future prospects for the circulating exosomes in sepsis as potential diagnostic and therapeutic agents.

2. Exosomes and their role in sepsis pathophysiology

Exosomes, found as local and systemic intercellular communicators [38–40], are small vesicles, ranging from 30 to 200 nm, which are present in a wide range of biofluids, and contain important cellular messages such as transcription factors, cytokines, RNA, growth factors and adhesion molecules [36,37]. In particular, exosomal-mediated transport mechanisms have been crucial to the initiation, development, and treatment of inflammation both locally and systematically [41,42]. In addition, circulating exosomes contain biomolecules that are considered to be important early molecular fingerprints and potential diagnostic markers of inflammation and disease states in a variety of diseases, particularly cancer and sepsis [42–44] Herein, we discuss the pathophysiology of sepsis in brief and the role of exosomes.

2.1. Sepsis pathophysiology

The pathophysiology of sepsis is considered to be an initial hyper-inflammatory state lasting only a few days, followed by a prolonged immunosuppressive phase [9]. The initial hyperinflammatory phase (cytokine storm) causes fever, refractory shock, hypoxia, and cardiac or pulmonary failure [9,12,45]. The up-regulation of pro-inflammatory pathways in this phase also causes the release of pathogen-associated molecules resulting in activated coagulation and complementary pathways [10,45]. Later, the prolonged immunosuppression leads to organ injury and sometimes organ failure [12]. This prolonged immunosuppressive state is related to numerous factors such as apoptotic depletion of immune cells, increased expression of negative costimulatory molecules, increased regulatory T (Treg) cell expression, expression of programmed cell death (PD)-1 on $CD4^+$ T cells, and cellular exhaustion [9, 46].

Host immune cells express pattern recognition receptors (PRPs) on extracellular surfaces and in the cytosol viz. Toll-like receptors (TLRs) and Nod-like receptors (NODs), respectively. TLRs are essential for initiation of host immune response in sepsis via pathogen detection, recognising pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [3], and activation of the complement system (which has been recognized as a potentiator of the inflammatory response (IR) during sepsis) [47]. The production of inflammatory cytokines is attributed to the detection of pathogens in the cytosol by NODs. This causes the formation of inflammasomes, stimulating the hyperinflammatory initial phase 'inflammatory state with activation of leucocytes, complement and coagulation pathways that underpin the endothelial, cellular and cardiovascular dysfunction that characterizes sepsis' [48]. On the other hand, the alteration of immune cells by unrestrained apoptosis is considered to be primarily associated with immunosuppression in sepsis. Sepsis rapidly triggers profound apoptosis in cells representing non-specific IR i.e. macrophages/monocytes, dendritic cells, Natural Killer (NK) cells, γδ T cells, and those representing the specific IR viz. $CD4^+$ T cells, and B cells. However, apoptosis of neutrophils is delayed, and Treg cells are more resistant to sepsis-induced apoptosis [9,49]. This indicates the sequential effects on apoptosis on the aforementioned cells, therefore highlighting the role of apoptosis in sepsis pathophysiology. Figure (3) represents the immunological dysfunction and essential components of the immunosuppression state in sepsis [45].

2.2. The role of circulating exosome in sepsis pathophysiology

Circulating exosomes, as mediators of intercellular communication (paracrine and endocrine), have a significant role in the pathophysiology of various diseases and pathological conditions, including cancer [41], neurodegenerative [50] and cardiovascular diseases [51,52], pneumonia and sepsis [53,54]. Exosomes are involved in the pathophysiology of these diseases via multiple mechanisms such as influencing the phenotype of the target cell, modulating immune responses, affecting cellular migration, promoting cancer metastasis and the spread of neurotoxic protein aggregates [55]. This section addresses the role of circulating exosomes in the hyperinflammatory state, the immunosuppressive state and ultimately in organ dysfunction within sepsis.

There is a recent surge in reports on the role of circulating exosomes in sepsis pathophysiology. In this regard, a sepsis mouse model using lipopolysaccharide (LPS) (which causes endotoxemia which mimics most aspects of sepsis) showed a 2-fold increase in the number of serum exosomes using total exosomal protein (CD63, CD81 and CD9) quantification [56], which indicates an increase in the release and decrease in the uptake of exosomes during the septic state [49,50]. Further, multiomic investigations that covers a wide range of exosomal biological mediators in sepsis serum exosomes was conducted by Li et al. (2021) and colleagues. This study provided a landscape of molecular dynamics in serum exosomes, where they identified total of 354 proteins, 195 mRNAs, 82 lncRNAs and 55 miRNAs that were differentially expressed in sepsis patients. They showed the changes in the molecular aspects of serum exosomes during the development of sepsis and proposed associated molecular mechanisms [57].

Furthermore, multiple research papers have been reported to clarify the role of the circulating exosomes in sepsis by studying their ability to induce inflammation, promote immunosuppression and sepsis progression as well as their role in end organ dysfunction.

Firstly, circulating exosomes have been shown to induce the hyper-inflammatory state of sepsis. Xu J et al., studied the effect of these exosomes on cytokine production. This study showed that circulating exosomes promote cytokine production (pro-inflammatory and immunosuppressive cytokine) [11]. Pro-inflammatory cytokines called Interleukins (IL) such as IL-1β, IL-2, IL-6 and Tumor necrosis factor α (TNFα) start to increase drastically in the early phase of sepsis [10] while anti-inflammatory cytokines such as IL-4 and IL-10 increase during the later stage in septic patients [10,58]. Furthermore, exosomes can also cause distress to macrophages, which in turn cause the stimulation of Nuclear factor kappa B (NF-κB) and increase in cytokine production (IL-1β, IL-6, IL-12 and TNFα) [59]. Similarly, others showed that exosomes were an important stimulus for the release of high-mobility group box-1 (HMGB-1) protein (a late lethal mediator of sepsis) from hepatocytes, which has significant implications in the pathogenesis of sepsis and other inflammatory disorders of the liver [60]. The ability of exosomal miR-92a-3p to activate alveolar macrophages in sepsis-induced acute lung injury [61]. It was found that exosomes derived from LPS-treated alveolar epithelial cells induced pulmonary inflammation through exosome shuttled miR-92a-3p, mediating crosstalk between alveolar cells and macrophages, which contributed to macrophage activation by inhibiting Phosphatase and tensin homolog protein (PTEN) expression and regulating the activation of the NF-κB signalling pathway [61]. Similarly, another study has revealed that high levels of macrophage-secreted exosomal Aminopeptidase N promotes epithelial cell necroptosis, inducing reactive oxygen species (ROS) production, mitochondrial dysfunction and the activation of the NF-κB pathway [62]. This suggests that the high levels of secreted Aminopeptidase N is associated with sepsis-induced acute lung injury and can ultimately predict the risk of poor outcomes in sepsis [62].

On the other hand, it has been shown that exosomes have a weakened ability to provoke an immune response which in turn protects the genetic cargo that they carry, therefore proving to have vital roles in the immunosuppressive state and progression of sepsis [21]. Monocyte derived exosomes may cause a down regulation in important protein networks including the immune response [63]. This study used human monocytes treated with exosomes. The monocytes were derived from sepsis patients and subjected to proteomic analyses. Results obtained from this study supports the notion that circulating exosomes contribute to immunomodulation during sepsis [63]. Furthermore, another study showed that miRNA contained in circulating exosomes are key mediators in the progression of sepsis and the development of septic lung injury [64]. Also, other role players in sepsis progression include platelet-derived exosomes containing miRNAs, which induce neutrophil extracellular traps (NETs) formation [65]. Increases in NETs formation has been linked to the development of multiple organ dysfunction during sepsis [11,65,66]. Moreover, high level of circulating (exosomal) CD14 has been proposed as an effective marker and a prognostic tool for mortality prediction in sepsis [66]. This finding has been supported by a recent study that showed P2X7 receptor induces the release of CD14 in extracellular vesicles. This reduces macrophage plasma membrane CD14 that functionally affects LPS, but not monophosphoryl lipid A, pro-inflammatory cytokine production [67]. The significant increase in the number of exosomes during sepsis has adverse effects on many organ systems such as the lungs, kidneys, heart and CNS [68]. Exosomal miR-30d-5p from neutrophils have proven to be able to induce macrophage polarization and pyroptosis and contribute to sepsis-related lung injury as reported by Jiao et al. (2021) via specific mechanisms as

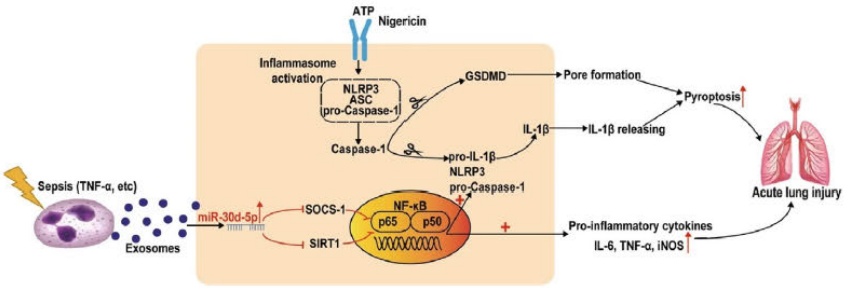

Figure (4). Schematic diagram representing the mechanism by which exosomal miR-30d-5p induces macrophage polarization and pyroptosis (a programmed cell death mediated by pro-inflammatory signals, such as infections, and is highly associated with inflammation. Adapted from Jiao et al. (2021) [69].

Table 1
Exosomal contents biomarkers for sepsis prognosis, diagnosis and progression.

Exosomal content	Biomarkers	Analysis technique	Isolation method	Reference
Proteins	- Serine palmitoyl transferase 3	Proteome analysis	Ultracentrifugation	[80]
Proteins	- DNA methyltransferase	Proteome analysis	Differential centrifugation	[81]
Proteins	- Selenoprotein P - Histidine-rich glycoprotein	Proteome analysis	Precipitation (ExoQuick kit)	[63]
Proteins	- zona-occludens-1	sodium dodecyl sulphate–polyacrylamide gel electrophoresis	Ultracentrifugation	[82]
Whole exosomes	- Plasma exosomes level	ELISA Kits	Precipitation (ExoQuick kit)	[83]
Nucleic acids	- hsa_circRNA_104,484 - hsa_circRNA_104,670	Gene Analysis and Polymerase chain reaction (PCR)	Ultracentrifugation	[84]
Nucleic acids	- miR-92a-3p	Gene Analysis and Polymerase chain reaction (PCR)	Ultracentrifugation	[61]
Nucleic acids	- miR-276–3p - miR-21–5p - miR-193a-5p	Gene Analysis and Polymerase chain reaction (PCR)	Precipitation (miRCURY exosome isolation kit)	[85]
Nucleic acids	- miR-193a-5p - miR-542–3p	Gene Analysis and Polymerase chain reaction (PCR)	Precipitation (miRCURY exosome isolation kit)	[86]

represented in Figure (4). This study proposed targeting this pathway as a therapeutic approach to control sepsis-related lung injury [69]. Similarly, a study by Mu X et al. and colleagues (2018) unveiled a new vascular redox signalling pathway related to cardiovascular dysfunction in sepsis and a novel functional role of endothelial podosomes in vascular dysfunction [70]. Contrary to this, Bao et al.,. (2022) show that exosomes play a pivotal role in the activation of neutrophil antithrombotic function, preventing the release of reactive oxygen species during sepsis [71]. Circulating exosomes have been proven to have an adverse effect on the immune system leading to the progression of sepsis, coupled to this, these exosomes also play a key role in the development of lung injury [64], liver disorders [60], bone defects and/or multiple organ dysfunction [11, 13,65,66].

In conclusion, the role of circulating exosomes in sepsis can be summarized as follow: 1) sepsis increases in the levels of circulating exosomes 2). They have the ability to increase the release of pro-inflammatory cytokines during sepsis. 3). They also down regulate the immune response and are involved in immunomodulation. 4). They may accelerate the development of organ dysfunction. The previously mentioned roles have attracted attention to the use of circulating exosomes as diagnostic tools, mortality predictors and therapeutic drug-delivery carriers for sepsis.

3. Diagnostic and prognostic potential of circulating exosomes in sepsis

Early sepsis diagnosis is of paramount importance to improve the clinical outcomes of patients, but this is often hindered by the lack of sensitive and specific biomarkers to aid in quick decision-making [72]. At present, sepsis diagnosis depends on clinical features, detection of inflammatory markers such as procalcitonin (PCT) and C-reactive protein (CRP) in the blood, and microbiological culture techniques, which are time-consuming and shows limited diagnostic potential in terms of identifying infectious origin [73–75]. Additionally, there is a general lack of significant prognostic tools for sepsis. Currently, clinical tools such as the Acute Physiology and Chronic Health Evaluation (APACHE II) and sequential organ failure assessment (SOFA) are widely used for the assessment and prognostication of sepsis [3,76], however, there is still a need for definite tools in order to improve sepsis outcomes and ultimately reduce mortality rates. Therefore, advanced definite and easily accessible biomarkers are required for swift sepsis diagnosis. Exosomes are an excellent example of biomarkers that include expression patterns, which may aid in stratifying patients into more homogeneous subgroups or develop targeted therapeutic interventions. However, different isolation methods have yielded significant effects on the concentration and composition of isolated exosomes [77–79]. Exosomal cargo contains various mediators which have been proven to be vital in the pathogenesis of sepsis; therefore, several studies published recently have proposed the exosomal content as promising biomarkers for sepsis prognosis, diagnosis and progression, as represented in Table 1.

Exosomal proteomic analysis as a diagnostic tool to monitor the progression of sepsis, using a simple and efficient protocol for exosome isolation has been reported by Xu et al. (2018). This study revealed that the sepsis disease progression is inversely linked with serine palmitoyl transferase 3, which has been implicated in the development of sepsis due to its involvement in sphingolipid metabolism [80]. This study identified proteomes using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The proteins were analysed using Kyoto Encyclopedia of Genes and Genomes (KEGG). Similarly, a study on the potential of the circulating exosomes expressing DNA methyltransferase (DNMTs) using Quantitative (q)PCR analyses to predict septic shock was reported by Dakhlallah et al. (2019). This study demonstrated that DNMT mRNA expression in plasma exosomes has a significant diagnostic and prognostic value that could differentiate patients with sepsis, and septic

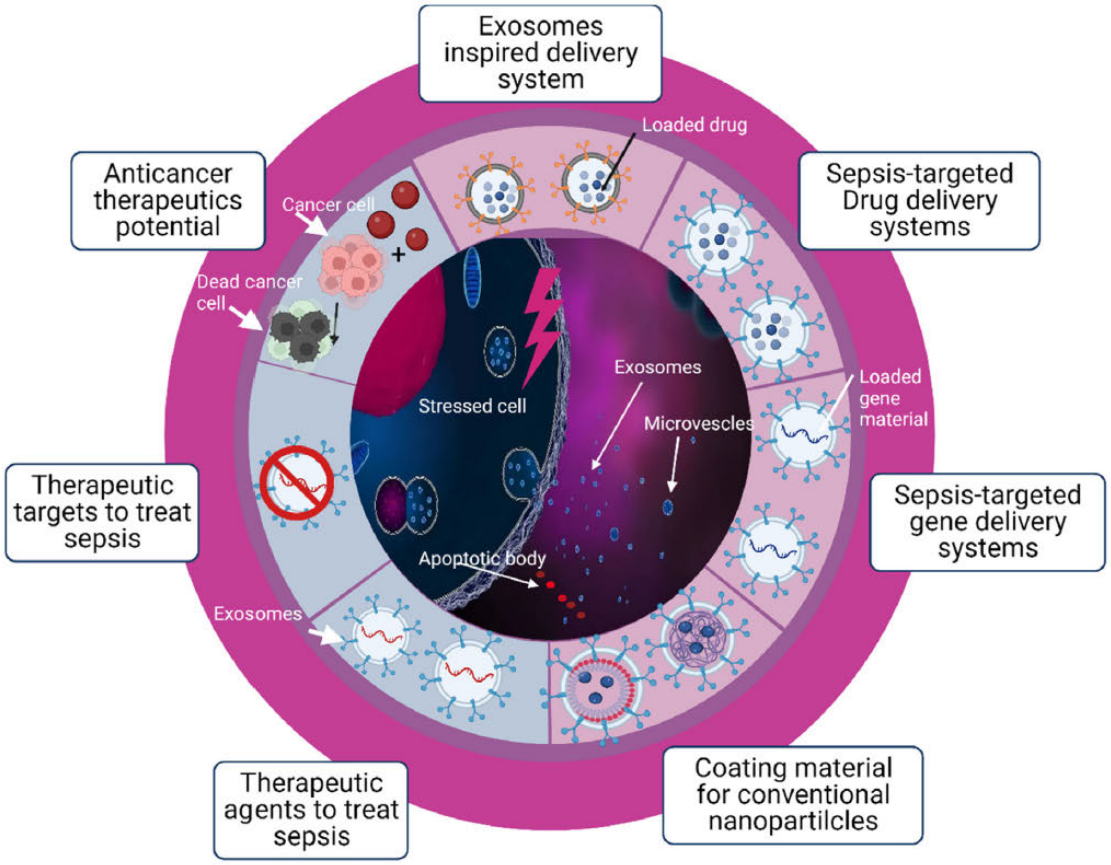

Figure (5). Schematic diagram illustrating the therapeutic and drug delivery potential of the sepsis exosomes and extracellular vesicles which include: 1) Exosome inspired delivery systems. 2) Sepsis-targeted drug delivery systems. 3) Sepsis-targeted gene delivery systems. 4) Coating material for conventional nanoparticles. 5) Therapeutic agents to treat sepsis. 6) Therapeutic targets to treat sepsis. 7) Anticancer therapeutic potential. (Created with BioRender.com).

shock from healthy individuals. This study proposes the use of the levels of exosomes in the plasma together with the exosome-DNMT ratio as a molecular diagnostic marker to distinguish septic shock patients from patients with or without sepsis [81]. Wisler et al. (2020) studied the proteomic pathway of monocyte-derived exosomes during sepsis and identified their immunoregulatory functions. This study determined proteomes using LS-MS/MS and analysis was performed using Hierarchical Clustering and heatmap visualization software from Genesis. Results from this study showed a down-regulation of several crucial protein networks in sepsis-derived exosomes [63]. These down-regulated proteins could serve as sepsis biomarkers.

Similarly, the exosome levels in plasma have been investigated by Im et al. (2020) as an indicator of the severity of organ dysfunction and mortality in patients with sepsis. Enzyme linked immunosorbent assay (ELISA) was used to determine exosome levels in 220 patients: with exosome levels in the control, sepsis, and septic shock groups trending upward (204 g/mL vs. 525 g/mL vs. 802 g/mL, $P < 0.001$), respectively. The receiver operating characteristic (ROC) analysis was performed and determined cut-off point of 809 μg/mL for the prediction of disease severity and mortality. This study concluded that high levels of plasma exosomes were significantly related to the organ failure severity and elevated mortality rate in critically ill sepsis patients [83].

Exosomal nucleic acids have also been explored as biomarkers for sepsis. In this regard, Reithmair et al. (2017) observed that sepsis and septic shock patients had upregulated exosome miRNAs (miR-276–3p, miR-21–5p, miR-193a-5p) which correlated with disease severity when compared to healthy volunteers. In addition, the predictive value of two exosome miRNAs were reported, viz. MiR-30a-5p and miR-125b-5p predicted patient survival together with serum 193a-5p [85]. Similarly, Hermann et al. (2020) reported that circulating exosomal miR-193a-5p and miR-542–3p can distinguish patients with infectious disease, in the context of community-acquired pneumonia or sepsis, from healthy volunteers. This study reports that EV-derived miR-1246 could be used to measure disease severity [86]. Furthermore, Tian et al. (2021) identified the exosomal human serum albumin circular RNA 104,484 (hsa_circRNA_104,484) and hsa_circRNA_104,670 as potential biomarkers for sepsis diagnosis. The study involved exosome isolation, observation and monitoring of the changes in the exosomal circRNA through various analysis techniques, gene analysis and construction of a circRNA-miRNA-mRNA regulatory network [84]. The above-mentioned studies used Real Time qPCR to quantify miRNA expression.

Recently, exosomal biomarkers have been developed to indicate sepsis susceptibility in other conditions, such as burn injuries. Exosomes from burn patients have significantly increased levels of zona-occludens-1, which signifies the distress of barrier tissue (epithelium and endothelium). Circulating exosomes were also elevated in burn patients developing sepsis compared to those with no infection [82].

Nevertheless, careful attention must be given to exosomal heterogeneity when investigating their potential as biomarkers. The heterogeneity of exosome subgroups is well recognized, and has been studied by Wu et al. (2021) through purification of exosomes using different surface markers. In this study, subpopulations of exosomes from septic mice have been isolated, which have contained distinct miRNA cargo patterns [87]. These findings have functional implications in disease progression since it can be postulated that the difference in cargo content within subpopulations could affect the recipient cells in a different manner by

Table 2
Exosomes-based therapies and delivery systems utilizing sepsis circulating exosomes.

Source of exosomes	Isolation/drug loading technique	Studied mechanism of action	Therapeutic outcomes	Reference
Septic mice Serum	Differential ultracentrifugation	- Induction of T-lymphocyte differentiation. - Augmentation of T-lymphocyte proliferation and migration	- Prevent tissue damages. - Prolong the survival of cecal ligation and puncture sepsis mice model.	[56]
Septic patients Plasma	Differential ultracentrifugation	Inhibition of T-lymphocyte apoptosis via down-regulation of proapoptotic gene Bad, active Caspase-3 and up-regulation of antiapoptotic gene Bcl-2 via hsa-miR-7-5p.	Reducing mortality rate of septic mice model.	[92]
LPS-stimulated tumor cells	Differential ultracentrifugation	- Improvement of endothelial functions. - Immune balance via inhibition of pro-inflammatory factors, and prevention of cytokine storms, involving regulation of seven major microRNAs	- Prevention of multiple Organ functional impairment. - Prolonging the survival of sepsis model mice.	[93]
LPS-stimulated macrophages	Density gradient centrifugation/ electroporation to load Cisplatin	upregulation of Bax and Caspase-3 in the apoptosis signalling pathway.	Substantial increase of cisplatin apoptosis rate and over all antitumor activity against lung cancer.	[98]

exosomes of the same subgroup. Therefore, the heterogenous nature of exosomes should be considered when investigating their biomarker value [88,89].

In summary, due to the protection afforded by the lipid bilayer of exosomes, these particles are stable in circulation and easily accessible with minimal invasion, through liquid biopsies. They contain bioactive molecular contents which participate in disease progression, providing an opportunity to utilize circulating exosomes for diagnostic and prognostic purposes and monitoring of patient's response to treatment.

4. Therapeutics and drug delivery potential of circulating exosomes

Recently, exosome-based therapeutics and drug delivery systems have been shown to be effective, revealing significant therapeutic improvement against a variety of diseases [40,90,91]. Circulating exosomes in sepsis have displayed significant therapeutic potential to improve the treatment options for sepsis patients as represented in Fig. 5 [56,65,83,92–94]. These exosomes have been utilized in developing exosome-based therapeutics (Table 2) and employed as therapeutic targets to modulate host immune responses [56,95,96] and inhibit immune cell apoptosis [60,92,96] during sepsis.

Circulating exosomes in sepsis have shown significant therapeutic potential against the immunosuppressive state of sepsis via inhibition of T-lymphocyte apoptosis. Deng et al. (2019) discovered the anti-apoptotic effect of sepsis patient's circulating exosomes on T-lymphocytes and validated their treatment potential in septic mice who showed enhanced survival rate [92]. Furthermore, sepsis exosomes displayed significant anticancer potential through their apoptotic action, several studies reported that sepsis exosomes and their contents (MiR-155, caspase-1, reactive oxygen species and reactive nitrogen species) could initiate cell apoptosis via several mechanisms, including activation of PRPs pathway, caspase-1, caspase-3 and NLRs [95–97]. Therefore, they could be employed as anticancer biological agents by enhancing their targeting ability to cancer cells via different surface modification approaches such as coating or attachment of targeting moieties and others.

The use of circulating exosomes in sepsis as therapeutic targets has been investigated by Essandoh et al. (2015). This study revealed that, the harmful consequences of the high level of serum exosomes during sepsis could be minimized via specific inhibition of exosomal generation and release. In this study, exosome generation inhibitor GW4869 was used to reduce the serum level of inflammatory cytokines, which protected the heart from sepsis related malfunctions and improved survival rate in both *in vitro* and animal sepsis models [99]. Later, Alarco' n-Vila et al. (2020) targeted exosomal CD14 release mechanism and explored whether activation of purinoreceptor 7 (P2X7) receptors (cellular receptors that mediate the release of exosomal CD14 into the blood circulation) could maintain high levels of exosomal CD14 following bacterial infection and prevent onset of sepsis [100]. Similarly, the exosomal protein complex of thioredoxin-interacting protein; and the neutrophil-lymphocyte ratio (NLR) family pyrin domain-containing 3 (which has a proven role in the activation of caspase-, IL-1β and IL-18 inflammatory mediators); have been investigated as sepsis therapeutic targets. In this regard, Wang et al. (2021) developed a small molecule (PSSM1443) that could disturb the stability of this complex (*in vitro*) and therefore, inhibited its activity and supressed sepsis inflammation [101]. However, there are several therapeutic targeting approaches that are yet to be explored, such as, competitive inhibition, biomimicry and other techniques.

On the other hand, exosomes could serve as an efficient drug delivery system with targeting ability to the affected organs [102,103]. Exosomes have the ability to avoid the endosomal pathway and lysosomal degradation and deliver cargo directly into the cytoplasm; which offers an advantage over other conventional nanoparticulate systems such as liposomes or polymeric nanoparticles. While exosomes (derived from various cancer cell lines and macrophages) have been extensively studied as efficient drug delivery systems for anticancer drugs, neurogenerative and cardiovascular agents and other biological molecules [22,23,104] with advanced targeting ability and treatment efficiency, similar concepts could be applied here in sepsis treatment.

Therefore, circulating exosomes could serve as effective therapeutic agents and targets for sepsis treatment [56] as well as therapeutic agents for cancer treatment [97]. However, the ability of circulating exosomes in sepsis to be utilized as biocompatible nano drug delivery systems to target sepsis microenvironments and eradicate resistant pathogens has yet to be explored.

5. Conclusion

Circulating exosomes and their components or molecular cargo have shown significant advances in the field of sepsis diagnosis and management. The implementation of these innovative agents as biomarkers, could provide non-invasive and real-time assessment of sepsis and its progression as well as a promising chance for patient stratification according to disease severity and treatment susceptibility. Despite the significant advances highlighted in this article, sepsis exosomal biomarkers are far away from clinical practice stages, as further investigations are required to address technical challenges associated with isolation, characterization and analysis of exosomes. In the same context, further understanding of exosomal biology, in term of the exosomal cargo heterogenicity, interaction with the recipient cells and numerous surface receptors, will boost the progression made in this field. Additional limitations related to diagnostic test specificity, such as lack of ROC and the Area Under Curve (AUC) analyses for the recently published biomarkers are also identified. It has become clear that orthodox approaches regarding biomarkers for sepsis need to make room for advanced molecular biotechnology and other new technologies, which are critically required for further understanding of sepsis, its progression, treatment and diagnosis. Applying novel technologies within the field of exosome

research for prognostic, diagnostic and possible future therapeutic interventions for sepsis within the framework of acceptable healthcare guidelines is highly recommended as a significant step forward to diagnosing and treating sepsis.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

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3 CHAPTER THREE: MANUSCRIPT TWO

Investigation of Exosomal Tetraspanin Profile in Sepsis Patients as a Promising Diagnostic Biomarker. (Published: Biomarkers, 2024, pages 78–89)

Bridging Text

The theoretical framework presented in publication one **(Chapter Two)** supports the scientific role of circulating sEVs in the pathophysiology of sepsis and subsequently its potential as a possible biomarker in the future and 'context of use' in the clinical setting. Therefore, this publication focused on an sEV isolation and characterization technique that is new, novel, and fully automated, as many disadvantages, such as high input sample amounts, a decrease in the quality and quantity of sEVs yielded and higher time consumption, have been identified in conventional methods. Furthermore, the findings of this study are significant as they identify specific tetraspanin colocalization patterns in sepsis patients, which could provide a foundation for further use of sEVs as possible biomarkers for sepsis. This was determined by:

(1.) Using a fully automated platform to provide quick, multi-level, comprehensive and accurate measurements for sEV analyses.
(2.) Identifying tetraspanin colocalization patterns specific to sepsis patients as possible biomarkers

NB. Formatting, tables/figures and referencing was done in accordance with the journal requirements.

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Investigation of exosomal tetraspanin profile in sepsis patients as a promising diagnostic biomarker

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ABSTRACT

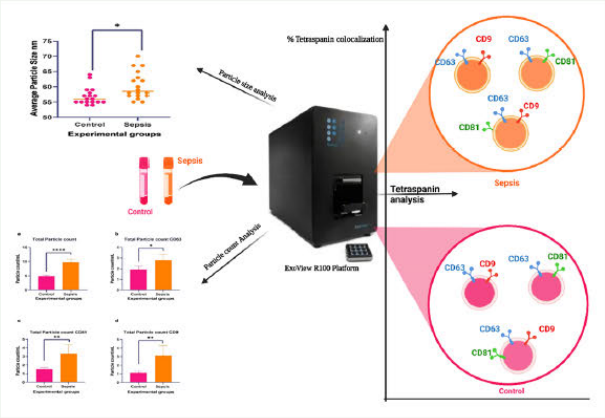
Introduction: Sepsis, a leading cause of mortality globally, has a complex and multifaceted pathophysiology which still requires elucidation. Therefore, this study aimed to analyze and quantify the number of exosomes in sepsis patients from a South African cohort using the ExoView (NanoView Biosciences, Boston, MA) platform.

Methods: Blood samples were collected from black South African patients attending the local Intensive Care Unit (ICU) hospital. Exosomes were isolated and characterize via TEM and CD63 ELISA kits. ExoView was used to determine particle count, particle size distribution and colocalization of different tetraspanin markers.

Results: Exosomal levels in sepsis patients were significantly higher compared to the control group ($p < 0.05$). Sepsis exosomes showed a homogenous size distribution ranging from 55 to 70 nm. Tetraspanin colocalization analysis revealed that sepsis exosomes have significantly higher CD63/CD9, CD63/CD81 and CD63/CD9/CD81 colocalization percentages than the control group.

Conclusion: This unique tetraspanin colocalization pattern of sepsis exosomes could serve as a potential sepsis biomarker. Further investigations are required to identify sepsis exosomal cargo signatures for further understanding of sepsis pathophysiology in order to develop effective diagnostics and treatments.

GRAPHICAL ABSTRACT



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1. Introduction

Sepsis is a highly complex and life-threatening disease caused by a dysregulated immune response to infection, leading to hyper-inflammation and end-organ dysfunction (Singer et al. 2016). Sepsis continues to be one of the leading causes of mortality globally and represents more than 50% of deaths in Intensive Care Units (ICU) (Markwart et al. 2020) and approximately 20% of overall global deaths (Rudd et al. 2020). In the USA, sepsis is a leading cause of in-hospital death and was the immediate cause of nearly 35% of in-hospital deaths (Rhee et al. 2019). Moreover, sepsis is considered to be a burden with a significant economic impact globally (Reinhart et al. 2017). The lack of substantive progress in research within the context of developing nations has heavily deepened this healthcare burden. The rapid and accurate detection of sepsis and factoring patient heterogeneity are crucial

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to reducing sepsis-related harmful effects and decreasing mortality rates across the spectrum (Agnello et al. 2021).

Sepsis is considered a heterogenous syndrome as the pathophysiology consists of a variety of physiologic and biochemical abnormalities (Nedeva et al. 2019, Pieroni et al. 2022). It is considered biphasic; the initial phase is regarded as a hyper-inflammatory phase, attributed to the host immune system's response to infection and the subsequent release of pro-inflammatory cytokines (Agnello et al. 2021). More than 50% of patients usually recover during this stage; however, a significant number of patients progress to the late immunosuppressive state (Brady et al. 2020). The immunosuppressive condition is characterized by immune cell depletion, cellular apoptosis, T-cell exhaustion, as well as imbalances in anti-inflammatory responses by the immune system (Wiersinga and VAN DER Poll 2022). These factors increase the risk of secondary infection and organ dysfunction/failure.

The complexity of the disease has led to problematic outcomes in terms of diagnosis and prognosis. Prompt and efficient diagnosis and administration of appropriate treatments are crucial to lower mortality rates and higher survival rates (Pieroni et al. 2022). Clinical biomarkers should ideally have the potential to diagnose sepsis, aid in severity predictions, and provide guidelines for clinicians in making appropriate and timeous treatment decisions. Additionally, the stratification of patients into homogenous groups depending on disease severity and treatment susceptibility should also factor in when identifying an ideal and precise clinical biomarker. Widely used clinical biomarkers for sepsis diagnosis, such as C-Reactive Protein (CRP) and procalcitonin, have played an essential role in sepsis management. However, limitations of these biomarkers, such as time-consuming, inability to identify infection origin and their limited diagnostic potential in terms of patient stratification, still exist. Despite the significant advances shown by the recently introduced innovative protein biomarkers, such as pro-adrenomedullin and presepsin, to overcome the challenges mentioned above, there is still an urgent need to develop novel biomarkers that can provide valuable information about this heterogenous population of sepsis patients. (Singer et al. 2016, Turgman et al. 2023).

Recently, small extracellular vesicles (exosomes) have attracted global interest due to their significant diagnostic and therapeutic potential for treatment of various diseases (Babaker et al. 2022, Khadka et al. 2022, Luo et al. 2021, Takeuchi and Nagai 2022, Geng et al. 2022, Yang et al. 2022). Exosomes are lipid bi-layered spherical vesicles with sizes rang–ng from 20–150nm (Luan et al. 2017, Zhou et al. 2020), containing lipids, transmembrane proteins and cytosolic proteins, nucleic acids and other cellular components (Yáñez-Mó et al. 2015, Li et al. 2018, Kalluri and Lebleu Valerie 2020). Exosomes are present in most cells, tissues and human body fluids such as plasma, urine, saliva, tears, gastrointestinal secretions, semen, and breast milk (Chen et al. 2019), enabling easy access to liquid biopsies. The biological functions of exosomes include antigen presentation, immune regulation, tissue development and remodelling, and cell-cell communication (Fais et al. 2016). Exosomes are usually internalized by the target cells, followed by cargo release (Gurung et al. 2021) to participate in various physiological and pathological processes, including cell homeostasis, immune response, and cancer progression. Exosomal cargo and surface proteins reflect the nature and physiological state of the cell from which it originates (Gurung et al. 2021). Exosomes contain DNA, RNA, proteins, and lipids specific to the cell of origin (Gurung et al. 2021, Luan et al. 2017, Zhou et al. 2020) and hence represent unique pathological changes. Therefore, exosomes have the potential to serve as novel biomarkers (Penfornis et al. 2016, Mathieu et al. 2019) that positively impact the diagnosis and treatment of various diseases (Fais et al. 2016, Luan et al. 2017, Breitwieser et al. 2022, Zhou et al. 2020).

The exosomal lipid bi-layer consists of several surface markers, including tetraspanins (CD63, CD9, CD37, CD81 and CD82) (Vlassov et al. 2012, Chaudhari et al. 2022), proteoglycans, and other transmembrane molecules (Breitwieser et al. 2022). The tetraspanin proteins are ubiquitously expressed on exosomes and are involved in cell adhesion, cell motility, and signal transduction processes. CD63 is the first characterized tetraspanin protein and has been more extensively studied than the other members of this protein group (Liu et al. 2020, Tippett et al. 2013). Interestingly, a study done by Breitwieser et al. (2022) approved exosomes from different cell origins have different patterns of tetraspanin surface colocalization and therefore, the functions of these exosomes are dependent on any changes in the composition of these surface markers (Breitwieser et al. 2022). Moreover, disease-specific variations in exosomal tetraspanin profiles have been reported for their diagnostic and patients stratification potential in rheumatoid arthritis (Rydland et al. 2023), viral infections (Ninomiya et al. 2021, Dogrammatzis et al. 2019) and cancers (Brzozowski et al. 2018, Odaka et al. 2022). However, to the best of our knowledge, these variations have not yet been studied for sepsis, therefore, investigating the effect of sepsis on the exosomal tetraspanin profiles would have a promising diagnostic, prognostic and patient stratification value.

Therefore, the aim of this study is to quantify and characterize circulating exosomes in South African sepsis patients and identify exosomal tetraspanin biomarkers using the cutting-edge technique provided by NanoView Biosciences, Boston, MA. The data from ExoView analysis of South African sepsis patients compared to healthy individuals, including particle counts, size distribution and tetraspanin colocalization, are herein reported. This study provides insight into the role of the circulating exosomes in sepsis. It considers a preliminary research that sheds light on using exosomal tetraspanin colocalization (for the first time) as a diagnostic biomarker for sepsis.

Clinical significance

- Sepsis is a global health threat with over 50% of ICU deaths attributed to it. With this alarming rate, there is no gold-standard for diagnosis with only clinical signs and symptoms used to diagnose the disease.

- In this study, we investigate the role of circulating exosomes as possible diagnostic tools for sepsis.
- There is an increased number of circulating exosomes in patients presenting with sepsis, however this is not substantial for the use of exosomes as potential biomarkers.
- In this study, we looked at the tetraspanin colocalization patterns, identifying specific patterns that are upregulated in sepsis patients.
- The use of these colocalization patterns as potential biomarkers is an ongoing area of research. However, this study identifies the tetraspanin colocalization of CD63 and CD9 to be abundant in sepsis patients, alluding to its potential as a diagnostic tool.

2. Materials and methods

2.1. Study population and sample collection

Our study population consists of Black South African patients from the ICU at Inkosi Albert Luthuli Hospital, a regional tertiary hospital in South Africa. Healthy individuals were used as the control group. Black South African patients diagnosed with sepsis according to The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Organ dysfunction can be identified as an acute change in total SOFA score ≥2 points consequent to the infection.) were recruited for this study. Participants with age less than 10 years, pregnancy, and comorbidities other than HIV were excluded from this study. In this study, according to declaration of Helsinki, we used two methods of informed consent, deferred consent, where patients had provided permission to use their blood in the study after they had recovered and next-of-kin proxy consent, where permission was provided by a family member/guardian when patients recruited for this study were critically ill and unable to consent at the time of sample collection. The treating physician took blood samples (8 mL) during the first 12 hours of admission into the ICU via an arterial line. Plasma was obtained and stored at −80 °C until the use date.

2.2. Exosome isolation

Exosome isolation was done using the total exosome isolation kit from plasma (Invitrogen by life technologies) as per the manufacturer's instructions. Briefly, 0.6 mL of clarified plasma was mixed with 0.3 mL of 1X Phosphate Buffered Saline (PBS). This solution was incubated at room temperature for 10 minutes with 0.18 mL of exosome precipitation reagent (supplied in the kit). The solution was centrifuged at 10,000 g for 5 mins to obtain the exosomes which were collected in the pellet at the bottom of the tube. The supernatant was carefully aspirated and discarded. The exosomes were then resuspended in 0.25 mL of 1X PBS and stored at −20 °C until further analysis.

2.3. Transmission electron microscopy (TEM)

Isolated exosomes were diluted in 1X PBS to obtain a 1:20 dilution ratio and placed onto nickel grides. The samples were negatively stained with uranyl acetate. The morphology of the particles were analyzes using the JEOL JEM 1400 transmission electron microscope (JEOL, USA) at a magnification of 20k and images were captured at 100 nm. Gatan Capture software (AMETEK. Inc, USA) was used to obtain images.

2.4. Quantification of circulating exosomes

The quantification of circulating exosomes was determined by the quantification of immunoreactive exosomal CD63 using an enzyme-linked immune absorbency assay (ExoELISA™, System Biosciences, Mountain View, CA), as per manufacturer's instructions. In brief, isolated exosomes were immobilized onto a microtiter plate with exosome binding buffer and was then incubated at 37 °C for 1 hour. The plate was then washed and incubated for 1 hour with the CD63 primary antibody. Following this, another wash step and incubation with the secondary antibody was done. The plate was then washed and incubated with the super-sensitive Tetramethylbenzidine ELISA substrate at room temperature for 15 minutes with agitation. The reaction was then terminated using the stop buffer and the absorbance was measured immediately at 450 nm. The number of exosomes/ml was obtained using an exosomal CD63 standard curve that was generated using the calibrated exosome standard that was supplied.

2.5. Exosome analyzes with ExoView (NanoView Biosciences, USA)

According to the manufacturer's instructions, size and fluorescent analyzes were performed using the ExoView R100 platform (NanoView Biosciences, USA). Briefly, samples were diluted in PBS and incubated on ExoView tetraspanin microchips in a sealed 24-well plate at room temperature overnight. The chips were washed three times and the ExoView Tetraspanin Labelling Antibodies that consist of anti-CD81 Alexa-555, anti-CD63 Alexa-488, and anti-CD9 Alexa-647 were added. The chips were then incubated with the antibodies, followed by another incubation step with the labelling solution. The chips were washed three times, rinsed in deionized water, and dried. All washing steps were conducted using the ExoView CW100 chip washer. The chips were then imaged with the ExoView R100 reader using the Exo Scan 2.5.5 acquisition software.

2.6. Statistical analyzes

All data analyzes and graphical representations were generated using GraphPad Prism 8.0 (C.A., La Jolla). Statistical significance was considered when $p < 0.05$.

3. Results

In this study, we aimed to include tetraspanin analyzes (CD63, CD81 and CD9) along with investigating exosomal concentration differences and other exosomal characteristics to understand the role of exosomes as sepsis biomarkers. Flow diagram and clinical characteristics of participants are presented in Figure 1 and Table 1, respectively. The patients for this study included sepsis patients that were HIV positive or presented with no other previously diagnosed co-morbidities. The HIV patients have all been on antiretroviral (ARV) drugs. Tetraspanin analyzes were performed using the ExoView R100 platform, which involves the immobilization of exosomes by the surface membrane proteins (CD63, CD81, CD9) on a microarray chip. Particle counts and particle size were determined using light interference. Immunofluorescent staining allowed the determination of tetraspanin composition at a single vesicle level. The ExoView R100 platform yielded multifaceted data, which we further discuss below.

3.1. Morphology and quantification of circulating exosomes

Isolated exosomes were analyzed using TEM for morphology and estimated size range. The total number of circulating exosomes was quantified using an exosome validated ELISA which detected the exosomal CD63 marker. The TEM analysis revealed a spherical shaped vesicle as well as the approximate size of the exosomes were within the range of 30-75nm (Figure 2b). The total number of circulating exosomes/ml was significantly higher in sepsis patients (1.39 x 10^{12}) when compared to healthy individuals (0.27 x 10^{11}) *(p = 0.0258)* (Figure 2a).

3.2. Exosomal levels and size distribution

Sepsis patients group showed a significant increase in the circulating exosomal level compared to the healthy individuals' group (p). This significant increase is represented by the total particle count captured by all probes and the average number of particles captured by each probe individually (Figure 3). The sepsis group showed a trend whereby all probes (viz. CD63, CD81, CD9) captured an approximately equivalent number of exosomes (particle count). In contrast, the control group CD63 probe captured a higher number of exosomes (compared to the other probes). Light interference was used to size particles down to 50 nm. The average size of particles within sepsis patients (55–70nm) is significantly larger than the average particle size of the control group (54–64nm) (Figure 4). The SP-IRS measurements showed homogenous size distribution among CD63, CD81, and CD9 captured exosomes with the average size of the control group of 57 nm while the average size of the sepsis patients of 60 nm (Figure 3). The exosomal average particle size of the control group was 57 nm, 56 nm, and 57 nm, and for the sepsis patients' group was 59 nm, 60 nm and 62 nm as captured by the CD63, CD81, and CD9 probes, respectively, as shown in Figure 5.

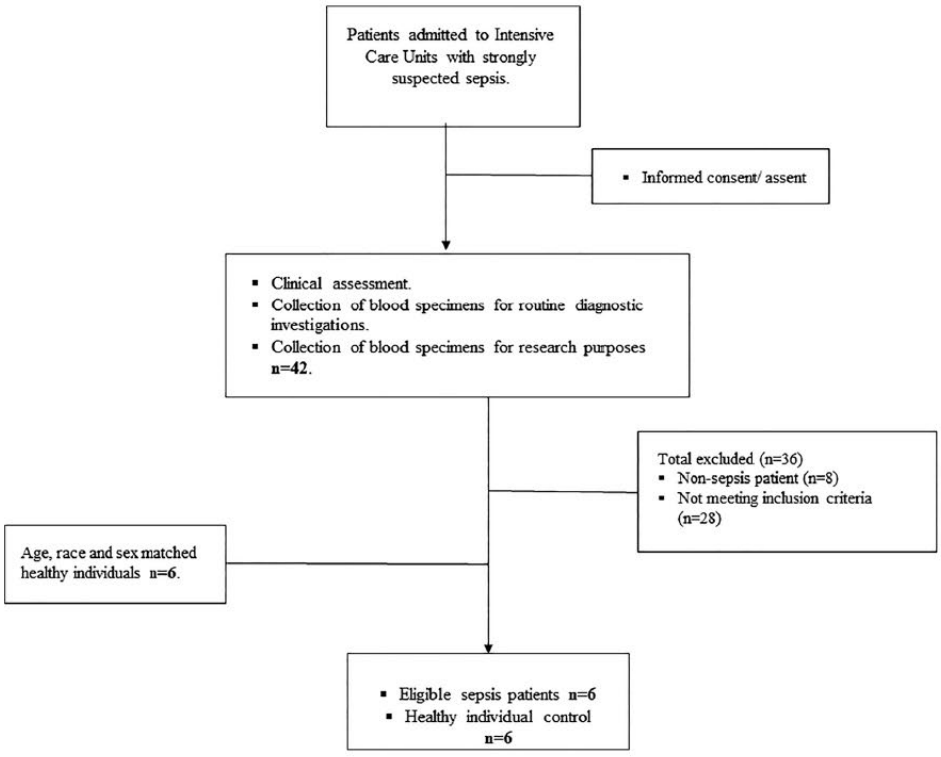

Figure 1. Participant's flow diagram.

3.3. Tetraspanin analyzes

Tetraspanin colocalization on the surface of exosomes at a single vesicle level has been investigated using immunofluorescent staining (Figure 6) to determine the colocalization percentage and colocalization trend.

3.4. Tetraspanin colocalization percentage

Tetraspanin colocalization analysis revealed that the exosomes captured by the CD63 probe from sepsis patients significantly increased the percentage of exosomal tetraspanin colocalisation compared to the control group. In this regard, sepsis patients showed that 15.18 ± 5.23% of these exosomes had two or more tetraspanin proteins colocalized on their surfaces (84.82 ± 5.24% had no colocalisation). In contrast, 94.1 ± 1.28% of the control group exosomes showed no tetraspanin colocalisation on their surfaces (single positive for only one tetraspanin). In comparison, only 5.9% ± 1.27 of the exosomes had stained positive for more than one tetraspanin.

On the other hand, exosomes captured by the other two probes (CD81 and CD9) showed an approximately equal tetraspanin colocalization percentage for both sepsis and control groups. Sepsis patients showed that 39.47 ± 6.45% of exosomes captured by the CD81 probe and 31.84 ± 4.65% of exosomes captured by the CD9 probe had two or more tetraspanin proteins colocalized on their surfaces. Similarly, control groups showed an equal colocalization percentage for both (40.5 ± 8.17% and 32.61 ± 7.91%, respectively).

Table 1. Clinical characteristics of participants.

Variables	Sepsis patients
Age range	*25–72 years*
Race	*African*
SOFA score range	*4–11*
Blood pressure (mmHg)	*129/71*
Heart rate (beats/min)	*102 ± 25.71*
Haemoglobin (g/mol)	*8.95 ± 1.22*
Respiratory rate (breaths/min)	*17.83 ± 4.99*
O_2 saturation (%)	*98.17 ± 2.56*
C-Reactive Protein (µg/mL)	*177.5 ± 130.98*
White cell count (x10_3per L blood)	*14.23 ± 10.76*
Platelet count (x10^3 /µL)	*243.17 ± 202.32*
Bilirubin (mg/dL)	*66.17 ± 126.52*
Creatinine (mg/dL)	*289.83 ± 249.67*
Mean arterial pressure (mmHg)	*89.83 ± 23.78*
HIV-positive sepsis patients	*3 patients*
HIV-negative sepsis patients	*3 patients*

Notes: All values are represented as the mean ± SEM.

3.5. Tetraspanin colocalization pattern analysis

Differences in the tetraspanin colocalization pattern between sepsis and the control group could shed light on their ability to interact with target cells and how they function. Therefore, this subsection investigates these differences. The colocalization pattern analysis for exosomes captured at the CD63 capture spot presented in Figure 7 showed that sepsis patients had a significantly higher percentage of CD63/CD9 (4.43 ± 2.27%), CD81/CD9 (3.03 ± 0.85%) and CD63/CD81/CD9 (2.07 ± 1.03%) colocalization compared to the control group CD63/CD9 (1.03 ± 0.50%), CD81/CD9 (2.02 ± 0.58%) and CD63/CD81/CD9 (0.80 ± 0.39%). On the other hand, CD63/CD81 colocalization showed an insignificant increase in the sepsis group (5.65 ± 4.50%) compared to controls (2.07 ± 0.38%).

The colocalization pattern analysis for exosomes captured at the CD81 capture spot (Figure 8) showed no significant differences between the sepsis patient and the control group. Sepsis patient's colocalization percentage recorded as follow: CD63/CD81 (14.80 ± 5.96%), CD63/CD9 (3.30 ± 0.64%), CD81/CD9 (14.08 ± 5.08%) and CD63/CD81/CD9 (7.27 ± 1.99%) while the control group showed a colocalization percentage of CD63/CD81 (12.29 ± 2.73%), CD63/CD9 (4.00 ± 1.17%), CD81/CD9 (15.80 ± 5.60%) and CD63/CD81/CD9 (7.77 ± 3.25%).

The colocalization pattern analysis for exosomes captured at the CD9 capture spot presented in Figure 9 showed that sepsis patients had a significantly higher percentage of CD63/CD9 (12.25 ± 3.76%) and a significantly lower percentage of CD81/CD9 (7.72 ± 2.88%) colocalization compared to the control group, which showed CD63/CD9 (4.08 ± 1.14%) and CD81/CD9 (15.93 ± 4.86%). While CD63/CD81 and CD63/CD81/CD9 colocalization showed an insignificant increase in the sepsis group (7.13 ± 3.23% and 4.68 ± 1.45%, respectively) compared to controls (5.18 ± 1.06% and 7.43 ± 2.96%, respectively).

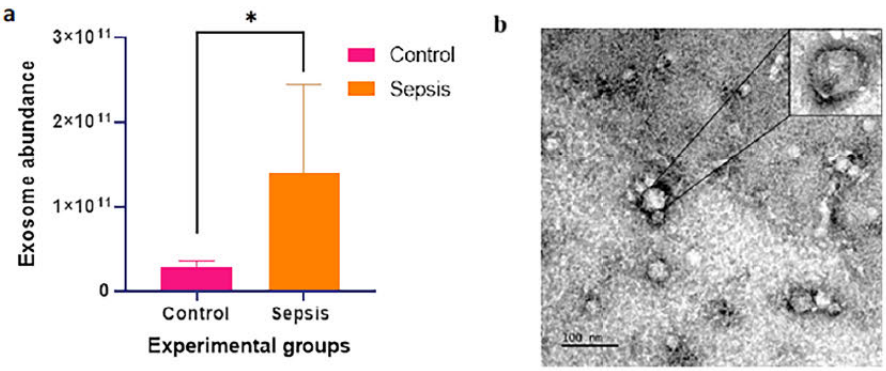

Figure 2. Identification and characterization of isolated exosomes in patients presenting with sepsis compared to healthy individuals. (a) Exosome abundance quantified using CD63 ExoELISA, shows a significant increase in the number of circulating exosomes in patients with sepsis when compared to the control group (control vs sepsis $p = 0.0258$). Exosome abundance plotted as the mean ± SD (b) Electron micrograph of isolated exosomes showing spherical shape and approximate size, scale bar 100 nm.

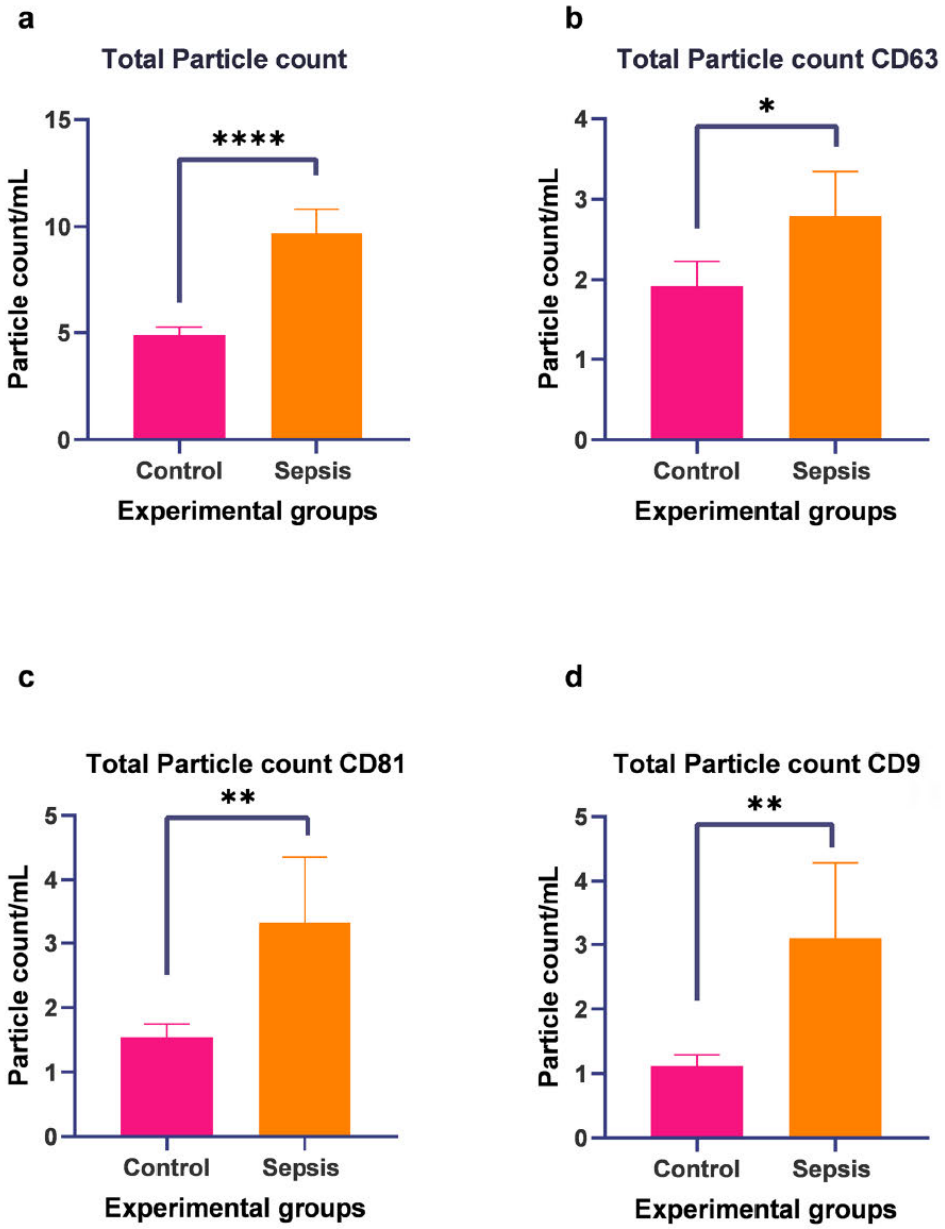

Figure 3. Comparison of total particle count between the control and sepsis patients groups and the average particle count captured by each probe. Comparisons were made using unpaired *t*-tests. (a) The total particle count captured by all probes (control vs sepsis $p < 0.0001$). (b) The total particle count captured by CD63 the probe (control vs sepsis $p = 0.007$). (c) The total particle count captured by the CD81 probe (control vs sepsis $p = 0.0021$). (d) The total particle count captured by the CD9 probe (control vs sepsis $p = 0.0022$). Data shown as particle count/mL, plotted in the graphs, are the mean ± *S.D* ($n = 6$ for each group).

Interestingly, we observed that Human Immunodeficiency Virus (HIV) positive sepsis patients have a higher number of CD9 positive exosomes when compared with HIV-negative sepsis patients (Figure 10). However, we could not conclude on the significance due to limitations in our study design that include small sample size and the effect of other variables such as SOFA score.

4. Discussion

As the healthcare burden of sepsis increases with every passing year, there is still a dire need for a reliable and clinically relevant biomarker. This burden is heavily increased in developing nations as there is a lack in the research available. In recent years, exosomes have gained much attention for their role as potential novel biomarkers and therapeutic applications; however, our knowledge of exosomal phenotypes is incomplete due to the complexity and limitations of various isolation and characterization methods. Previously reported studies have analyzed exosomal tetraspanin profiles in various diseases such as cancer and viral infections, these studies have suggested that these variations aids in the progression of the disease and has the potential to be utilized as a diagnostic marker. As far as we are aware, these

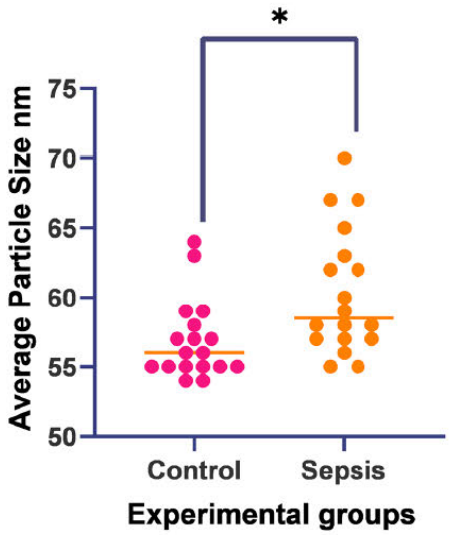

Figure 4. Comparison of average particle size between the control group and sepsis patients using an unpaired *t*-test ($p = 0.0187$). Sepsis patients showed a significant increase in size. Data shown as particle size in nm, plotted is the mean ± *S.D.*, ($n = 6$ for each group).

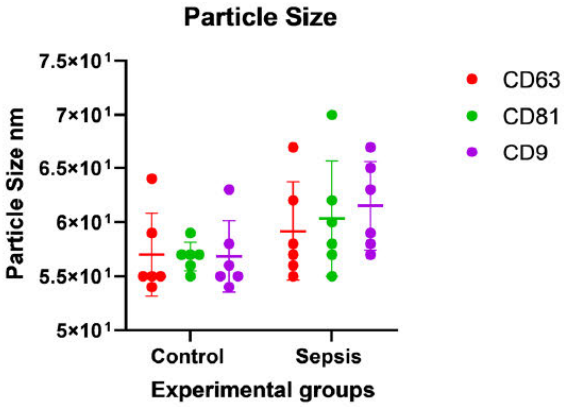

Figure 5. Comparison of average particle size captured by each tetraspanin probe (CD63, CD81, CD9). Comparisons were made using multiple *t*-tests; however, no significant differences were observed ($p > 0.05$). Particle size shown in nm, plotted is the mean ± *S.D.*, ($n = 6$ for each group).

variations have not been studied in sepsis. In this study, we aimed to quantify and characterize circulating exosomes and tetraspanin markers in South African sepsis patients to provide further understanding of their role in sepsis as prognostic, diagnostic and therapeutic tools. The increase in the percentage of CD63 and CD9 colocalization patterns in sepsis patients observed in this study may be useful in investigating exosomes and their surface epitopes as potential biomarkers.

Over the past decade, there have been tremendous advances in exosomal isolation and characterization methods, which have contributed to a better understanding of the role of exosomes in disease diagnosis, progression and treatment (Yang et al. 2019). Despite significant advances in the conventional exosome isolation methods (differential ultracentrifugation, ultrafiltration, precipitating agents, immunoaffinity capture, microfluidics, size-exclusion chromatography and commercial kits), some disadvantages have also been identified, including increased time consumption, high sample input, high cost and lower yield (quantity and quality of exosomes). Recently, a novel platform, ExoView (NanoView Biosciences, Boston MA), has been introduced to overcome the limitations mentioned earlier for the isolation and provide an advanced option for the characterization and quantification of exosomes and exosomal tetraspanin biomarkers (Deng et al. 2022). This fully automated platform provides quick, multi-level, comprehensive and accurate measurements for exosome particle size analysis, exosome count, exosome phenotyping, and biomarker colocalization utilising a relatively low sample input. Importantly, results obtained from the ExoView R100 are reproducible as the fully automated platform eliminates the bias related to downfalls of other isolation methods associated with aspiration processes. This is crucial for the identification and development of reliable biomarkers.

The ExoView R100 platform allows for an easy and helpful way to obtain data. ExoView uses the single-particle interferometric reflectance imaging sensor (SP-IRIS) technique to collect simple automated data (Deng et al. 2022). This platform allows for the isolation, characterization and quantification of exosomes that are fluorescently detected by tetraspanin antibodies (CD63, CD81 and CD9).

In this study, we look into circulating exosomes in South African sepsis patients. Previously, studies demonstrated that mice injected with lipopolysaccharide (LPS), which mimics the symptoms of sepsis, had elevated levels of exosomes present in their serum compared to normal control mice (Gao et al. 2019). Increased exosome levels were also observed in the media of LPS-challenged cells (Ti et al. 2015). Our study's results indicate elevated exosome levels in sepsis patients compared to healthy individuals. Data from quantification and characterization techniques confirmed a higher exosome abundance in sepsis patients. Microscopy confirmed the morphology and size of isolated exosomes, TEM images (Figure 2) show spherical, bi-layered vesicles with an approximate size range of 50-90nm. These results are consistent with a study that associated the increased particle counts in patients presenting with septic shock with the severity of organ failure (Im et al. 2020). This study also includes a positive relationship between exosome levels and SOFA scores. Therefore they indicate that exosomes may also play a role in predicting mortality in septic shock patients (Im et al. 2020).

The elevated levels of exosomes in sepsis patients alludes to a role for exosomes in the pathophysiology of sepsis. Further work is required to elucidate the potential of exosomes as diagnostic and prognostic tools for sepsis. We also performed tetraspanin analyzes to shed more light on this role. Tetraspanins such as CD63, CD81 and CD9 are regularly considered surface markers for extracellular vesicles (Andreu and Yáñez-Mó 2014). These tetraspanins are transmembrane proteins belonging to a protein superfamily. These proteins organize membrane microdomains termed tetraspanin-enriched microdomains by creating clusters and interacting with numerous transmembrane and cytosolic proteins (Charrin et al. 2009, Andreu and Yáñez-Mó 2014). A study in 2021

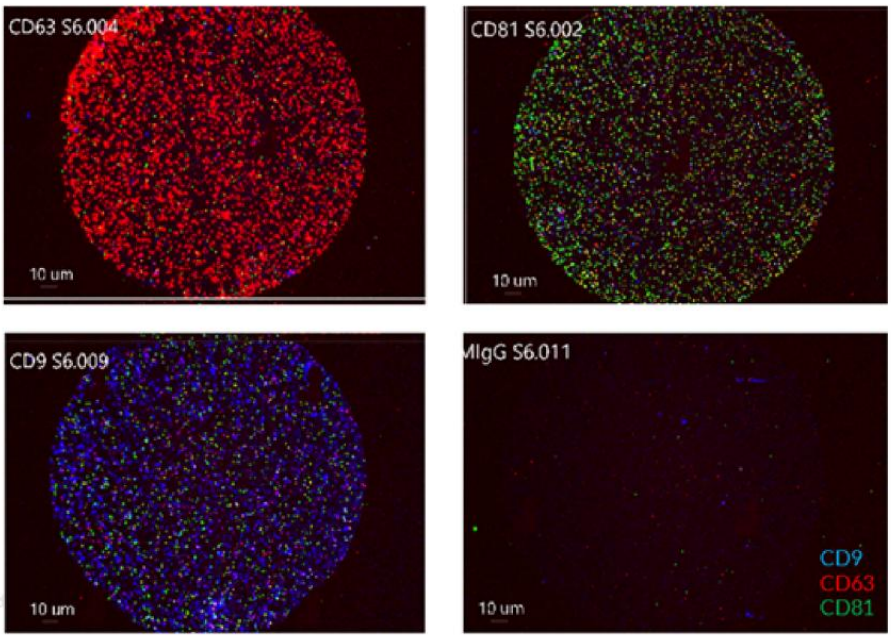

Figure 6. A Representative fluorescent image for each capture spot. Fluorescent image of exosomes captured by the CD63 probe (a), CD81 probe (b), CD9 probe (c) and IgG (d).

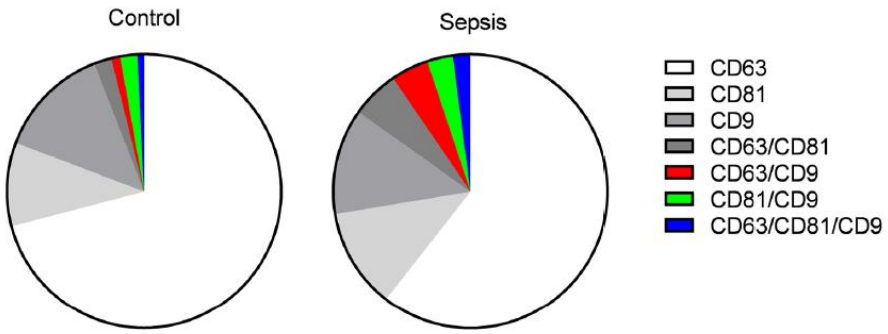

Figure 7. Depicts the tetraspanin colocalisation of exosomes using the fluorescent channel for CD63. The data shown represents the tetraspanin colocalisation percentage (%) and plotted is the mean percentage for the control group and sepsis patients. Significant increases in colocalization patterns include CD63/CD9 (control *vs* sepsis $p = 0.004$), CD81/CD9 (control vs sepsis $p = 0.037$) and CD63/CD81/CD9 (control vs sepsis $p = 0.018$), depicted in red, green and blue respectively, ($n = 6$ for each group).

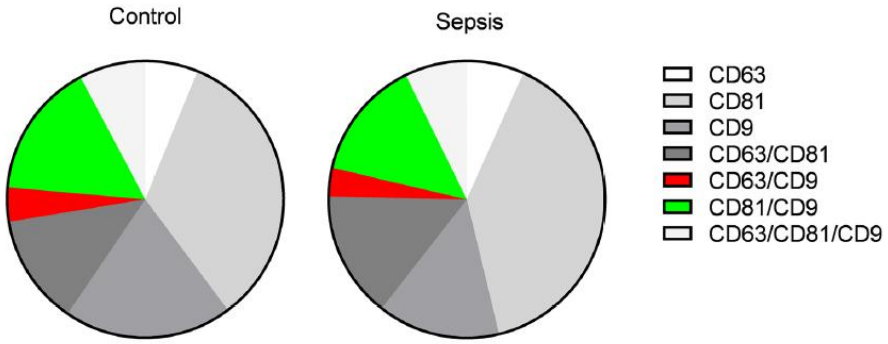

Figure 8. Depicts the tetraspanin colocalization of exosomes using the fluorescent channel for CD81. The data shown represents the tetraspanin colocalization percentage (%) and plotted is the mean percentage for the control group and sepsis patients. No significant values were observed *($p > 0.05$)* ($n = 6$ for each group).

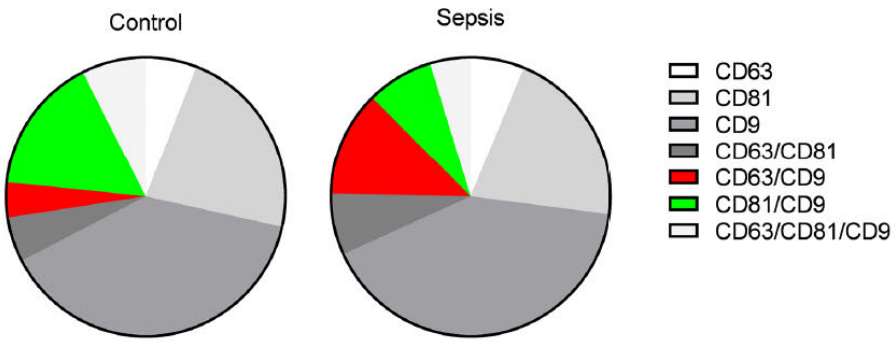

Figure 9. Depicts the tetraspanin colocalization of exosomes using the fluorescent channel for CD9. The data shown represents the tetraspanin colocalization percentage (%) and plotted is the mean percentage for the control group and sepsis patients. Significant increases in colocalization patterns includes CD63/CD9 (control vs sepsis $p < 0.0001$), CD81/CD9 (control vs sepsis $p = 0.005$), ($n = 6$ for each group).

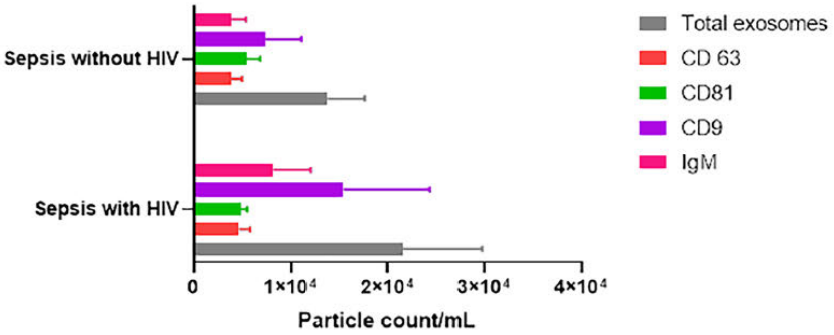

Figure 10. A Representation of the total number of exosomes captured by the CD9 probe in sepsis patients who are HIV positive vs sepsis patients who are HIV negative ($p > 0.05$).

showed that extracellular vesicles from different sources (cultured cells and human serum) have varying tetraspanin profiles suggesting that extracellular vesicles subpopulations occur with distinctive tetraspanin composition (Mizenko et al. 2021).

Fluorescently labelled antibodies provided detailed data on CD63, CD81 and CD9 captured exosomes. A 2021 study revealed that higher levels of exosomal CD63 are associated with the severity of organ failure (Im et al. 2021). Consistently, we found that particle counts were significantly higher among all capture probes in sepsis patients. Further extensive analysis showed that colocalization patterns using the CD63 and CD9 capture probes were significantly greater in these patients. A study by Breitwieser et al., affirmed the use of the ExoView R100 platform to show that colocalization can differentiate the exosomal origin from different cells (Breitwieser et al. 2022). Therefore, this suggests a cell type-specific tetraspanin heterogeneity for the commonly used surface markers.

Additionally, we have observed that patients presenting with sepsis who are also HIV positive, have a higher number of CD9 positive exosomes, however these results are inconclusive due to limitations in our study design. Chronic co-morbidities such as HIV, cancer, lung diseases, etc. are present in approximately 60% of patients with sepsis and can influence the outcomes and progression in these patients (Esper et al. 2006). In sepsis, chronic co-morbid medical conditions and advanced age, depending on the level of immunosuppression, could increase infection rates and accelerate organ failure in these patients, ultimately increasing mortality rates (Yang et al. 2010). Targeting HIV as a co-morbidity for sepsis and investigating the effects HIV has on the progression and outcomes of sepsis is of great importance globally, especially in South Africa. In South Africa, there are approximately 8 million people living with HIV and remains a national health care issue with no definitive treatment. A study in 2021 investigated the clinical role of CD9 positive exosomes in cancer and HIV patients as well as HIV patients with cancer (Dimitrakopoulos et al. 2021). Exosomes were isolated using a commercial kit and quantified using a double-sandwich ELISA technique. The results from this study showed a higher number of exosomes in HIV and cancer patients when compared to healthy individuals suggesting possible prognostic value of CD9 positive exosomes (Dimitrakopoulos et al. 2021). It has also been reported that exogenous CD9 expression increases the production of exosomes and has a role in virion formation in HIV (Böker et al. 2018). Further investigations are required to fully understand the role of exosomes in HIV and the effects co-morbid medical conditions has on the progression of sepsis.

We have observed that tetraspanin colocalization patterns could serve as potential biomarkers due to the significant increases observed in sepsis patients. In addition, our results highlight that within sepsis patients, particles that stained positive for CD63 and CD9 on both the CD63 and CD9 capture probes may be of increasing interest in exploring the potential of exosomes as possible biomarkers. Based on our

results, it was observed that CD63-positive particles are abundant. However, there is also significance in the number of CD81 and CD9 positive particles. Overall, the role of tetraspanin proteins in sepsis is an ongoing area of research. Further studies are required to understand their role and functions in this heterogeneous disease fully. Future approaches should include the investigation of tetraspanin protein differences between sepsis and Systemic Inflammatory Response Syndrome (SIRS) patients, in order to shed light on the pathological processes associated with the progression of sepsis. This study proposes a promising potential for the exosomal tetraspanin colocalization pattern to be biomarkers and/or therapeutic targets for diagnosing and treating sepsis. The expression patterns observed in this study could possibly support stratification of patients into more homogenous groups which could aid in more timeous diagnosis and initiation of treatments. This study also provides information on colocalization patterns that could be specific to HIV positive sepsis patients, further research is required to properly conclude on these results and observe the changes in expression patterns during HIV and other co-morbidities. This could facilitate the use of exosomes as prognostic markers. We have provided a pre-liminary basis to implicate exosomes in the pathophysiology of sepsis and to identify patterns specific to the disease, further research is required to investigate the exosomal cargo of sepsis patients to interpret and improve exosome-based diagnostic tools and therapeutic approaches correctly.

The main limitations of this study were the sample size that could influence the study statistical power and the high cost of using the ExoView R100 platform. Therefore, further investigations involving larger sample size and lower cost-analysis platform such as flow cytometry using the appropriate fluorescent probes are highly recommended.

5. Conclusion

This study highlights different tetraspanin colocalization patterns in sepsis patients that could serve as a potential biomarker for sepsis. Further research is required to identify the distinctive colocalization pattern for patient stratification in sepsis in order to optimzse sepsis therapeutic intervention.

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Ethical approval

Ethical approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004587/2022) and the South African Department of Health (NHRD: KZ_202107_008).

Authors' contribution

Roushka Bhagwan Valjee: Sample collection, Investigation, Methodology, Conceptualization, Formal Analysis, writing of the original draft. Irene Mackraj: Conceptualization, Writing revision and editing, Provision of resources and Supervision. Roshila Moodley: Writing revision and editing, Provision of resources. Usri H. Ibrahim: Investigation, Formal Analysis, writing of the original draft preparation, reviewing, and editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, [Usri Ibrahim], upon reasonable request.

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4 CHAPTER FOUR: MANUSCRIPT THREE

Characterizing the surface marker profiles of circulating small extracellular vesicles in sepsis patients. (Submitted: Smart Materials in Medicine)

Bridging Text

Our previous study in publication two **(Chapter Three)** sheds light on tetraspanin colocalization patterns and the potential of sEVs as biomarkers for sepsis. The surface membrane of sEVs is composed of a variety of markers, alluding to surface marker heterogeneity and disease-specific sEV subtypes. Additionally, with sepsis being a multifaceted pathological state, the presence of co-morbidities adds to the burden of developing biomarkers for sepsis. We, therefore, evaluated the surface marker profiles in 4 cohorts (viz. healthy controls, hypertension, sepsis patients with no pre-existing co-morbidities and sepsis patients with previously diagnosed hypertension). Hypertension is a global health burden, and the presence of hypertension increases the risk of developing sepsis due to various factors such as increased inflammation and a compromised cardiovascular system. This was determined using the MACSPlex flow cytometric analysis which allows the identification of 37 different markers present on the surface of isolated sEVs. The findings from this study identify specific surface marker profiles in the aforementioned pathological states, which lends greater significance to the use of sEVs as potential biomarkers for sepsis.

NB. Formatting, tables/figures and referencing was done in accordance with the journal requirements.

Characterizing the surface marker profiles of circulating small extracellular vesicles in sepsis, hypertension and sepsis with hypertension.

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Data availability statement:

The data that support the findings of this study are available from the corresponding author, [Usri Ibrahim], upon reasonable request.

Abstract

Sepsis is one of the leading causes of mortality worldwide. Pathogenesis heterogeneity, together with no "gold standard" for diagnosis, drives research efforts in biomarker identification. Moreover, the burden of pre-existing co-morbidities, specifically hypertension, has complicated the clinical picture and obscured the diagnosis of sepsis. Small extracellular vesicles (sEVs) have recently been identified as possible biomarker candidates for various diseases, including sepsis, due to their important roles in intercellular communication and inflammation. Recently, circulating sepsis sEVs abundance, cargo contents and surface tetraspanin proteins have been studied as possible sepsis biomarkers. Therefore, this study aimed to investigate the biomarker potential of sEV surface markers profile in sepsis, hypertension and hypertensive sepsis. This was done using the MACSPlex exosome kit (Miltenyi Biotech) which utilized flow cytometry to identify 37 different surface markers on isolated sEVs. Specific profiles were identified in hypertension, sepsis and hypertensive sepsis states showing differentially expressed surface markers when compared to healthy controls or with other experimental groups. These results show that there are disease specific surface marker profiles in these pathological states. Altogether, the identified profiles can facilitate diagnostic and/or therapeutic progress in sepsis and hypertension.

Keywords

Sepsis, hypertension, small extracellular vesicles, surface marker, diagnosis, MACSPlex.

Highlights

- This study investigates the surface marker profiles of sepsis, hypertension and with both conditions combined.
- This was done using the MACSPlex exosome kit which identifies 37 surface markers on isolated small extracellular vesicles.
- The findings of this study show that there are disease-specific surface marker profiles on small extracellular vesicles isolated from sepsis, hypertension and sepsis with previously diagnosed hypertension.
- These profiles aid in the identification and classification of disease-specific small extracellular vesicle subsets in sepsis and hypertension.
- The identified profiles add to the understanding of sepsis pathogenesis and may be of interest in biomarker development for sepsis.

4.1 Introduction

Sepsis and septic shock are among the leading causes of mortality, with mortality rates of sepsis being 15-25% and mortality rates of septic shock as high as 30-40% [1, 2]. In 2017, the World Health Organization (WHO) identified sepsis as a global health priority [3]. In that year, The Global Burden of Disease Study reported approximately 48.9 million sepsis cases and 11 million sepsis-related deaths are caused annually, representing a staggering 20% of all global deaths and the cause of 1 in 5 deaths worldwide [4, 5]. The Surviving Sepsis Campaign issued the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), which defines sepsis as life-threatening organ dysfunction caused by a dysregulated immune response to infection, with organ function being evaluated by the Sequential Organ Failure Assessment (SOFA) score [6, 7]. Approximately 15% of all patients with sepsis experience septic shock [4, 8]. The pathophysiology of sepsis is highly heterogeneous involving complex processes and various systems driven by the initial stimuli/pathogen introduced into the immune system [9-11]. The increase in pro- and anti-inflammatory pathways initiates the vast release of cytokines, mediators and pathogen-related molecules [10, 12]. This subsequently leads to the activation of coagulation pathways and other complement cascades. The development of sepsis varies significantly among individuals [13, 14]. With this, the prognosis and outcome depend upon the various elements specific to the ill individual, such as existing or newly developed chronic illnesses. These chronic illnesses include but are not limited to hypertension, diabetes, chronic renal disease, vascular disease, HIV and TB [13, 15]. This variation still requires further elucidation in order to develop precise and accurate diagnostic and therapeutic tools.

Hypertension, defined as 2 or more measurements systolic blood pressure (SBP) >140 mmHg or diastolic blood pressure (DBP) >90 mmHg, continues to be a significant problem across the globe [16]. The most recent World Health Organization (WHO) report from 2017 indicated that hypertension prevalence was higher among adults in low- and middle-income countries than in high-income countries [16, 17]. Blood pressure (BP), determined by various factors, including blood volume and cardiac output, is controlled by the interaction of the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system (SNS) and the immune system [18]. Alterations of these factors may directly or indirectly cause an increase in the mean BP and/or variability which in time may lead to end organ failure [19]. Systemic endothelial dysfunction is an important factor in the pathology of chronic hypertension. Hypertensive patients have increased levels of reactive oxygen species which in turn causes

the disruption of protective nitric oxide excretion by endothelial cells [20]. It has been hypothesized that systemic endothelial dysfunction increases the predisposition to developing sepsis [20, 21]. Specifically, patients with chronic hypertension are at a higher risk of developing sepsis in part due to shared endothelial dysfunction including increased oxidative stress with systemic inflammation [22]. Hypertension is one of many pre-existing chronic illnesses observed in patients with sepsis [23]. These chronic illnesses add to the difficulty of developing possible biomarkers for sepsis as the physiological systems and pathways affected by these pathological states vary within the pathophysiology of sepsis.

Currently, a biomarker is defined as an indicator of a particular disease state that can be measured and monitored [24, 25], which traditionally have included nucleic acids, proteins and peptides, hormones, growth factors, antibodies and more [24, 25]. Biomarkers are used to track the progress, the effects of treatment, and serve as an indicator of a disease state [26], making them critical for monitoring heterogenous diseases. They also help patients through early diagnosis and are prognostic, thereby guiding therapeutic interventions clinically. For a biomarker to be deemed useful, several criteria need to be fulfilled [26, 27]. It should be specific to the disease with minimum false positives, and minimally invasive to obtain. The most important factor is that it should give information on the prognosis of the disease and be an effective measure of the outcome of treatment [27]. In the past decade, small extracellular vesicles (sEVs), such as exosomes, have emerged as a novel and potent tool in understanding the development and status of various diseases [28]. These small vesicles, measuring 30-200 nm in diameter, can be found in most bodily fluids including blood, urine, amniotic fluid, malignant ascites fluids, synovial fluid, and breast milk [29-31]. Ongoing research has shown that sEVs play a significant role in intercellular communication [32, 33]. This is achieved by means of the selection and packaging of proteins, mRNA, miRNA, and bioactive lipids into sEVs [34]. The fact that the contents of sEVs reflect the state of the cell of origin has prompted researchers to investigate their potential as biomarkers [28]. To investigate the biomarker potential of sEVs, phenotyping of sEV groups is crucial in understanding their role in pathological states. Our previous study showed that tetraspanin (CD63, CD81 and CD9) colocalization patterns in sepsis could serve as potential biomarkers for sepsis [35]. Primarily, the functions of tetraspanins include the formation, and sorting of cargo as well as the uptake and release of sEVs [36]. The formation of tetraspanin webs leads to a variety of surface profiles on the membrane of sEVs [36]. This is caused by the interaction of tetraspanins with other tetraspanins and surface markers present on the membrane of sEVs. Furthermore,

proteins are either encapsulated within the lumen or embedded on the surface of sEVs therefore enabling subtyping of these sEVs without destroying their structure [37]. These surface proteins are indicative of sEV biogenesis, secretion, protein-protein interactions, and recipient cell targeting. It is, therefore, vital to study these surface proteins in the pathology of sepsis.

The present study includes a comparative analysis of surface marker profiles of sEVs from South African patients diagnosed with sepsis with no pre-existing co-morbidities and sepsis patients previously diagnosed with hypertension. This study aimed to provide an understanding of the effects of hypertension on the pathophysiology of sepsis and investigate the surface marker profiles in these disease states. This was performed by multiplexed flow cytometric bead assay (MACSPlex), which to the best of our knowledge is the first such study. Using this technique, this study provides valuable insights into the molecular distinctions between sepsis and sepsis with hypertension. These specific surface marker profiles may serve as potential biomarkers for diagnostic stratification and therapeutic targeting.

4.2 Materials and methods

4.2.1 Ethics statement

Regulatory ethical and institutional approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004587/2022) and the South African Department of Health (NHRD: KZ_202107_008). Patients were recruited from Inkosi Albert Luthuli Central Hospital and King Edward VIII Hospital in Durban, KwaZulu-Natal.

4.2.2 Study population and sample collection

Our study population consists of Indian and Black South African patients from the ICU at Inkosi Albert Luthuli Hospital and King Edward VIII Hospital. Patients were recruited based on the following inclusion criteria; patients presented clinical manifestations consistent with sepsis and/or septic shock, were >10 years of age and included women who were not pregnant at the time of recruitment. The patient clinical characteristics are provided in a table shown as the mean ± SD (supplementary data, table 1). The experimental study groups include patients with sepsis and/or septic shock with previously diagnosed hypertension, including those patients on hypertensive medications except any calcium channel blocker (n=12), additionally a group consisting of patients with sepsis and/or septic shock with no pre-existing co-morbidities (n=12). In this study, we used 2 methods of informed consents, deferred consent

where patients had provided permission to use their blood in the study after they had recovered and next-of-kin proxy consent where permission was provided by a family member/guardian. This was done as patients recruited for this study were critically ill and unable to provide consent at the time of sample collection. This study used 2 control groups consisting of age, gender and race-matched individuals with hypertension (n=12) and a group of healthy individuals with no chronic illnesses (n=12). Blood samples (8ml) were taken by the treating physician during the first 12 hours of admission into ICU, via an arterial line. Blood samples were taken in vacutainer tubes treated ethylenediaminetetraacetic acid (EDTA) (Becton, Dickinson and Company, SA) and plasma was obtained by centrifugation for 15 minutes at 3500 x g. The plasma was stored at -80°C until the date of use. Plasma samples were used for analysis within 3 months of collection.

4.2.3 sEV isolation

sEV isolation was done using the total exosome isolation kit from plasma (Invitrogen by Life Technologies, CA, USA) as per the manufacturer's instructions. Briefly, 0.6mL of clarified plasma was mixed with 0.3mL of 1X Phosphate Buffered Saline (PBS). This solution was incubated at room temperature for 10 minutes with 0.18mL of exosome precipitation reagent (supplied in the kit). The solution was centrifuged at 10 000 g for 5 mins to obtain the sEVs which were collected in the pellet at the bottom of the tube. The supernatant was carefully aspirated and discarded. The sEVs were then resuspended in 0.25mL of 1X PBS and stored at -20ºC until further analysis.

4.2.4 sEV characterization

The quantification and size distribution of sEVs were performed using Nanoparticle Tracking Analysis (NTA) with the NanoSight500 NTA 3.2 Nanoparticle Tracking Analysis Release, Version Build 0069 instrument equipped with a sCMOS camera type and a Blue405 laser (Malvern Panalytic, UK). Briefly, a particle distribution of between 10 and 100 particles per image was obtained by the dilution of samples in 1X PBS. Samples were mixed for uniform particle dispersion before being added into the chamber (temperature: 25°C and viscosity: 0.86 cP). Samples were introduced into the instrument, videos were recorded at a camera level of 12, a camera shutter speed of 20ms and a camera gain of 600. These parameters were kept constant among all samples. The mean, mode and median particle sizes and concentration of

particles were determined via video analysis. The size of the sEVs was represented as the mean particle size ± SEM. Furthermore, the morphology of isolated sEVs was analysed using Transmission Electron Microscopy (TEM) using the protocol provided in our previously published study (supplementary data, figure 1) [35].

4.2.5 Quantification of circulating sEVs

The quantification of circulating sEVs was determined by the quantification of immunoreactive exosomal CD63 using an enzyme-linked immune absorbency assay (ExoELISA™, System Biosciences, Mountain View, CA), as per the manufacturer's instructions. In brief, isolated sEVs were immobilized onto a microtiter plate with exosome binding buffer and were then incubated at 37ºC for 1 hour. The plate was then washed and incubated for 1 hour with the CD63 primary antibody. Following this, another wash step and incubation with the secondary antibody were done. The plate was then washed and incubated with the super-sensitive Tetramethylbenzidine ELISA substrate at room temperature for 15 minutes with agitation. The reaction was then terminated using the stop buffer and the absorbance was measured immediately at 450nm. The number of sEVs/ml was obtained using an sEV CD63 standard curve that was generated using the calibrated sEV standard (R^2 =0.9696, y=1E-11x) that was supplied.

4.2.6 Bead-Based Multiplex Flow Cytometry Assay

Isolated sEVs were subjected to bead-based multiplex exosome flow cytometry assay using the MACSPlex exosome kit, human (Miltenyi Biotec) using the short protocol as per the manufacturer's instructions. A concentration of 10µg of EVs was used for each sample, this was then diluted to a final volume of 120µl for each sample. The sample was added to a 1.5mL tube followed by 15µl of MACSPlex Exosome Capture Beads (containing 39 different antibody-coated bead subsets) and 15µl of the detection antibody cocktail (containing CD63, CD81 and CD9 antibodies). The tubes were then incubated for 1 hour at room temperature protected from light on an orbital shaker at 450rpm. Following the incubation, 500µl of the MACSPlex Buffer was added to each tube and centrifuged at 3000 x g for 5 minutes at room temperature. Approximately 500µl of the supernatant was carefully aspirated and discarded, followed by a second addition of 500µl of the MACSPlex buffer to each tube. The tubes were incubated for 15 minutes at room temperature protected from light on an orbital shaker at

450rpm followed by centrifugation at 3000 x g for 5 minutes at room temperature. Approximately 500µl of the supernatant was aspirated and discarded and the solution was resuspended by pipetting up and down as well as by vortexing the tubes for 30 seconds. Flow cytometric analyses were performed with the Cytek Northern Lights flow cytometer (Cytek Biosciences B.V., Amsterdam, Netherlands). Approximately 350µl (final sample volume diluted in MACSPlex buffer) was loaded to and acquired by the instrument, resulting in approximately 15 000 single bead events being recorded. The 39 bead populations were distinguished by the FITC and PE (excited by the blue laser) channels, sEVs were detected using the APC (excited by the red laser) channel. The Spectroflo® (Cytek Biosciences B.V., Amsterdam, Netherlands) software was used for data acquisition from each sample. FlowJo software (v10, FlowJo LLC) was used to analyze flow cytometric data. Median fluorescence intensity (MFI) for all 39 capture bead subsets were background corrected by subtracting respective MFI values from matched non-EV blank controls which contained the sEV capture beads, antibody detection cocktail and water, as well as the MFI of the corresponding isotype controls (mIgG1, REA).

4.2.7 Statistical analysis

All data analyses and graphical representations were generated using GraphPad Prism 8.0 (La Jolla, CA, USA). The data was analyzed using an ordinary one-way ANOVA followed by the Tukey's multiple comparisons test. Statistical significance was considered when $p < 0.05$.

4.3 Results

4.3.1 Concentration and size determination of circulating sEVs.

To define disease specific sEV profiles samples from sepsis and sepsis with hypertension patients were collected within the first 12 hours post admission, and the healthy control and hypertensive groups were donated by volunteers who were age, gender and race matched. The sEVs were isolated using the total exosome isolation kit from plasma (Invitrogen by Life Technologies, CA, USA) as per the manufacturer's instructions, in order to facilitate a comparative analysis among the four groups. The size distribution of isolated sEVs was analysed using Nanoparticle Tracking Analysis. The isolated sEVs were within the accepted

size range of 30-200nm. sEVs isolated from sepsis patients (average size 147.7nm), hypertensive patients (average size 147.5nm) and sepsis patients with hypertension (average size 162.3nm) were significantly larger when compared to healthy controls (average size 84.8nm) (p= 0.027, 0.028, 0.009 respectively; shown in Figure 1a). A validated CD63 ExoELISA was used for the quantification and concentration of isolated sEVs (Figure 1b) as equivalent concentrations of sEVs are crucial for consistent comparisons of sEV surface markers using multiplex bead-based flow cytometric analysis [36]. The particle count/mL was significantly higher in patients with sepsis ($1.21 \times 10^{11} \pm 1.9 \times 10^{10}$), and sepsis with hypertension ($2.46 \times 10^{11} \pm 6.72 \times 10^{10}$) when compared to healthy controls ($2.36 \times 10^{10} \pm 0.29 \times 10^{10}$) (p= 0.005, <0.0001 respectively). sEV abundance was also significantly higher in sepsis with hypertension patients ($2.46 \times 10^{11} \pm 6.72 \times 10^{10}$) when compared to sepsis ($1.21 \times 10^{11} \pm 1.9 \times 10^{10}$) and hypertensive ($7.32 \times 10^{10} \pm 5.45 \times 10^{10}$) patients (p=0.0005, <0.0001 respectively). There was also a trend observed, showing a higher particle/mL in hypertensive patients when compared with healthy controls, however, this was not statistically significant (p=0.24).

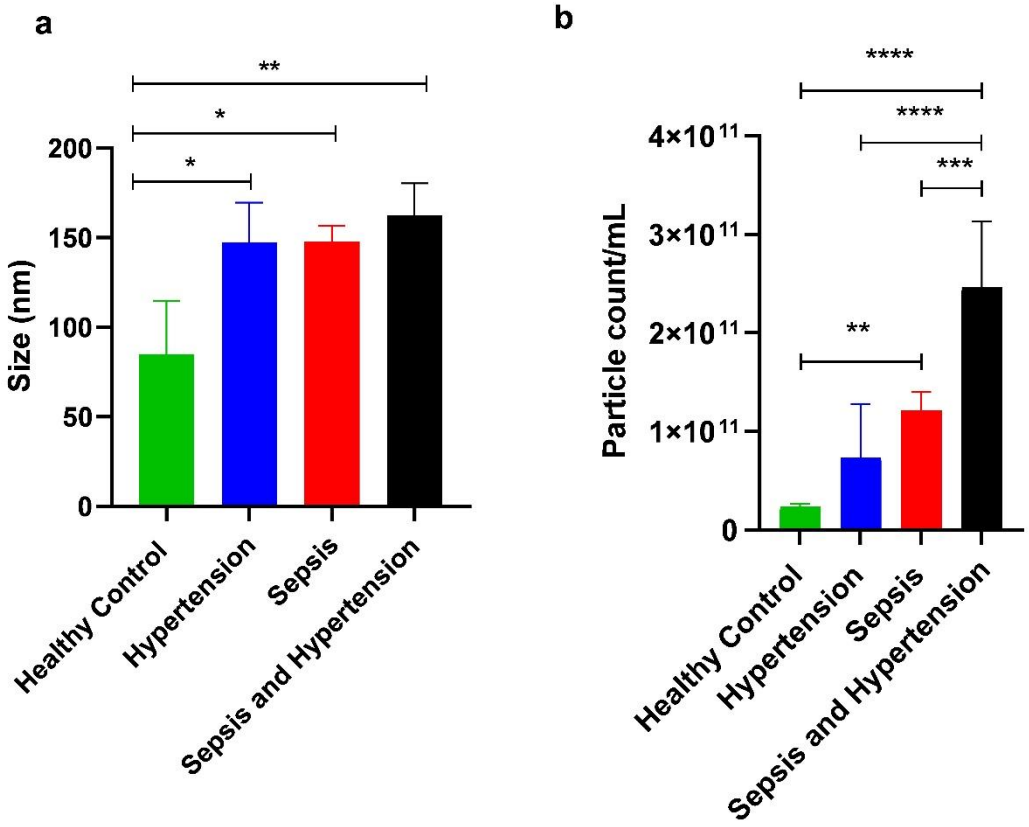

Figure 1. Average particle size and abundance of isolated sEVs. **A).** Results from NTA showing the average particle size of isolated sEVs among different groups, sEVs are significantly larger

in sepsis, hypertension and sepsis with hypertension groups when compared to healthy controls (p= 0.027, 0.028, 0.009 respectively). **B).** CD63 ExoELISA results showing particle count/mL among groups, sepsis and sepsis with hypertension groups showed a significant increase in sEV concentration when compared to healthy controls (p= 0.005, <0.0001 respectively). The sepsis with hypertension group showed a significant increase in sEV concentration when compared to the sepsis and hypertension groups (p=0.0005, <0.0001 respectively). Graphs were plotted as the *mean* ± *SEM*.

4.3.2 Surface protein profiling of isolated sEVs

To study the surface marker profiles, isolated sEVs were analyzed using a multiplex bead-based flow cytometric assay. The MACSPlex assay allows a simultaneous, semi-quantitative detection of 37 different sEV surface markers (and 2 internal isotope controls) in a single sample using dyed bead populations, each specific to an antibody that recognizes these surface markers. Beads are differentiated by their specific fluorescence characteristics (FITC VS PE) and the binding of sEVs is detected using APC-conjugated antibodies targeting the 3 major tetraspanins (CD63, CD81 and CD9). The gating strategy and identification of bead populations are shown in Supplementary data Figure 2.

In this study, we analyzed the populations of sEVs in plasma samples among four groups (healthy controls, hypertension, sepsis and sepsis with hypertension) with 12 samples per group. The Median Fluorescence Intensity (MFI) was determined, and each sample MFI was corrected with a non-EV blank control. The MFI are plotted as the mean ± SEM. Surface markers with an MFI allophycocyanin (APC) signal lower than that of the isotype controls were considered non-detected, and no symbols are presented in the figures (Figures 2a and 2c). For the purpose of this study, four comparisons were made; 1. healthy controls vs hypertension, 2. healthy controls vs sepsis, 3. hypertension vs sepsis and hypertension and, 4. sepsis vs sepsis and hypertension.

4.3.2.1 Hypertension and sepsis groups.

Firstly, surface marker profiling consisting of all 37 markers in each of the four groups was done in order to provide a baseline for further analysis (Figures 2a, 2b, 2c, 4a). The results from this study highlighted markers that are significantly increased or uniquely present within each experimental group. The analysis of control vs hypertension and control vs sepsis revealed that there are 7 surface markers (CD4, CD105, CD25, ROR1, CD209, CD146, CD142) in the hypertension group and 6 markers (CD4, CD105, CD56, HLA-ABC, CD209, CD45) in the

sepsis group that are significantly increased or uniquely present when compared to the healthy control group.

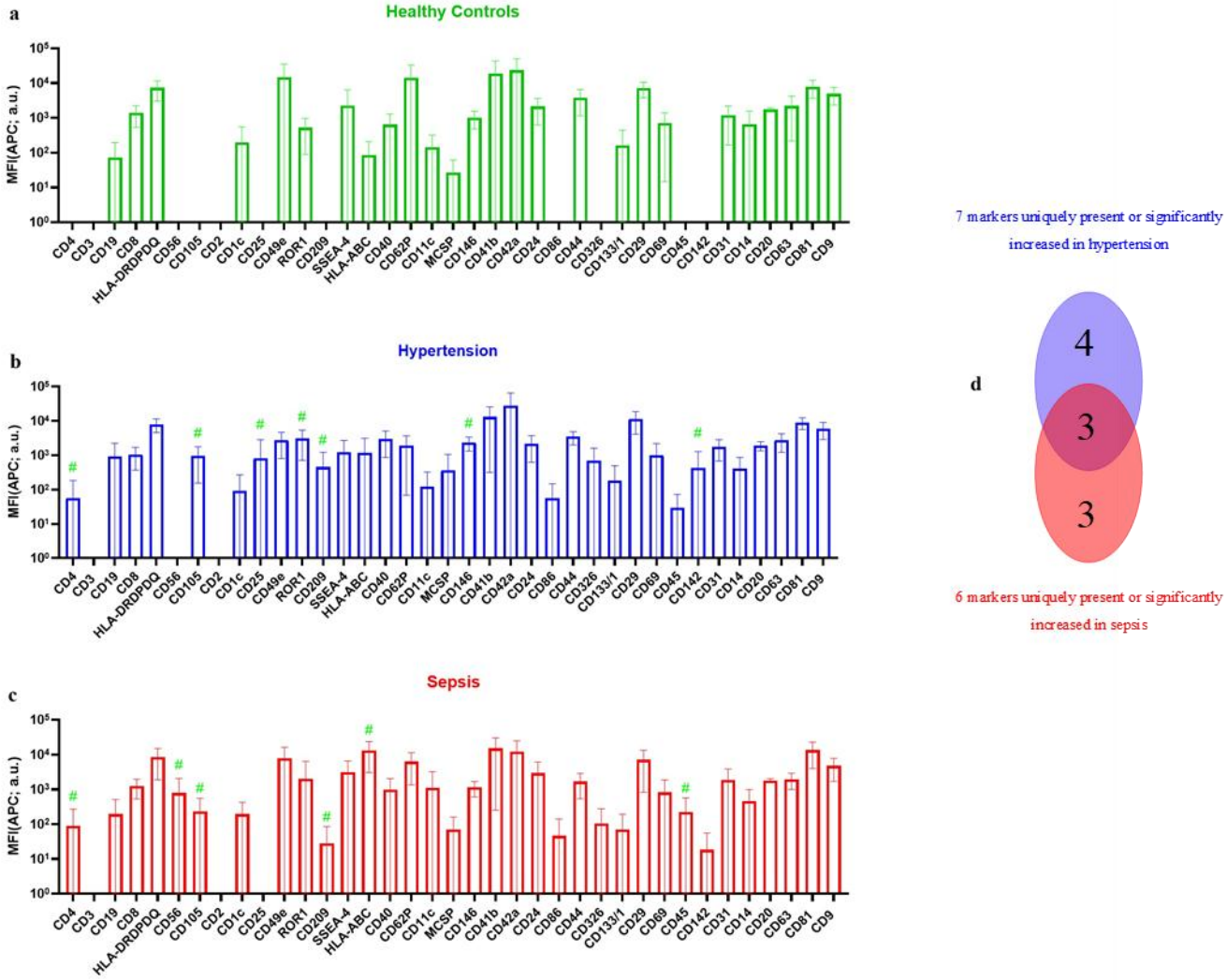

Figure 2. Surface marker profiles of healthy controls (a), hypertension (b) and sepsis (c) groups (# shows markers that are significantly increased or uniquely present in groups when compared to healthy controls) n=12 per group. 2d Venn Diagram depicting unique and common markers in the hypertension and sepsis groups when compared to healthy controls. a.u = arbitrary unit.

Three of the 7 and 6 markers detected in the hypertension and sepsis group respectively were in common between the groups (Figure 2d), however not detected in healthy controls (CD4, CD105, CD209). In the hypertension group, ROR1 ($p=0.005$) and CD146 ($p<0.05$) were significantly increased when compared to the healthy controls, whereas CD25 and CD142 were exclusively detected (Figure 3a). Exclusively detected markers in the sepsis group include

CD56 and CD45, with HLA-ABC (p=0.018) being the only marker significantly increased when compared to healthy controls (Figure 3c). Surface markers which were not significantly increased or uniquely present in the hypertension and sepsis groups, when compared to healthy controls, are provided in Supplementary data Tables 2 and 3, respectively.

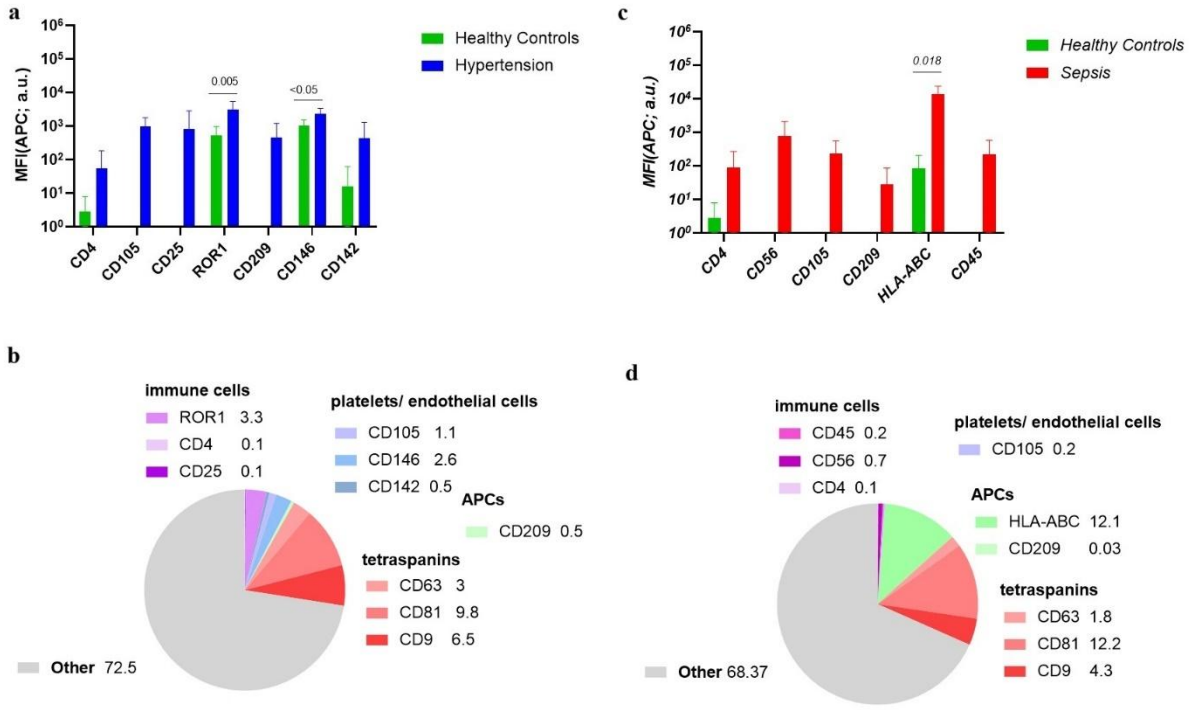

Figure 3. Surface markers which are differentially or uniquely expressed in the hypertension group (a) and sepsis group (c) when compared to healthy controls, n=12 per group. Bars indicate the MFI APC signal that is plotted as the mean ± SEM. Statistical significance was determined using Tukey's multiple comparisons test. Bars without significance depicted indicate that these surface markers were non-detected in healthy controls but present in the respective groups. Percentage expression of differentially and uniquely expressed surface markers in the hypertension group (b) and sepsis group (d), grouped according to known/potential cellular origins. "Other" in grey represents surface markers that were non-significantly different or below the isotope threshold in each group. a.u= arbitrary unit.

Thereafter, MFI values of each bead population was calculated into a percentage of the total signal to provide the percentage expression of the total signal for markers significantly

increased or uniquely present, as well as the tetraspanins (CD63, CD81 and CD9), in the hypertension and sepsis groups. The surface markers were differentiated into groups based on their known/potential cellular origins, namely immune cells, platelets and endothelial cells and antigen-presenting cells (APCs) in the hypertension and sepsis groups. In the hypertension group, 3.5% (CD4- 0.1%, CD25- 0.1% and ROR1- 3.3%) of the total MFI signal is attributed to sEVs which may have originated from immune cells (T cells and B cells). The MFI signal from CD105 (1.1%), CD146 (2.6%) and CD142 (0.5%) are indicative of cellular origins from platelets and endothelial cells, while CD209 (0.5%) hints towards the presence of antigen-presenting cell-derived sEVs (Figure 3b). In the sepsis group, sEVs derived from immune cells account for 1% of the total MFI signal (CD4- 0.1%, CD56- 0.7% and CD45- 0.2%), whereas 0.2% of the signal from CD105 is indicative of endothelial cell-derived sEVs in sepsis. The HLA-ABC and CD209 correspond to 12.7% and 0.03% respectively point towards sEVs originating from APCs (Figure 3d).

4.3.2.2 Sepsis with hypertension group.

The surface marker profiling of the sepsis with hypertension group was done to provide a baseline for further comparisons (Figure 4a). The 2 comparisons made were hypertension vs sepsis with hypertension and sepsis (with no previously diagnosed co-morbidities) vs sepsis with hypertension (previously diagnosed). There are 37 detectable markers used in this assay, of these, 20 (CD4- $p<0.0001$, CD8- $p=0.001$, CD56- $p=0.01$, CD2- $p<0.01$, CD1c- $p=0.002$, CD11c- $p=0.002$, CD45- $p=0.001$, CD105- $p=0.04$, CD49e- $p=0.008$, CD62p- $p=0.0005$, CD41b- $p=0.0007$, CD42a- $p=0.006$, CD31- $p<0.05$, HLA-DRDPDQ- $p<0.05$, CD86- $p=0.009$, CD14- $p<0.0001$, HLA-ABC- $p=0.0007$, SSEA-4- $p=0.02$, CD44- $p=0.004$, CD29- $p=0.02$) surface markers were significantly increased in sepsis with hypertension when compared to the hypertension group (Figure 5a). The comparison between the sepsis with hypertension group and the sepsis group revealed significant increases in 21 (CD4- $p<0.0001$, CD8- $p=0.006$, CD56- $p=0.02$, CD2- $p=0.02$, CD1c- $p<0.01$, CD25- $p<0.05$, CD45- $p=0.004$, CD105- $p=0.008$, CD49e- $p=0.03$, CD62p- $p=0.001$, CD41b- $p=0.002$, CD42a- $p=0.004$, CD31- $p<0.05$, CD146- $p<0.05$, HLA-DRDPDQ- $p<0.05$, CD86- $p=0.007$, CD14- $p<0.0001$, CD40- $p=0.04$, MCSP- $p=0.04$, CD44- $p=0.0004$, CD29- $p=0.004$) surface markers (Figure 6a). Seventeen of these markers were common in the sepsis with hypertension group when compared to the hypertension and sepsis groups (Figure 4b). Surface markers which were not differentially

expressed in the sepsis with hypertension group when compared to the hypertension and sepsis groups are provided in Supplementary data Table 4 and 5 respectively.

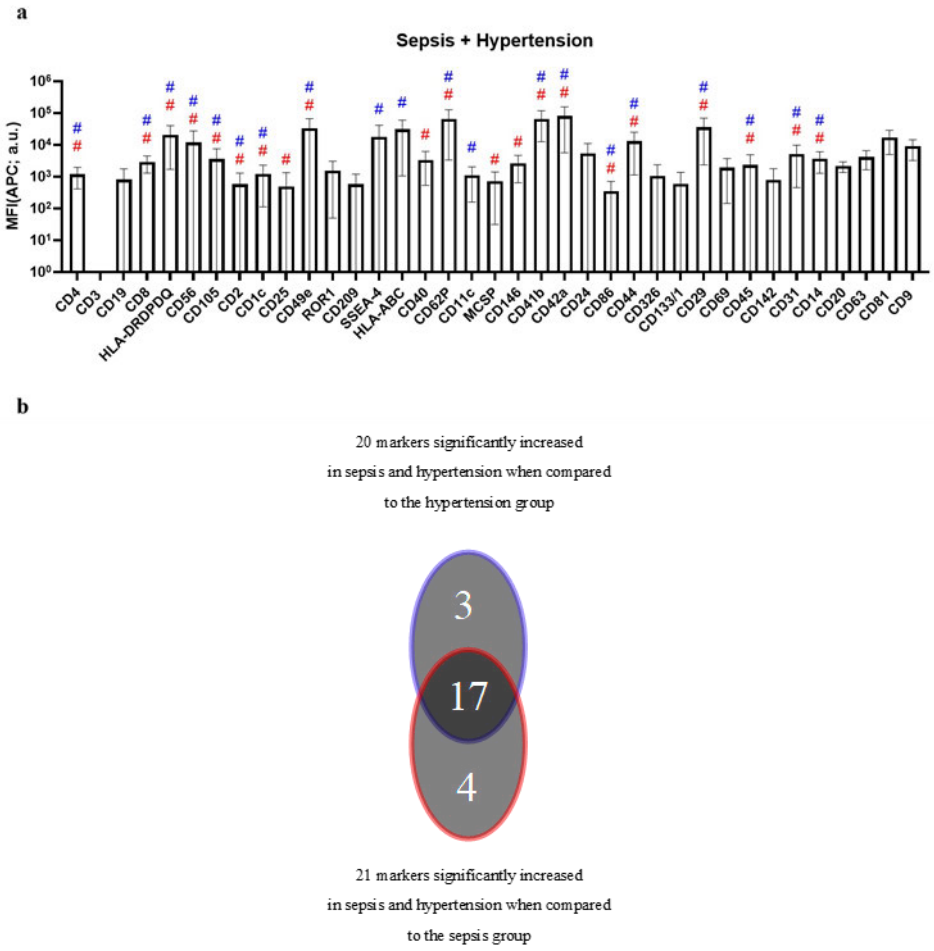

Figure 4. Surface marker profile of sepsis with hypertension group (a) n=12. Markers that are significantly increased when compared to the hypertension group (depicted by #) and the sepsis group (depicted by #). 2b Venn Diagram depicting unique and common markers in the sepsis with hypertension group when compared to the hypertension and sepsis groups. a.u = arbitrary unit.

There are 3 surface markers (CD11c, HLA-ABC and SSEA-4-) specific to sepsis with hypertension when compared to the hypertension group. Furthermore, the percentage expression of the total MFI signal revealed that the cellular origins of the sEVs detected in the sepsis with hypertension group when compared to the hypertension group include immune cells, platelets and endothelial cells, APCs, epithelial cells and stem cells (Figure 5b). The immune

cell-derived sEVs is attributed to MFI signals of CD45 (0.5%), CD11c (0.2%), CD1c (0.3%), CD2 (0.1%), CD56 (2.6%), CD4 (0.3%) and CD8 (0.6%), indicating that these sEVs may originate from T cells, B cells and other myeloid cells. Approximately 56.6% of the total MFI signal accounted for platelet and endothelial-associated markers, including CD49e (7.4%), CD105 (0.8%), CD62p (14.6%), CD41b (14.6%), CD31 (1.1%) and CD42a (18.1%), suggesting that platelet derived sEVs are highly abundant in sepsis with hypertension when compared to the hypertension group. Moreover, sEVs corresponding to antigen-presenting cells (CD86- 0.1%, HLA-DRDPDQ- 4.7%, CD14- 0.8% and HLA-ABC- 6.9%), epithelial cells (CD29- 3% and CD44- 8%) and stem cells (SSEA-4- 4%) were detected. Overall, the MFI signal of the significantly increased surface markers in sepsis with hypertension when compared to the hypertension group accounts for 87.7% (excluding tetraspanins) of the total MFI signal.

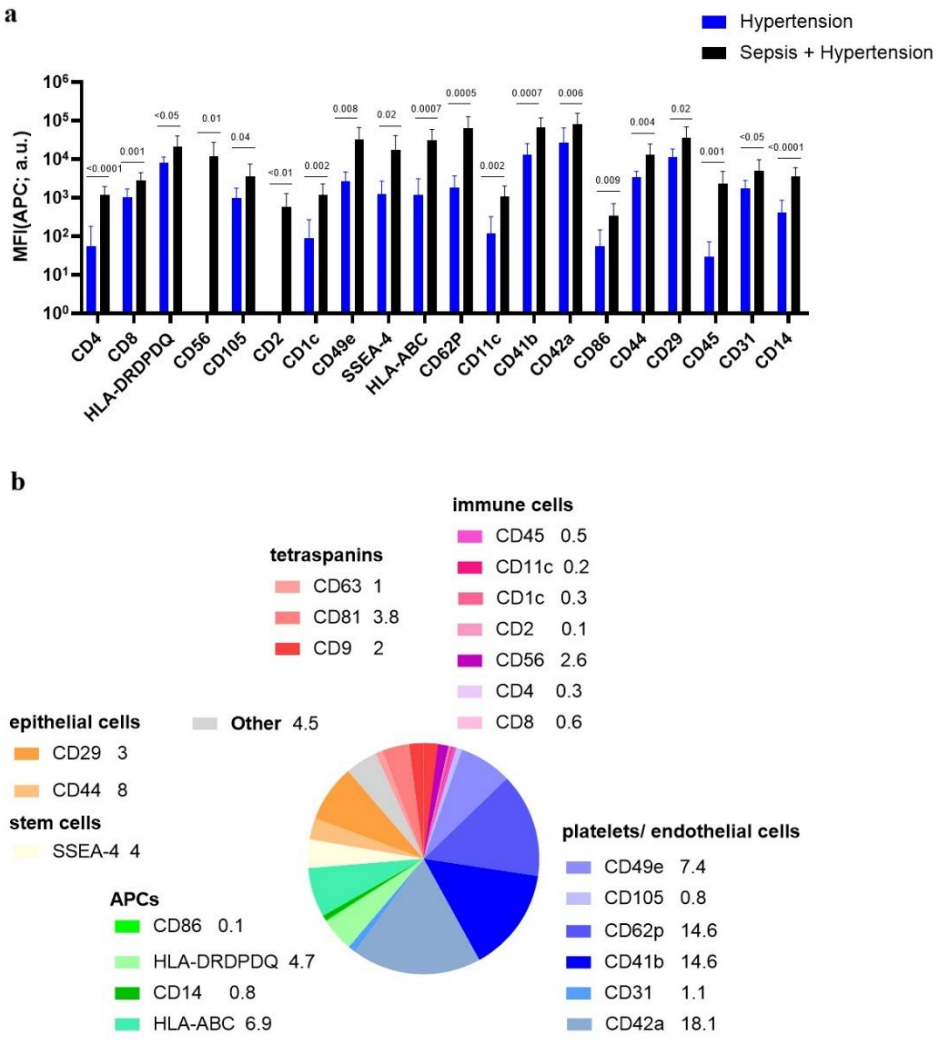

Figure 5. Surface markers which are differentially expressed in sepsis with hypertension when compared to the hypertension group (a) n=12 per group. Bars indicate the MFI APC signal that is plotted as the mean ± SEM. Statistical significance was determined using Tukey's multiple comparisons test. Percentage expression of differentially expressed surface markers in the sepsis with hypertension group (b), grouped according to known/potential cellular origins. "Other" in grey represents surface markers that were non-significantly different or below the isotope threshold in each group. a.u= arbitrary unit.

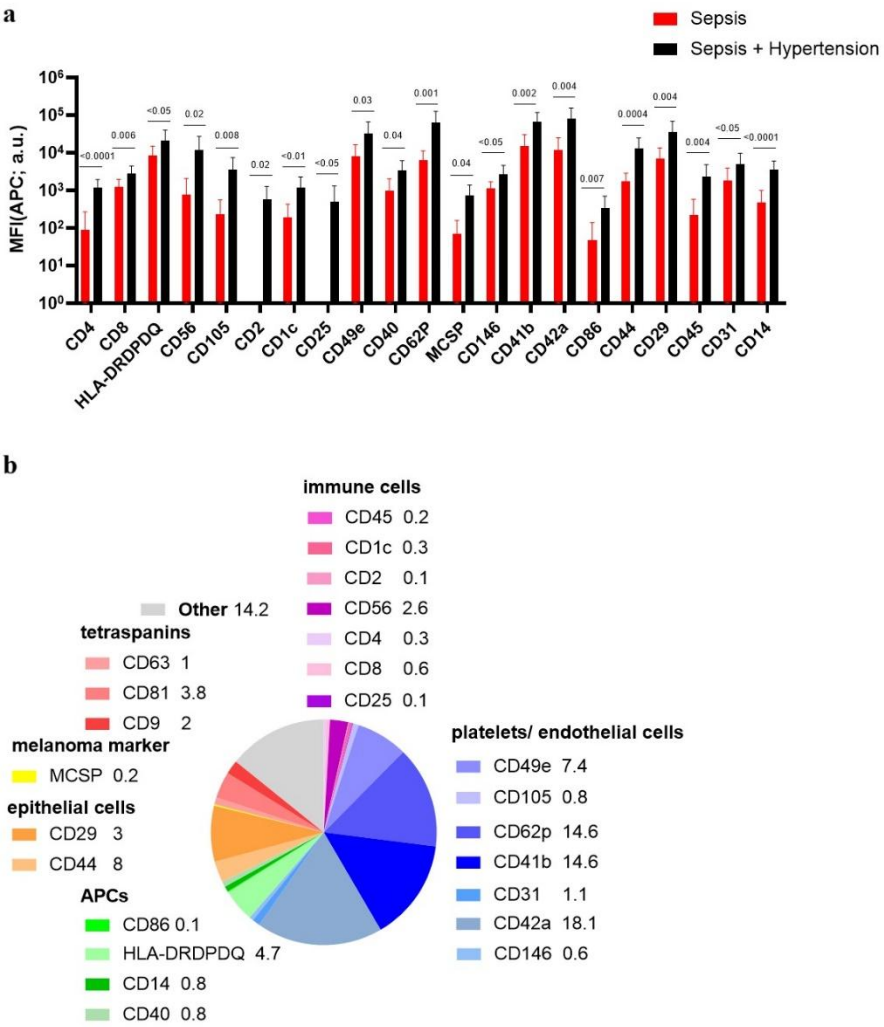

Figure 6. Surface markers which are differentially expressed in sepsis with hypertension when compared to the sepsis group (a) n=12 per group. Bars indicate the MFI APC signal that is plotted as the mean ± SEM. Statistical significance was determined using Tukey's multiple comparisons test. Percentage expression of differentially expressed surface markers in the sepsis with hypertension group (b), grouped according to known/potential cellular origins.

"Other" in grey represents surface markers that were non-significantly different or below the isotope threshold in each group. a.u= arbitrary unit.

The significant increase in the expression of 4 surface markers (CD25, CD146, CD40 and MCSP) showed to be unique to the sepsis with hypertension group when compared to the sepsis group. Moreover, 79% of the total MFI signal is attributed to the expression of the significantly increased surface markers in the sepsis with hypertension group when compared to the sepsis group (Figure 6b). Platelet and endothelial derived sEVs accounts for 57.2% of the total MFI signal (i.e CD49e- 7.4%, CD105- 0.8%, CD62p- 14.6%, CD41b- 14.6%, CD31- 1.1%, CD42a- 18.1% and CD146- 0.6%). The MFI signal corresponding to CD45 (0.5%), CD1c (0.3%), CD2 (0.1%), CD56 (2.6%), CD4 (0.3%), CD8 (0.6%) and CD25 (0.1%) is indicative of the presence of sEVs derived from immune cells such as T cells, suggesting that there is an exacerbated effect of sepsis with previously diagnosed hypertension on the immune response/system when compared to the sepsis group. APC-derived sEVs accounts for 6.5% of the MFI signal (i.e CD86- 0.1%, HLA-DRDPDQ- 4.7%, CD14- 0.8% and CD40- 0.8%, whereas sEVs orginating from epithelial cells correspond to the expression of CD29 (3%) and CD44 (8%).

4.4 Discussion

In this study, the MACSPlex exosome human kit (Miltenyi Biotec) was used to identify specific surface marker profiles in the pathological states of sepsis, hypertension and sepsis with pre-existing hypertension. Our study design included 4 comparisons, i.e. healthy controls *vs* hypertension, healthy controls *vs* sepsis, hypertension *vs* sepsis with hypertension and sepsis *vs* sepsis with hypertension, and found that there were 7, 6, 20 and 21 surface markers that were differentially expressed respectively. To the best of our knowledge, these surface marker profiles have not been studied in these pathological states, and this is the first such study. In recent years, the role of sEVs in intercellular communication and the potential impact on the target cell has lent support to the notion that disease-specific sEVs may serve as effective biomarkers [32, 38]. The molecular composition of sEVs is considered to be highly heterogeneous thus providing distinct 'fingerprints' or signatures of the cargo, and importantly the surface marker profiles sharing characteristics with the tissues of origin [39].

There are various clinical challenges faced when diagnosing sepsis with existing hypertension, as these pathological states share several overlapping symptoms due to common pathological

pathways making it difficult to distinguish between the two and accurately diagnose sepsis. The study provides valuable insights into the molecular distinctions between sepsis and sepsis with hypertension through surface marker profiling of sEVs.

When comparing hypertension to healthy controls, there were 4 surface markers (CD146, CD25, CD142, and ROR1) specific to hypertension. These markers (CD25 and ROR1) are predominantly found on immune cells, as these surface markers play pivotal roles in the immune system through cell survival and differentiation [40-42] and cell proliferation [43]. CD25 is a marker for T cell activation, which forms the alpha chain for the interleukin-2 (IL-2) receptor, playing a crucial role in immune homeostasis [40, 42]. While the expression of ROR1 in normal tissues is usually low or absent, it is suggested that Receptor Tyrosine Kinase-like Orphan Receptor 1 (ROR1) is a marker of various forms of cancers and may help detect renal malignancies early [44, 45]. The results from this study may indicate that surface marker ROR1 may be expressed by B cells in hypertensive patients, as a previous study suggests that anti-ROR1 may be used as treatment for various B-cell malignancies [46]. In the pathophysiology of pulmonary hypertension, the infiltration of T cells and B cells to the pulmonary vessels, in turn, promotes vasoconstriction and lung vascular remodelling [47-49]. This indicates the activation of inflammatory pathways and immune dysregulation in hypertension [47]. On the other hand, CD146 is highly expressed on endothelial cells, playing a crucial role in angiogenesis [50], as well as promoting inflammation and fibrosis [51]. Endothelial dysfunction arising from dysfunctional angiogenesis and changes in endothelial cells is a hallmark characteristic of hypertension pathophysiology [47, 52]. Endothelial dysfunction, together with the formation of fibrin by extrinsic and intrinsic coagualtion pathways, contribute to the prothrombic state and clot formation in hypertensive patients [53, 54]. CD142 also known as Tissue Factor has a crucial role in the activation of the blood clotting system [55]. The unique presence of CD142 and significant increase in CD146 in the hypertension group is indicative of the involvement of angiogenic and endothelial pathways, and ultimately end organ damage in hypertension. These results show that sEV profiles in the hypertension group may provide molecular evidence for the pathways specific to the pathophysiology of hypertension.

When comparing the sepsis group to healthy controls, a total of 3 surface markers (CD56, CD45 and HLA-ABC) are exclusive to the sepsis group, with a significant increase in HLA-ABC and the unique presence of CD56 and CD45. In comparison to localized infections, sepsis is a complex and multifaceted dysregulation of the refined immunological response between

inflammation and anti-inflammation. The sepsis definition refers to a “dysregulated host response to infection” [6], highlighting that the innate and adaptive immune systems play a vital role in the development of sepsis [10]. CD45 plays a central role in the immune response and function in sepsis by T cell activation and signaling [56], as well as cell proliferation [57]. When exposed to an inflammatory trigger, CD45 is differentially expressed, indicating pathophysiologic and diagnostic implications in sepsis [58, 59]. Natural killer (NK) cells play a crucial role in the hyper-inflammatory state of sepsis, subsequently promoting tissue damage and organ failure. CD56, expressed on NK cells, contributes to inflammation in sepsis by secreting interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [60]. While the presence of HLA-ABC, found on all nucleated cells, is indicative of the involvement of various somatic cells in the pathophysiology of sepsis, it has been linked to the immune pathway, playing crucial roles in immune response and the genetic predisposition to the immune response to infection [61, 62]. The presence of these surface markers is indicative of the role of the immune system and response in the pathophysiology of sepsis, and may provide molecular evidence that there is an increased stimulation of the immune system during sepsis. These results provide evidence that the combination of these surface markers may be exploited for further investigations into biomarker development for sepsis.

When analyzing the hypertension and sepsis groups, there were 3 surface markers (CD4, CD105 and CD209) present in both groups, however, absent in healthy controls. The presence of common makers indicates that there are common pathways involved in both pathologies, particularly pertaining to the inflammatory response, endothelial function and organ damage/dysfunction. CD4, expressed on T cells, plays a role in orchestrating an immune response by the production of cytokines (IL-17) which is crucial for the proliferation, differentiation and function of other immune cells, such as B cells [63]. The increase in the production of IL-17 and IL-17-producing T cells has been reported in sepsis and hypertension respectively [64, 65]. These increases lead to a reduced ability to mount an effective immune response when exposed to a secondary infection and the invasion of end-organ tissues [64, 65]. Furthermore, the increase in the production of cytokines implies that CD4 plays a role in inducing inflammation in both hypertension and sepsis. The initiation of immune responses is also a key role of CD209, however, CD209 also plays an important role in pathogen recognition [66]. The results from this study indicate that CD209 potentially contributes to the development and progression of hypertension and sepsis by promoting inflammation and effects on immune cell function. Endothelial dysfunction is characteristic of hypertension, however it has been

suggested that stimulation of endothelial cells exacerbates inflammation through the release of pro-inflammatory molecules in the pathology of sepsis [67]. CD105, a component of the TGF-β receptor complex, is expressed on endothelial cells and is crucial for angiogenesis (formation of new blood vessels) [68]. The presence of CD105 in the hypertension and sepsis groups in the current study, while being non-detected in healthy controls, points towards roles in the inflammatory response and endothelial dysfunction in both diseases. Hence, sepsis and hypertensive sEVs may contribute to both inflammatory and angiogenic sides of hypertension and sepsis pathophysiology, potentially contributing to disease severity.

Following this, two significant comparisons were made in order to identify and analyze the surface marker profiles of sepsis in the presence of a co-morbidity. We first compared the surface marker profiles of sepsis with pre-existing hypertension to the hypertension group. Interestingly, there were 20 (CD4, CD8, CD56, CD2, CD1c, CD11c, CD45, CD105, CD49e, CD62p, CD41b, CD42a, CD31, HLA-DRDPDQ, CD86, CD14, HLA-ABC, SSEA-4, CD44, CD29) out of the 37 surface markers that were significantly increased in sepsis with pre-existing hypertension. We then compared the surface marker profiles of sepsis with pre-existing hypertension to the sepsis (with no pre-existing co-morbidities) group. There were 21 (CD4, CD8, CD56, CD2, CD1c, CD25, CD45, CD105, CD49e, CD62p, CD41b, CD42a, CD31, CD146, HLA-DRDPDQ, CD86, CD14, CD40, MCSP, CD44, CD29) out of the 37 surface markers that were significantly increased in the sepsis with pre-existing hypertension group. We then analyzed the two comparisons and found that there were 17 surface markers in common, significantly increased in the sepsis with pre-existing hypertension when compared to the sepsis and hypertension groups. The surface markers that correspond to immune cells include CD4 [69], CD8 [70], CD56 [71], CD2 [72], CD1c [73] and CD45 [74], whereas there are surface markers indicative of platelet and endothelial functions, including CD42a [75], CD31 [76], CD41b [77], CD62p [78], CD105 [79], and CD49e [80]. These surface marker results also point towards APC-derived sEVs (CD86 [81], HLA-DRDPDQ [82] and CD14 [83]) and sEVs originating from epithelial cells (CD29 [84] and CD44 [85]). This is indicative that sepsis with pre-existing hypertension exacerbates or amplifies the immunological and endothelial pathways as well as the cells associated with these pathways, when compared to hypertension and sepsis, potentially to the heightened vulnerability and complexity observed in these patients. The observed results also signify that sepsis with pre-existing hypertension stimulates other pathways and cells in the body.

There were 3 surface markers (CD11c, HLA-ABC, and SSEA-4) unique to the sepsis with pre-existing hypertension group when compared to the hypertension group, and there were 4 surface markers (CD25, CD146, CD40, and MCSP) unique to the sepsis with pre-existing hypertension group when compared to sepsis. These findings imply that hypertension not only shares common pathogenic pathways with sepsis but also introduces specific molecular signatures, which may serve as possible contenders for biomarker investigations for diagnostic stratification. The heterogeneity of surface marker profiles underscores the necessity for personalized approaches to treatment and highlights the importance of considering pre-existing conditions like hypertension when evaluating and managing sepsis.

The interpretation of the results showed that CD56 was elevated in the sepsis group when compared to healthy controls, and further increased in sepsis with hypertension when compared to the sepsis group. This suggests that CD56 may be of particular interest for exploitation as a possible candidate for a biomarker of sepsis and sepsis with previously diagnosed hypertension. Additionally, the detection of unique markers (CD2) solely in sepsis with hypertension suggests additional molecular alterations associated with the hypertensive state that may influence disease severity and progression.

The sEV markers are indicative of EV biogenesis, protein interactions and cell targeting. sEVs released by cells during pathological states have distinct compositions within their cargo and on their surface which may serve as markers of these states. Compared to the cargo of sEVs, these markers can provide copious, stable and precise information on EV processes and pathological states, therefore providing diagnostic and prognostic factors. Traditionally, a haematology analyzer used for complete blood counts is used for the diagnosis of sepsis. In a blood test, changes in cellular components of the hematopoietic system or soluble factors are measured. The current analysis and the study of EVs allow for the examination of signals from a wider range of sources. The analysis of surface markers on sEVs provides evidence of wide-range cell activation sepsis. Biomarker investigations progress when the difference in a parameter is statistically significant, biologically meaningful, and clinically useful, with effect sizes and Area Under the Curve metrics of Receiver Operating Characteristic Curves strong enough to improve the prediction of diagnosis. The results of this study further adds to the value of sEVs as diagnostic markers along with the fact that these vesicles can be easily and non-invasively obtained by liquid biopsies.

4.5 Conclusion

Overall, this study demonstrates a specific surface protein profile for the pathological states of sepsis (with no previously diagnosed co-morbidities), hypertension and sepsis with hypertension. The use of sEVs as biomarkers has recently gained great interest as disease biomarkers due to their roles in cellular communication and are present in bodily fluid which makes them easily obtainable by liquid biopsies. Our study aimed to provide further information on the heterogeneity of the surface markers present on these sEVs. Surface markers may be of greater interest as biomarkers as they provide information on the cell source while the cargo is reflective of deeper pathogenic mechanisms. Future investigations should focus on elucidating the functional roles of these distinct markers, their contribution to disease pathophysiology, and their potential utility in clinical settings. Additionally, expanding the sample size and integrating functional assays could provide a more comprehensive understanding of the biological significance of these molecular differences, ultimately enhancing patient stratification and tailored interventions. The surface marker profiles described in this study aid in classification and to an extent further understanding of the pathogenesis of sepsis by identifying the cells (and by inference the systems) involved in various aspects and mechanisms of the pathogenesis of sepsis. These profiles can facilitate the identification and classification of disease-specific sEV subsets for the development of biomarkers for sepsis.

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4.7 Disclosure of Interest

The authors report no conflict of interest.

4.8 Credit author statement

Roushka Bhagwan Valjee: Sample collection, Investigation, Methodology, Conceptualization, Formal Analysis, Writing of the original draft and Editing. **Usri H. Ibrahim:** Investigation, Formal Analysis, Writing of the original draft preparation, Reviewing, and Editing. **Manu Vatish:** Conceptualization, Provision of resources. **Wei Zhang:** Investigation, Methodology, Formal Analysis, Reviewing and Editing. **Irene Mackraj:** Conceptualization, Writing revision and editing, Provision of resources and Supervision.

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4.10 Supplementary Data

Supplementary Data

Characterizing the surface marker profiles of circulating small extracellular vesicles in sepsis, hypertension and sepsis with hypertension.

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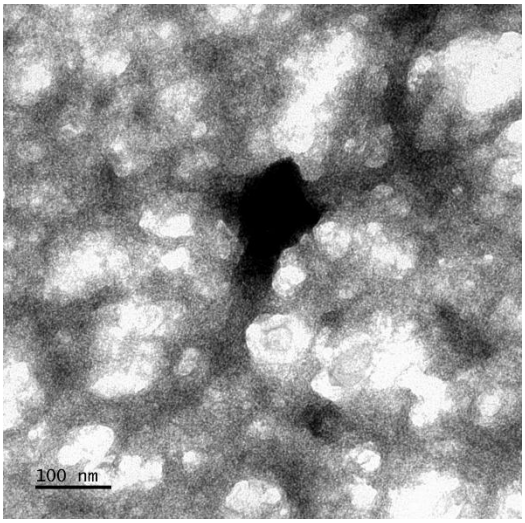

Figure 1. TEM analysis shows an example of the morphology of isolated sEVs. Spherical vesicles with an approximate size range of 30-150nm were observed.

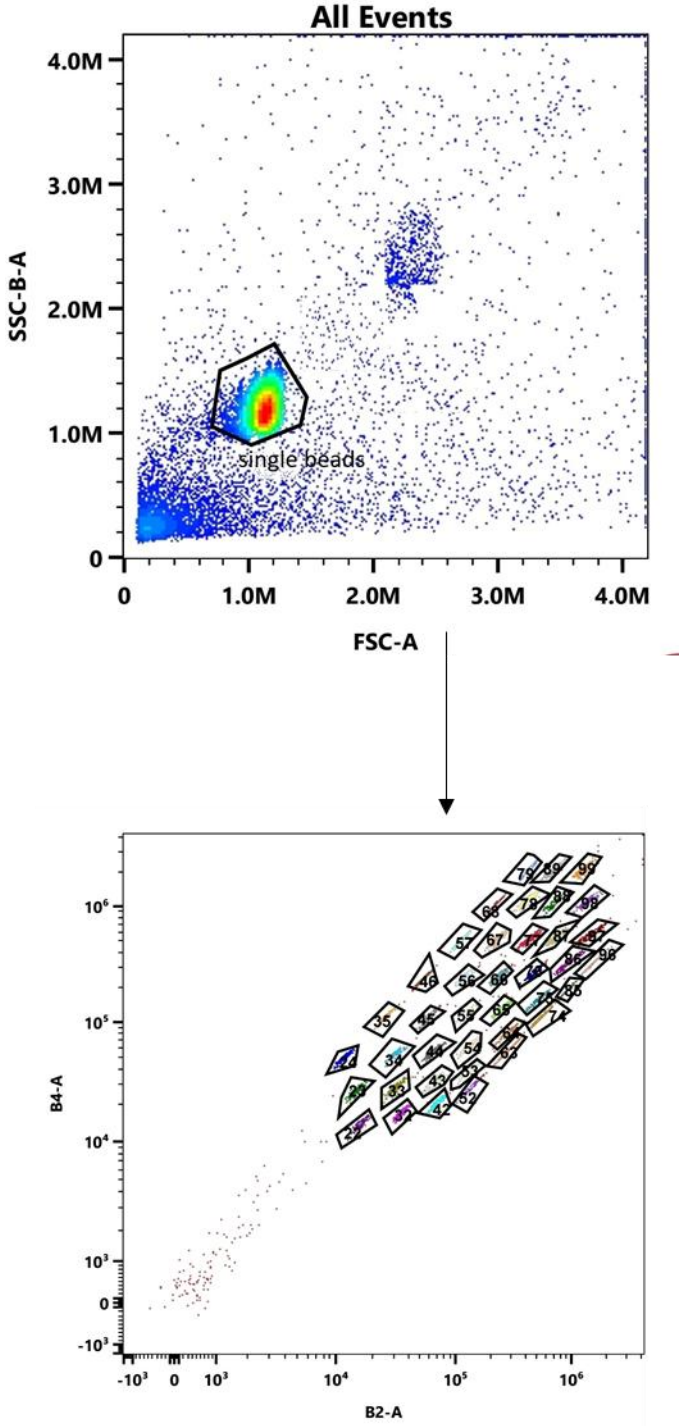

Figure 2. The gating strategy applied for gating of single beads, followed by gates to identify the 39 distinct capture bead populations are shown. All bead populations were identified according to the manufacturer's recommendations.

Table 1 Clinical characteristics of participants

Variables	Sepsis Patients	Sepsis patients with hypertension
Age range	*20-71 years*	*31-84 years*
Race	*Indian (50%)* *African (50%)*	*Indian (30%)* *African (70%*
SOFA score range	*2-12*	*4-15*
Blood pressure (mmHg)	*Systolic 118±15.8* *Diastolic 75±17.3*	*134±20.3* *87±10.8*
Heart rate (beats/min)	*133±25.58*	*102±23.32*
Haemoglobin (g/mol)	*10.02±1.72*	*8.27±1.71*
Respiratory rate (breaths/min)	*22.83±8.12*	*19.67±6.12*
O_2 saturation (%)	*98.75±1.55*	*96.92±6.76*
C-Reactive Protein (µg/mL)	*193.58±114.61*	*195.33±105.95*
White cell count (x10⁹per L blood)	*14.22±10.81*	*12.92±8.18*
Platelet count (x10^3 /µL)	*129.83±75.82*	*169.42±108.89*
Bilirubin (mg/dL)	*39.35±92.43*	*14.25±13.77*
Creatinine (mg/dL)	*106.25±68.61*	*319.1±254.95*
Mean arterial pressure (mmHg)	*89.42±20.55*	*83.58±18.30*

Notes: All values are represented as the mean±SD

Table 2 The median fluorescent intensity (MFI) of markers expressed in the control group vs hypertensive group. These markers are not considered significant as $p>0.05$. Data is expressed as mean ± SEM

Markers	MFI (APC; a.u.)		P-value
	Control (n=12)	Hypertension (n=12)	
CD3	0	0	-
CD19	71,6±40,2	909,0±399,0	0.15
CD8	1393,7±259,8	1026,3±208,0	0.85
HLA-DRDPDQ	7341,7±1425,3	7958,4±1073,5	0.99
CD56	0	0	-
CD2	0	0	-
CD1c	197,6±112,7	90,3±59,8	0.98
CD49e	14720,2±6202,7	2752,0±618,3	0.54
SSEA-4	2300,9±1367,1	1240,7±459,4	0.99
HLA-ABC	86,2±49,6	1168,8±618,1	0.99
CD40	642,6±246,8	2974,4±745,0	0.09
CD62P	14345,3±5461,3	1874,2±571,0	0.83
CD11c	143,8±53,7	121,3±61,2	0.99
MCSP	26,7±11,6	360,3±218,9	0.5
CD41b	18833,8±7818,7	6357,7±1249,8	0.83
CD42a	23534,2±8520,5	17468,1±5246,3	0.99
CD24	2129,2±451,8	2175,4±468,0	>0.99
CD86	7,9±7,9	55,6±29,0	0.95
CD44	3828,2±806,6	3448,5±435,3	0.99
CD326	50,1±50,1	681,6±304,2	0.41
CD133/1	162,5±88,8	180,2±94,2	0.99
CD29	7156,1±1143,4	11350,5±2294,0	0.96
CD69	712,1±220,6	980,0±358,4	0.96
CD45	0	28,9±13,7	>0.99
CD31	1189,6±341,4	1754,8±358,6	0.97
CD20	1748,7±61,3	1910,2±173,7	0.89
CD14	660,6±269,9	403,5±147,2	0.97
CD63	2223,8±603,9	2716,7±454,1	0.92
CD81	7940,0±1424,2	8989,5±1065,3	0.99
CD9	4931,5±809,9	5923,4±921,3	0.93

Table 3 The median fluorescent intensity (MFI) of markers expressed in the control group vs sepsis patients. These markers are not considered significant as $p>0.05$. Data is expressed as mean ± SEM

Markers	MFI (APC; a.u.)		P-value
	Control (n=12)	Sepsis (n=12)	
CD3	0	0	-
CD19	71,6±40,2	196,6±100,5	0.99
CD8	1393,7±259,8	1253,8±227,3	0.99
HLA-DRDPDQ	7341,7±1425,3	8451,8±1972,2	0.99
CD2	0	41,4±41,4	0.99
CD1c	197,6±112,7	194,0±78,5	>0.99
CD25	0	0	-
CD49e	14720,2±6202,7	7955,6±2533,5	0.86
SSEA-4	2300,9±1367,1	3170,0±1147,0	0.99
CD40	642,6±246,8	980,3±323,6	0.98
CD62P	14345,3±5461,3	6347,7±1579,2	0.95
CD11c	143,8±53,7	491,8±200,4	0.50
MCSP	26,7±11,6	70,6±29,9	0.99
CD146	1012,9±168,4	1150,3±179,6	0.99
CD41b	18833,8±7818,7	15266,5±4334,2	0.99
CD42a	23534,2±8520,5	12157,1±4136,6	0.94
CD24	2129,2±451,8	2980,1±913,8	0.93
CD86	7,9±7,9	46,8±29,6	0.97
CD44	3828,2±806,6	1709,3±336,9	0.85
CD326	50,1±50,1	104,8±58,9	0.99
CD133/1	162,5±88,8	69,9±39,0	0.97
CD29	7156,1±1143,4	7099,7±1889,9	>0.99
CD69	712,1±220,6	829,5±319,9	0.99
CD142	15,6±15,6	18,4±12,4	>0.99
CD31	1189,6±341,4	1866,1±611,1	0.94
CD20	1748,7±61,3	1808,3±79,5	0.99
CD14	660,6±269,9	468,3±166,7	0.99
CD63	2223,8±603,9	1969,5±292,2	0.99
CD81	7940,0±1424,2	13480,7±2853,8	0.43
CD9	4931,5±809,9	4792,5±929,2	0.99

Table 4 The median fluorescent intensity (MFI) of markers expressed in the hypertensive group vs patients with sepsis and hypertension. These markers are not considered significant as $p>0.05$. Data is expressed as mean ± SEM

Markers	MFI (APC; a.u.)		P-value
	Hypertension (n=12)	Sepsis and Hypertension (n=12)	
CD3	0	0	-
CD19	909,0±399,0	811,7±282,7	0.99
CD25	56,1±56,1	492,1±265,9	0.16
ROR1	3053,0±828,1	1549,8±500,3	0.14
CD40	2974,4±745,0	3372,1±948,8	0.97
MCSP	360,3±218,9	720,4±207,8	0.38
CD146	2343,0±312,0	2649,6±634,4	0.94
CD24	2175,4±468,0	5410,9±1766,8	0.13
CD326	681,6±304,2	1043,1±398,9	0.78
CD133/1	180,2±94,2	586,7±247,6	0.18
CD69	980,0±358,4	1925,9±594,1	0.33
CD142	431,4±267,8	803,3±292,8	0.59
CD20	1910,2±173,7	2126,3±234,1	0.77
CD63	2716,7±454,1	4109,5±745,4	0.29
CD81	8989,5±1065,3	16840,1±3964,1	0.17
CD9	5923,4±921,3	8919,2±1802,7	0.28

Table 5 The median fluorescent intensity (MFI) of markers expressed in sepsis patients vs patients with sepsis and hypertension. These markers are not considered significant as $p>0.05$. Data is expressed as mean ± SEM

Markers	MFI (APC; a.u.)		P-value
	Sepsis (n=12)	Sepsis and Hypertension (n=12)	
CD3	0	0	-
CD19	196,6±100,5	811,7±282,7	0.37
ROR1	748,3±205,4	1549,8±500,3	0.58
SSEA-4	3170,0±1147,0	17739,8±7350,4	0.06
HLA-ABC	13382,0±3635,2	30620,8±8534,7	>0.99
CD11c	491,8±200,4	1089,3±294,4	0.11
CD24	2980,1±913,8	5410,9±1766,8	0.33
CD326	104,8±58,9	1043,1±398,9	0.09
CD133/1	69,9±39,0	586,7±247,6	0.06
CD69	829,5±319,9	35697,9±10056,8	0.21
CD142	18,4±12,4	803,3±292,8	0.06
CD20	1808,3±79,5	2126,3±234,1	0.54
CD63	1969,5±292,2	4109,5±745,4	0.04
CD81	13480,7±2853,8	16840,1±3964,1	0.79
CD9	4792,5±929,2	8919,2±1802,7	0.08

5 CHAPTER FIVE: SYNTHESIS

5.1 Synthesis

Sepsis is a clinical syndrome, caused by an uncontrolled immune response following a severe infection [29]. It subsequently leads to general inflammation in the body, resulting in organ damage and failure. The pathophysiology of sepsis is considered multifaceted, involving various physiological systems [122]. Implementation of timeous, aggressive initial treatments is crucial for combatting the progression of sepsis and lowering mortality risks [123]. There is currently no gold standard for the diagnosis of sepsis, therefore the identification of definitive, specific and early biomarkers is vital and also a challenging area of research [124]. sEVs and exosomes have been implicated in the pathophysiology of sepsis based on the altered levels of exosomes in sepsis patients [125]. These vesicles carry unique cargo based on the cells they emanate from and are composed of a distinct molecular barcode in the form of surface protein markers [81]. As mediators of intercellular communication, sEVs have been shown to play a role in inflammation by the induction of pro-inflammatory cytokines [7]. This has aided in forming the rationale for evaluating sEVs as possible biomarkers for sepsis.

In publication one **(Chapter Two)**, the theoretical framework surrounding the role of circulating sEVs as diagnostic biomarkers for sepsis, highlights the advances made in the field of exosomes in sepsis diagnosis and management. Sepsis causes an increase in circulating sEVs, and this can exacerbate the hyper-inflammatory state of sepsis by increasing the release of pro-inflammatory cytokines and can also down-regulate the immune response possibly accelerating the development of organ dysfunction. From the perspective of the previously mentioned roles, the potential biomarker application of circulating sEVs were critically reviewed and evaluated, as well as the possibility of exosomes as therapeutic and drug delivery carriers. This highlighted that sEVs, their contents and surface composition do have the potential to diagnose sepsis and aid in disease progression and mortality predictions. This, therefore, formed a basis for our subsequent experiments.

sEVs are small spherical vesicles with a lipid bi-layer [5]. The lipid bi-layer consists of various surface markers, including but not limited to tetraspanins [90]. The tetraspanins that are commonly used to identify sEVs are CD63, CD81 and CD9 [126]. Therefore, publication two **(Chapter Three)** focused on isolating and quantifying circulating sEVs in sepsis patients as well as analyzing the tetraspanin colocalization of these sEVs. We and others have reported altered levels of circulating sEVs in sepsis. However, these differential levels of sEVs do not

qualify as biomarkers of sepsis as there are disease-specific sEVs containing various cargos and surface markers. Additionally, the incomplete knowledge of sEV phenotypes is due to the complexity and limitations of isolation and characterization methods.

Conventional sEV isolation methods have various identified disadvantages including cost and time consumption. Additionally, conventional isolation methods yield low quality and quantity of sEVs despite the many technical approaches employed for cleaning out impurities such as lipoproteins (e.g., size exclusion chromatography). Recently the ExoView R100 platform has emerged, allowing for a simpler method for obtaining sEV data. It uses the single-particle interferometric reflectance imaging sensor (SP-IRIS) technique to obtain automated data [127]. Isolation, characterization and quantification of sEVs are detected using fluorescence. These sEVs are detected by tetraspanin (CD63, CD81 and CD9) antibodies. This platform allows for a quick, multi-level and accurate determination of exosome particle size, count, phenotyping and tetraspanin colocalization whilst using a moderately low sample input, however disadvantages of the ExoView platform such as the high cost could pose an issue for translating the use of this assay in daily clinical practices. A study published in 2024, reported that the use of the ExoView platform for the direct quantification and simultaneous characterization of EVs is advantageous as the assay requires a minimum sample input and takes no longer that 3 hours to conducts, providing evidence that this analysis may aid in facilitating possible translation into routine clinical practices [128]. This particular study utilized ExoView for the understanding and monitoring of renal disease and function [128]. Furthermore, a study highlights that there are disadvantages of the ExoView platform however, these disadvantages preponderate over the advantages of other characterization methods such as NTA. This study reported that even though NTA is significantly cost-effective and convenient for daily use in comparison to the ExoView platform, the results generated from the ExoView platform are significantly more accurate [129]. In this study, the size measurements using NTA were overestimated by 2-fold [129]. The use of the ExoView R100 platform was affirmed to show that colocalization patterns differ based on sEV origin from different cells [130]. Therefore, our current study has provided the rationale for the involvement of sEV colocalization patterns in sepsis pathophysiology. With the abundance of CD63, CD81 and CD9 positive particles in sepsis patients, we reported distinct colocalization patterns observed in sepsis patients, indicating disease-specific tetraspanin heterogeneity for the commonly used markers. These results highlight that the colocalization patterns observed may be of increasing interest in exploring the potential of sEVs as biomarkers for sepsis. Additionally, they provide information

on the colocalization patterns that could be specific to HIV-positive sepsis patients, placing importance on targeting co-morbidities in sepsis biomarker research.

Clinical disorders associated with an increased risk of developing a disease are known as co-morbidities [131]. Co-morbidities such as HIV, cancer, chronic hypertension and lung diseases can influence the outcomes and progression of sepsis, ultimately increasing organ failure and the risk of mortality [132]. Alongside the burden of co-morbidities in the field of sepsis research, an array of markers is present on the surface of sEVs. These surface markers are involved in protein-to-protein interactions and recipient cell targeting [82]. The aforementioned tetraspanins act as intermediaries for the formation of a variety of surface marker profiles on the sEV membrane [126]. It is therefore possible to subtype these sEVs without destroying their structure. This was the motivation for our subsequent experiments to analyse the surface marker profiles in sepsis patients who have a pre-existing co-morbidities (hypertension).

In manuscript three **(Chapter Four)**, the biomarker potential of sEVs in sepsis was explored by analyzing the surface marker profiles in 4 study groups (viz. healthy controls, hypertension, sepsis patients with no pre-existing co-morbidities and sepsis patients with previously diagnosed hypertension). This was done using a multiplexed flow cytometric bead assay (MACSPlex), which identifies 37 different surface markers on isolated sEVs. Patients with chronic hypertension are at a greater risk of developing sepsis due to commonalities in the pathologies including endothelial dysfunction, oxidative stress and systemic inflammation [133]. Although there is substantial evidence on the individual surface markers in the pathology of sepsis [134-136] and in hypertension [137-139], there has been no scientific evidence linking the surface marker profiles when these two pathological states co-exist. The data indicates that there are specific surface marker profiles in sepsis (with no pre-existing co-morbidities), hypertension and sepsis with previously diagnosed hypertension, also providing insight into the differentially expressed surface marker profiles when compared to healthy controls or with the other study groups. These surface markers have roles in cytokine signalling [140], endothelial dysfunction [141], and activation of the immune response [142], contributing to the pathogenesis of hypertension and sepsis. Along with aiding in the further understanding of sepsis pathology, the identified surface marker profiles could possibly be exploited in the identification of disease-specific sEV subsets, adding further value to the use of sEVs as possible biomarkers for patient stratification and use in personalized medicine.

5.2 Conclusion

The multifaceted and complex pathophysiology of sepsis still requires further elucidation. This has added to the difficulty of identifying and developing reliable biomarkers for the early diagnosis of sepsis. Previously researched biomarkers such as lactate and CRP are not specific and have not been approved as the primary biomarkers of sepsis within clinical settings. Recent studies on sEVs provide theories that these vesicles can be used as prognostic and diagnostic biomarkers for sepsis. These small vesicles comprised of a vast array of surface markers present on the surface membrane and sEV phenotypes are still not fully understood. These surface markers are of great value in EV research as they are reflective of the cells from which these sEVs emanate. The novelty of the current study arose from the novel and innovative techniques used as well as the South African sepsis cohort incorporating a cohort of patients with a widely diagnosed co-morbidity (hypertension). Furthermore, unique surface marker signatures were identified in sepsis and hypertension, and this study provided evidence linking the surface marker signatures when these two pathological states co-exist.

The outcomes of this unique study have identified novel surface marker profiles and tetraspanin colocalization patterns in sepsis patients, providing insights into the role of sEVs in the pathology of the disease, thus aiding patient stratification in sepsis. In addition, the colocalization patterns highlight the disease-specific variations of the commonly used surface markers. Importantly, the surface marker profiles provide further insight into the pathophysiology of sepsis as these markers are involved in various mechanisms in the pathological state. The sEVs released during any pathological state have diverse compositions within their cargo and unique signatures on the surface membrane. Therefore, the findings of this study add to the value of sEVs as possible biomarkers of sepsis, which will aid diagnostically and prognostically in the high-care clinical setting.

5.3 Recommendations and Limitations

The presented findings support the idea of using sEVs as possible biomarkers of sepsis; however, these studies are limited, and further investigations are required. Limitations of this study include a small sample size in Chapter Three, as the study was underpowered for making a substantial comparison between HIV-positive and HIV-negative sepsis patients. All samples

used in this study were taken within the first 12 hours post-admission; however, it would be beneficial to investigate the surface marker profiles of sEVs during different stages of disease, adding further value to the potential of sEVs as biomarkers of sepsis. The current study did not include the effects of other variables (such as sex and SOFA score) on the colocalization patterns and surface marker profiles of sepsis; studies including these effects are recommended. Further complex in vivo and in vitro studies are required to clarify some of the precise biological processes of sEVs in the pathophysiology of sepsis and sepsis with previously diagnosed co-morbidities (such as HIV and hypertension). Studies that include the tetraspanin and surface marker difference between sepsis and Systemic Inflammatory Response Syndrome (SIRS) patients for the understanding of the role of sEVs during the progression of the disease are recommended. Moreover, understanding the differences in the colocalization patterns and surface marker profiles of ICU patients who are suspected of having sepsis but are found not to, compared to patients diagnosed with sepsis would be a favourable comparison. Patients suspected of having sepsis are the cohort of interest in the development of sepsis biomarkers. Intensive analyses of the cargo of the isolated sEVs are also required to justify the use of these vesicles as candidate biomarkers. Furthermore, the addition of currently researched biomarkers of sepsis, such as lactate and CRP, to sEV tetraspanin patterns and surface marker profiles could possibly aid in the identification of a combined biomarker panel. Research pertaining to biomarker development requires validation through investigations in a larger, independent cohort using a particular cutoff value, as well as sensitivity and specificity calculations for the potential biomarker of interest.

5.4 Summary of Findings

This study has provided theoretical and experimental evidence supporting the use of sEVs as potential biomarkers for sepsis. The major findings of this study can be summarized as follows:

- Commonly used markers for the identification of sEVs (CD63, CD81 and CD9) were analyzed and showed that CD63/CD9 positive exosomes were abundant in sepsis patients.
- Specific tetraspanin colocalization patterns were identified in sepsis patients among all three (CD63, CD81 and CD9) positive probes.
- The abundance of CD63, CD81 and CD9 positive sEVs and the specific tetraspanin colocalization patterns provided evidence that sEV subpopulations have specific tetraspanin compositions and is of increasing interest in exploring the potential of sEVs as biomarkers for sepsis.
- Further analysis into the surface markers of sEVs showed that there are disease-specific surface marker profiles present on the surface membrane of sEVs, which can facilitate the identification of disease-specific sEV subsets as possible biomarkers of sepsis.
- Among the four comparisons, i.e., healthy controls vs hypertension, healthy controls vs sepsis, hypertension vs sepsis with hypertension, and sepsis vs sepsis with hypertension, there were 7, 6, 20, and 21 surface markers that were differentially expressed, respectively.
- The surface marker profiles will aid in the further understanding of sepsis by identifying the cells involved in the pathogenesis of sepsis.

6 CHAPTER SIX: REFERENCES

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7 CHAPTER SEVEN: APPENDICES

7.1 Human Ethics Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

28 October 2022

Miss Roushka Bhagwan (214516599)
School of Lab Med & Medical Sc
Westville

Dear Miss Bhagwan,

Protocol reference number: BREC/00004587/2022
Project title: Investigating the biomarker potential of circulating exosomes in patients presenting with Sepsis
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 28 October 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 28 October 2022. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 13 December 2022.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

7.2 Human Ethics Approval Recertification

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 November 2024

Miss Roushka Bhagwan (214516599)
School of Laboratory Medicine & Medical Science
Westville

Dear Miss Bhagwan,

Protocol reference number: BREC/00004587/2022
Project title: Investigating the biomarker potential of circulating exosomes in patients presenting with Sepsis
Degree: PhD

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**28 October 2024**
Expiration of Ethical Approval:	**27 October 2025**

I wish to advise you that your application for recertification for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC). The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 10 December 2024.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof S Singh
Chair: Biomedical Research Ethics Committee

7.3 Hospital Approval (Inkosi Albert Luthuli Central Hospital)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Amended letter
17 June 2021

Prof I Mackraj
Department of Human Physiology
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear I Mackraj

PROTOCOL: Characterizing Trophoblastic Debris in Pregnant Women.
REF: BE036/12

We wish to advise you that your response to queries to BREC letter dated 10 June 2021 has been noted by a subcommittee of the Biomedical Research Ethics Committee. Your application for amendments listed below received on 11 May 2021 for the above study has now been **approved** by a subcommittee of the Biomedical Research Ethics Committee.

Amendments noted and approved:

1. Addition of a sepsis cohort.
2. Additional Investigators: Ms R Bhagwan (PhD Student) and Ms C Heeralall (PhD)
3. Addition of Pregnant women who are COVID Positive
4. To include the testing of placenta for changes as outlined.

The committee will be notified of the above approval at its next meeting to be held on 13 July 2021.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

KWAZULU-NATAL PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

DIRECTORATE:

INKOSI ALBERT LUTHULI CENTRAL HOSPITAL
OFFICE OF THE MEDICAL MANAGER
Private Bag X03, Mayville, 4058
800 Vusi Mzimela (Bellair) Road, Mayville,4091
Tel: 031 240 1059 Fax: 031 240 1005 Email: Ursula.john@ialch.co.za

Reference: BE 036/12
Enquiries: Medical Management

1 July 2021

Prof I Mackraj
Department of Human Physiology
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Prof Mackraj

RE: PERMISSION TO CONDUCT RESEARCH AT IALCH

I have pleasure in informing you that permission has been granted to you by the Medical Manager to conduct research on: **Characterizing Trophoblastic Debris in Pregnant Women.**

Kindly take note of the following information before you continue:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Kindly ensure that this office is informed before you commence your research.
4. The hospital will not provide any resources for this research.
5. You will be expected to provide feedback once your research is complete to the Medical Manager.

[redacted]

Dr L P Mtshali
Medical Manager

KWAZULU-NATAL PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

DIRECTORATE:

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Private Bag X03, Mayville, 4058
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Tel: 031 240 1059 Fax: 031 240 1005 Email: Ursula.john@ialch.co.za

1 July 2021

Prof I Mackraj
Department of Human Physiology
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Prof Mackraj

Re: Approved Research: Ref No: BE 036/12: Characterizing Trophoblastic Debris in Pregnant Women.

As per the policy of the Provincial Health Research Committee (PHRC), you are hereby granted permission to conduct the above mentioned research once all relevant documentation has been submitted to PHRC inclusive of Full Ethical Approval.

Kindly note the following.

1. The research should adhere to all policies, procedures, protocols and guidelines of the KwaZulu-Natal Department of Health.
2. Research will only commence once the PHRC has granted approval to the researcher.
3. The researcher must ensure that the Medical Manager is informed before the commencement of the research by means of the approval letter by the chairperson of the PHRC.
4. The Medical Manager expects to be provided feedback on the findings of the research.
5. Kindly submit your research to:

   The Secretariat
   Health Research & Knowledge Management
   330 Langaliballe Street, Pietermaritzburg, 3200
   Private Bag X9501, Pietermaritzburg, 3201
   Tel: 033395-3123, Fax 033394-3782
   Email: hrkm@kznhealth.gov.za

[redacted]

Dr L P Mtshali
Medical Manager

7.4 Hospital Approval (King Edward VII Hospital)

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

KING EDWARD VIII HOSPITAL,

Private Bag X02

Congella

4013

[redacted]

INTENSIVE CARE UNIT

CRITICAL CARE

Date: 2023/06/27	
To: Dr B Bilenge, Senior Medical Manager	From: Dr D Singh (HCU)

RE: Specimens for PhD

Dear Dr Bilenge

Miss Bhagwan wishes to collect specimens from patients in ICU.

This has been cleared by Bioethics at UKZN. I am happy to support.

I will request clearance from hospital management as well.

Kind regards

Dr D Singh [redacted]

Head Clinical Unit

APPROVED [redacted]

7.5 South African Department of Health Approval

KWAZULU-NATAL PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

DIRECTORATE:

Physical Address: 330 Langalibalele Street, Pietermaritzburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3180/ 3123 Fax: 033 394 3782
Email:

Health Research & Knowledge Management

NHRD Ref: KZ_202308_022

Dear Ms R Bhagwan
(UKZN)

Approval of research

1. The research proposal titled '**Investigating the biomarker potential of circulating exosomes in patients presenting with Sepsis'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at King Edward VIII and Inkosi Albert Luthuli Central Hospital.

2. You are requested to take note of the following:
   a. ***Kindly liaise with the facility manager BEFORE your research begins.***
      This is to ensure that conditions in the facility are conducive to the conduct of your research. These include, but are not limited to, an assurance that the numbers of patients attending the facility are sufficient to support your sample size requirements, and that the space and physical infrastructure of the facility can accommodate the research team and any additional equipment required for the research.
   b. *All research conducted in KwaZulu-Natal must comply with government regulations relating to Covid-19. These include but are not limited to: regulations concerning social distancing, the wearing of personal protective equipment, and limitations on meetings and social gatherings.*
   c. *Please ensure that you provide your letter of ethics re-certification to this unit, when the current approval expires.*
   d. *Provide an interim progress report and final report (electronic and hard copies) when your research is complete to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za*
   e. *Please note that the Department of Health shall not be held liable for any injury that occurs as a result of this study.*

For any additional information please contact Mr. X Xaba on 033-395 2805.

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Provincial Health Research Committee
Date: 15/11/2023

GROWING KWAZULU-NATAL TOGETHER

7.6 UKEV Forum 2022 invitation letter

SCHOOL *of* BIOLOGICAL SCIENCES

The University of Edinburgh
Ashworth Laboratories
The King's Buildings
Charlotte Auerbach Rd
Edinburgh EH9 3FL

Telephone +44 131 651 3575
Email a.buck@ed.ac.uk

3rd November 2022

Dear Roushka

Visit to Edinburgh to attend the UKEV Forum 2022

I am pleased to invite you to attend the UKEV Forum 2022, as detailed below.

1st-2nd December 2022, Royal College of Physicians of Edinburgh, 11 Queen Street, Edinburgh, EH2 1JQ
UKEV Forum 2022 - UK Society for Extracellular Vesicles

This is the annual conference of the UK Society for Extracellular Vesicles (EVs), which will be held as an in-person event this year at the historic Royal College of Physicians in the heart of the city of Edinburgh, Scotland. With keynote speakers, Graça Raposo, Guillaume Van Niel, Bernd Giebel and Hailing Jin, there will be sessions on EV biogenesis, EVs across biological systems, in vivo models & imaging of EVs as well as translational and therapeutic aspects of EVs. There will be opportunities for short talks, posters and lightning talks for delegates and industrial sponsors will be showcasing the latest in EV technologies. Early career researchers will be able to attend a training workshop before the main meeting and extensive networking and social events are scheduled to complement the exciting scientific programme.

[redacted]

Prof. Amy Buck
Head of Institute of Immunology & Infection Research
www.bucklab.org

7.7 Abstract in UKEV Forum 2022 Programme and Abstract Book

Abstracts

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Circulating exosomes in South African sepsis patients: An investigation to identify and quantify tetraspanin exosomal markers.

Roushka Bhagwan, Usri Hassan, Irene Mackraj
University of KwaZulu-Natal, Durban, South Africa

Abstract

Sepsis, a leading cause of mortality globally, has a complex and multifaceted pathophysiology which still requires elucidation.

The aim of this study was to analyse and quantify the number of exosomes in sepsis patients from a South African cohort using the Exoview (NanoView Biosciences, Boston MA) platform with a view to identify unique signatures in the cargo.

We recruited Black South African patients from the local hospital ICU. Plasma was collected and analysed using the ExoView platform. ExoView was used to isolate and characterize exosomes in terms of tetraspanin markers, particle size distribution, particle shape and colocalization of different tetraspanin markers.

In our cohort of patients, we found that the level of exosomes was higher than that of our control group ($p<0.05$). These exosomes were within the size range of 50-75nm. There is a high colocalization between CD9 and CD81 markers across most samples. CD9 positive exosomes indicate a higher number of isotopes on surface compared to other markers.

Our findings show that circulating exosomes in sepsis patients have a potential to elucidate some of the symptoms and the pathophysiology of sepsis. The increase in the number of circulating exosomes have the potential to be implemented to monitor the progression of sepsis. Further investigations are required to identify and quantify exosomal cargos to further understand the pathophysiology of sepsis and to develop effective diagnostics and treatments for sepsis.

UKEV
Society

7.8 English Editor Certificate

St Charles College,
Harwin Road,
Scottsville
Pietermaritzburg 3201
Tel: 083 593 2855
admin@kznlanguageinstitute.com
www.kznlanguageinstitute.com

Registration number: 131 804 NPO

Certificate of editing 11 December 2024

Name:

Roushka Bhagwan-Valjee

Title:

Investigating the biomarker potential of circulating small extracellular vesicles in patients presenting with sepsis.

This serves to confirm that the above document, with the exception of Chapters two, three and four, was edited substantively by a member of the KZN Language Institute's professional English language editing team. The document was returned to the author with tracked changes and comments intended to correct errors and to clarify meaning. It was the author's responsibility to attend to these changes.

J. Kerchhoff

Director of the KwaZulu-Natal Language Institute

KZN Language Institute - Transforming Words