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Assessment of Presepsin, Calprotectin & Bacterial causes in critically ill adult patients with sepsis

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

By

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﴿يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ﴾

صدق الله العلي العظيم

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Supervisors Certification

We certify that this M.Sc. thesis titled:

Assessment of Presepsin, Calpotectin &bacteria causes in critically ill adult patients with sepsis

Was prepared under our supervision in the College of Medicine/ University of Karbala, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology.

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Dedication

Our glorious prophet Mohammed & his household, the good and pure (Allah blessings be upon him & his household).

My lovely father,

The kind heart mother

My dear brother & sisters

My lovely husband

My lovely daughter.

I dedicate this work

Hijran Tawfiq

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Summary

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. It had very high morbidity and mortality rates and is a public health problem worldwide.

Sepsis was characterized as a systemic inflammatory response syndrome (SIRS) to infection, with three levels of severity: sepsis, severe sepsis, and septic shock. Many inflammatory markers were investigated in sepsis with different diagnostic utility as C.reactive protein & Procalcitonin. Presepsin and calprotectin are new markers which are not studied in Iraq.

Different pathogens could be associated with sepsis development as fungi, viruses, parasites and bacteria. Knowing the responsible pathogen of sepsis is fundamental for subsequent management approach.

The aim of the study is to evaluate the serum levels of Presepsin & Calprotectin in patients with septicemia as well as comparing the diagnostic utility of these biomarkers to the traditional used diagnostic methods in septicemia. Also assessing the etiological pathogenic causes of septicemia.

A forty clinically diagnosed patients with adult sepsis were included in the study. The patients were (20) males and (20) females with age ranged between (17-70). In addition to 40 healthy control participants with matched age and gender for patient group. Serum levels of Presepsin & Calprotectin were measured using commercial research ELISA kits.

Calprotectin and presepsin serum levels were elevated in patients' group compared to control with significant result for calprotectin (P=0.019, P=0.066) respectively. Presepsin show a significant diagnostic utility. Non-significant result for both biomarkers levels in sepsis subclasses: sever sepsis, septic shock

& sepsis (49.75±15.7, 59.53±18.8, 36.40±8.14) respectively for calprotectin, presepsin levels in septic shock, severe sepsis &sepsis (476.62±150.7, 551.88±161.87, 137.96±30.85) respectively.

Out of the 40 enrolled subjects, 24 patients had a confirmed microbial etiology of sepsis, candida infection in one patient, gram-positive (7) and Gram-negative (16) with non-significant association with study markers levels.

Current stady showed that, both calprotectin and presepsin elevated in septic patients and presepsin could be a good biomarker with high specificity for diagnosis of sepsis. In addition, Gram negative *Salmonella typhi* was the most prevalent microorganism obtained from blood culture of these patients.

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List of Abbreviations

Code	Words
ALP	ALP Alkaline phosphatase
AST	Aspartate transaminase
CBC	CBC Complete blood count
CD14+	CD14+ Clusters for differentiation 14
CD16+	CD16+ Clusters for differentiation 16
CRP	CRP C-reactive protein
DAMPs	Danger-associated molecular patterns
DIC	Disseminated intravascular coagulation and multiple organ
EDTA	EDTA Ethylene Di amine Tetra Acetic Acid
ELISA	ELISA Enzyme –Linked Immunosorbent Assay
ICU	Intensive care unit
IL-1ra	Interleukin-1 receptor antagonist

IL-6	IL-6 Interleukin-6
IMCU	Intermediate care unit
LPBs	lipopolysaccharides binding proteins
LPSs	Lipopolysaccharides
MODS	Multiple organ dysfunction syndrome
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PaO2	Partial pressure of oxygen
SBP	Sugar blood pressure
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential Organ Failure Assessment
SPSS	SPSS Specific Software Statistical Package for the Social Sciences
SS	Severe sepsis septic
Sx	Septic shock
TGFβ	Transforming growth factor β
TMB	TMB Tetra methyl benzidine
Tregs	Regulatory T cells

Chapter one
Introduction & literature Review

1.1 Introduction

Sepsis is a prevalent and potentially fatal infection in the intensive care unit that needs prompt and efficient antibiotic therapy. The most prevalent cause is bacterial infections, however viruses and fungi can also develop in people with comorbid illnesses and immunosuppression(Rhee et al., 2019)**.** Fever, tachycardia, and tachypnea are classic sepsis symptoms, a scattered inflammatory response produced by microbial infections. At least one organ malfunction has been associated with severe sepsis. When severe sepsis is combined with multiple organ system failures, the condition is known as septic shock(*Obaid et al., 2020*)**.**

This life-threatening organ failure caused by a dysregulated host response to an infection is a medical emergency for which early detection, suitable, and prompt therapies are critical in reducing mortality and morbidity*(Rosenqvist, 2021*)**.** Although there are numerous criteria for defining organ failure during sepsis, using 3 guidelines (the third iteration of the international consensus diagnostic definitions of sepsis)of Sepsis-related Organ Failure (SOFA) score to do so. The new concept gives us a better grasp of sepsis pathogenesis and more precise diagnostic criteria(*Mayr et al., 2014*),(Kolesnichenko et al., 2021)**.**

Septic shock is described as sepsis with circulatory collapse, which is the most severe kind of sepsis. "Adequate routine microbiologic cultures (including blood) should be obtained before beginning antimicrobial therapy in patients with suspected sepsis or septic shock when doing so outcomes in no significant delay in the start of antimicrobials," the recommendation for making a definitive diagnosis changed(*Pepic et al., 2021)***.**

Both Gram-positive bacteria and Gram negative bacteria represent the most commonly causative pathogens in sepsis patients. *Staphylococcus aureus, Enterococcus species, and Streptococcus pneumoniae* were the most prevalent

Gram-positive organisms had been identified, whereas *E.coli, Klebsiella pneumoniae, Acinetobacter baumannii, and Pseudomonas* species were the most common Gram-negative organisms(*Pepic et al., 2021)* The primary test for sepsis is still b lood culture, albeit the findings are generally not available for 24 to 48 hours after the collection but still, the gold standard for diagnosis of bacterial sepsis, and the bacterial culture results can provide information for determining the microbiological etiology of an illness, assisting in the selection of suitable empiric antibiotic treatment*(Holub et al., 2018).*

Calprotectin is a 36 kDa member of the S100 family of proteins. It is derived predominantly from neutrophils and has direct antimicrobial effects and a role within the innate immune response*(Ayling & Kok, 2018*)**.** Presepsin is a circulating molecule fragment derived from sCD14 and serves as mediator of lipopolysaccharid response against infectious agents. More recent studies have presented presepsin as a valuable potential biomarker for early diagnosis of sepsis(*Moustafa et al., 2021*).

Aim of the study

The goal of the study is to evaluate the diagnostic utility of Presepsin& Calprotectin as biomarkers compared to the traditional used diagnostic methods in addition to investigate bacterial pathogens causes of septic patients, these were achieved by the following objectives :

1.Bacterial isolation and identification by culture , biochemical tests and viteks system .

2.Determination of calprotectin and presepsin in patients and control by ELISA test .

1.2. Literature Review

1.2.1 Definition of Sepsis:

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. It has very high morbidity and mortality rates and is a public health problem worldwide(Singer *et al., 2016*).

Many of the components of the innate immune response that are normally concerned with host defenses against infection can, under some circumstances, cause cell and tissue damage and hence multiple organ failure(Cohen, *2002*).

Sepsis has a mortality rate greater than that of acute coronary syndrome or ischemic vascular stroke(Tusgul *et al., 2017).*

1.2.2History

Sepsis was first mentioned by scriptures in Ancient Greece. The word sepsis comes from the Greek word *"sepo"*, which means *"I rot"*, and has its first use in medical context in Homer's poems(Bhargavi Sindhuja, 2020).

Sepsis is developed as a result of an infection, which can come about as a result of poor hygiene. However, they never considered transmission by person-to-person contact, and therefore missed the general theory of infectious, the past 30 years have seen an increased focus on how to better understand and treat sepsis. In the early 1990s, a conference was held to come to some kind of consensus as to what sepsis was defined as(Finfer & Machado, 2016). This was modified by the 2001 conference but ultimately lead to the development of a distinction between an infection, sepsis, severe sepsis, and septic shock. In 1964, a Boston surgeon called Dr. Edward Frank published a management strategy for septic shock (Mayglothling *et al., 2011*).

The strategy included continuous monitoring of systemic arterial pressure, central venous pressure, cardiac output, urinary output, blood volume, blood chemistries, gases, pH, and electrolytes. Some of these, such as blood monitoring and urinary output, are still used today. He also recommended finding the cause of the infection, which is still done today, aided by the discoveries of antibiotics by Alexander Fleming earlier that century. Newer management techniques included volume resuscitation with crystalloid, use of stress dose steroids when sensible, oxygen delivery, and recognition and management of dysfunction of the central nervous system(Funk *et al., 2009)*.

1.2.3 Epidemiology of Sepsis

Severe sepsis remains a leading cause of morbidity and mortality in intensive care units. Severe sepsis is associated with a mortality rate of 30%, which rises to 40–60% if exacerbated by septic shock, in these severe systemic infections, the normally tightly controlled balance between the inflammatory, coagulatory, and neuroendocrine systems is lost. Systemic release of numerous inflammatory mediators, such as cytokines, anaphylatoxins, factors of coagulation, and fibrinolysis, has long been recognized as a major event in the pathophysiology of sepsis(Walke*y et al., 2011*).

Epidemiological studies from the United States of America and from Europe have shown that sepsis is a widely prevalent syndrome, with either steady or slightly decreasing rates of morbidity and of mortality in recent decades(Jaimes, *2005)*.

Probably the most often quoted article on the epidemiology of sepsis is the 2001 publication by Angus and colleagues, which used administrative data to estimate that there were 751,000 cases (3.0 per 1,000 population) in the United States each year, resulting in more than 200,000 deaths(Finfer & Machado, 2016).

The greater than 200,000 in-hospital cardiac arrests that occur in the United States annually, between 30,000 and 60,000 occur in patients with underlying sepsis. These patients are less likely to survive than cardiac arrest victims without sepsis(Morgan *et al., 2017*).

Epidemiology case studies using administrative hospital data have reported both growing incidence and declining mortality rates associated with severe sepsis in several different countries but mainly in the USA, Australia and New Zealand (*Yébenes et al., 2017)*.

The incidence of sepsis in Sweden is not known, but the incidence of severe sepsis is estimated to be at least 200 per 100,000 inhabitants and that of septic shock to be more than 30 per 100,000 inhabitants. According to the annual report Cause of Death in Sweden published by the National Board of Health and Welfare, 1,042 individuals died from sepsis during 2012 (Janols, 2014).

The mortality rate has declined over the years, but even if the patients do not directly die of sepsis, the survivors have an increased risk of death in the following 5 years and may suffer from persistent physical and cognitive dysfunction. The reasons for this are not known, but they are probably multifactorial(Fleischmann-Struzek e*t al.*, 2018*)*.

No reports for adult sepsis in Iraq but there are several studies on neonatal sepsis, as for prevalence of 54.67% documented in past study in Duhok city(Mohamed et al., 2020).

1.2.4 Etiology & Classification of Sepsis

Adult septicemia is microbial growth in the blood of adult accompanied with clinical manifestations, bacterial infections remain the primary cause of pathogenic sepsis, viruses and fungi comprise a meaningful percentage of sepsis etiologies*(*Dolin *et al., 2019)*.

Sepsis was characterized as a systemic inflammatory response syndrome (SIRS) to infection, with three levels of severity; sepsis, severe sepsis, and septic shock, this definition arose from the belief that sepsis shares an underlying inflammatory pathway with other insults, such as trauma or pancreatitis table 1.1 & figure 1.2(Cortés-Puch & Hartog, 2016).

Sepsis and septic shock, caused by Gram-negative and Gram positive bacteria, fungi, viruses, and parasites, have become increasingly important over the past decades, *Staphylococcus aureus and S. epidermidis* being responsible for more than half of the cases of sepsis due to Gram-positive bacteria, the increasing septicemia rates are probably caused by the increasing use of catheters and other invasive equipment, by chemotherapy, and by immunosuppression in patients with organ transplants or inflammatory diseases(Van Amersfoort *et al., 2003)*.

The most Bacterial pathogens that associated with increase mortality included *Pseudomonas aeruginosa, Escherichia coli ,Klebsiella pneumoniae and Staphylococcus aureus*(*Laurie et al., 2010)*. Patients were considered to have a bloodstream infection due to Enterobacteriaceae if any blood culture obtained within 48 hours of developing sepsis, severe sepsis, or septic shock were positive for *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella granulomatis, Proteus mirabilis, Proteus vulgaris, Enterobacter aerogenes, Enterobacter cloacae, Enterobacter sakasakii, Serratia marcescens, Citrobacter freundii, Citrobacter koseri, Citrobacter amalonaticus, Edwardsiella tarda, Hafnia alvei, Morganella morganii, Pantoea agglomerans, Plesiomonoas shigelloides, Providencia stuartii, Providencia rettgeri, Salmonella enterica, Shigella dysenterii, Shigella flexneri, Shigella sonnei, Shigella boydii, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Ewingella americana, or Kluyvera spp*.

Table 1.1: Sepsis cases and definition: Criteria for the systemic inflammatory response syndrome, sepsis severe sepsis & septic shock(Cajander, 2017).

Term	**Criteria**
SIRS	Two out of the following four criteria :temperature > 38 C or 36 heart rate < 90 /min Hyperventilation evidenced by respiratory rate> 20 /min or arterial co2 lower than 32 mmHg white blood cell count> 12 cell /L or lower than 4 cells/L
Sepsis	SIRS Criteria with presumed or proven infection
Severe sepsis	Sepsis with organ dysfunction
Septic shock	Sepsis with hypotension despite adequate fluid resuscitation

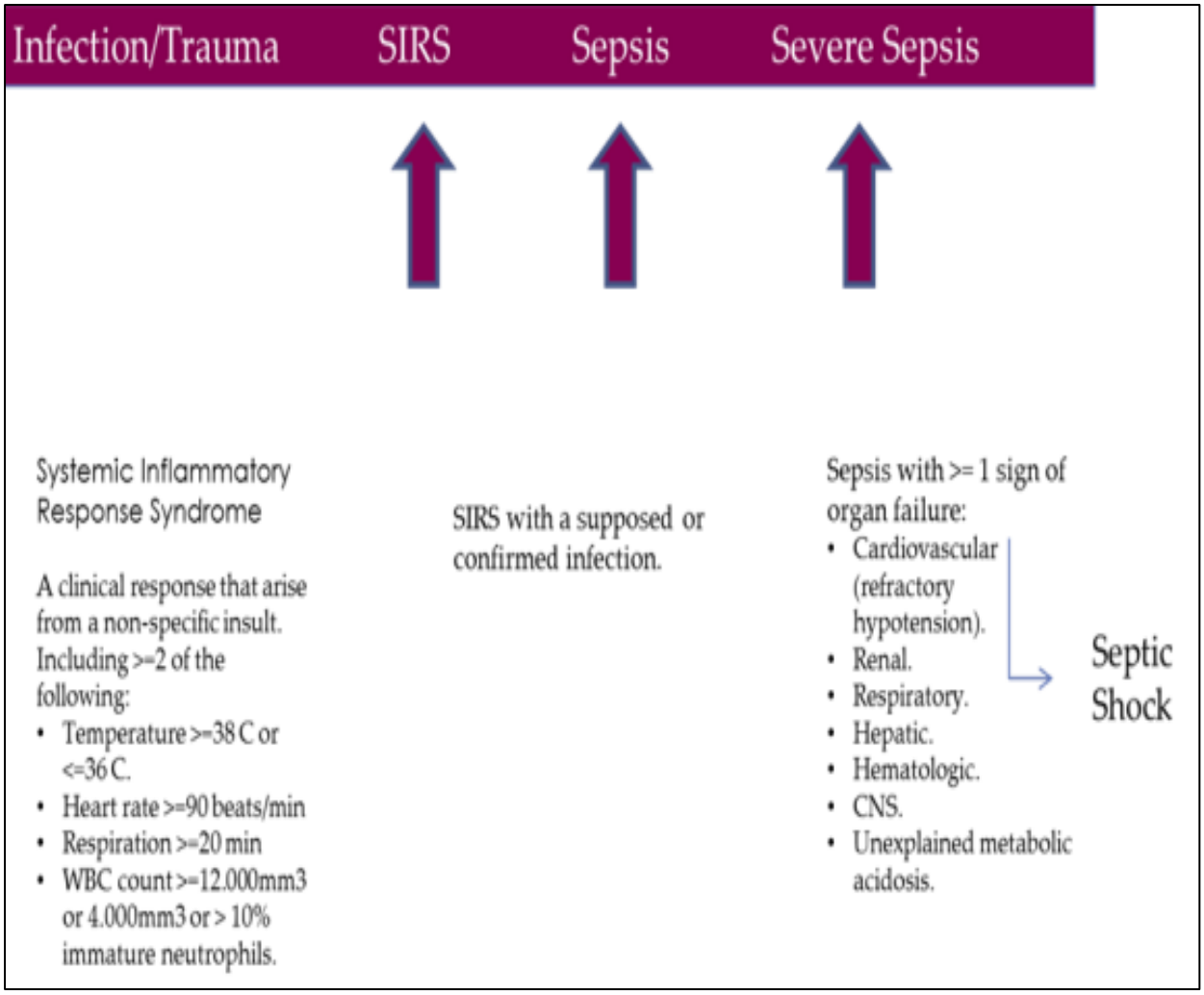

Figure 1.1: Definitions of SIRS, sepsis, sever sepsis and septic shock(*Almuhayawi, 2016)*.

1.2.5 Risk Factor for Adult Sepsis

Risk factors for sepsis include age, gender, the presence of invasive devices (eg, urinary catheters), and chronic medical conditions (eg, chronic obstructive pulmonary disease)(*Cohen, 2002*).

1.2.6 Clinical Picture of Sepsis:

The septic syndrome, acute lung injury, and multiorgan failure are closely tied to one another because bacterial cell walls can activate inflammatory

mediators, such as interleukin-1 and tumor necrosis factor, which can in turn lead to the septic syndrome and inflammatory injury to the lung, Clinical features more than serum markers, have been the best predictors of whether lung injury will follow sepsis*(Niederman & Fein, 1990)*.

Sepsis syndrome was specifically defined by signs and laboratory tests reflecting infection or inflammation plus evidence of a deleterious systemic effect (hypotension, reduced systemic vascular resistance, or unexplained metabolic acidosis)*(Montgomery et al., 1985)*.

There are clinical and laboratory characteristics to be considered in the diagnosis of sepsis, severe sepsis or septic shock. These include fever, hypothermia, level of consciousness and inflammatory parameters*(Silva et al., 2010).*

Sepsis develops from the spread of a localized infection. It is characterized as a severe and often fatal clinical syndrome that involves a systemic inflammatory response to infection and manifests with microvascular dysfunction, derangements in coagulation, and ultimately multi-organ dysfunction. Sepsis is diagnostically defined as having a suspected infection plus at least one characteristic symptom such as temperature change, tachycardia, respiratory distress, altered mental status, hypotension, white blood cell count changes, presence of elevated biomarkers, or microvascular dysfunction*(Patel et al., 2019).*

Because of inadequate specificity and sensitivity, the recent sepsis (Sepsis-3) guidelines recommend avoiding use of SIRS criteria and proposed a new definition; a life-threatening organ dysfunction caused by a dysregulated host response to infection (A new measure, SOFA Score, quick sequential (sepsis-related) organ failure assessment)*(Raveendran et al., 2019).*

It is crucial to rapidly identify sepsis so that adequate treatment may be initiated. Accordingly, the Sequential Organ Failure Assessment (SOFA) and

the quick SOFA (qSOFA) scores are used to evaluate intensive care unit (ICU) and non-ICU patients, respectively (Koch *et al., 2020*).

1.2.7Immuno Pathophysiology of Sepsis

There is a great deal known about the pathophysiology of sepsis, sepsis is an extreme response to inflammation(Rittirsch *et al., 2008*).

The first step in the initiation of the host response to the pathogen is the activation of innate immune cells, constituted primarily by macrophages, monocytes, neutrophils, and natural killer cells. This occurs via the binding of pathogen-associated molecular patterns, such as bacterial endotoxins and fungal β-glucans to specific pattern recognition receptors, on these cells(Gyawali *et al., 2019).*

In the cross-talk between the systems of pattern-recognition, complement and coagulation there is abundance of counter-regulatory mediators aiming to inflammatory cytokines like IL-10, IL-1 receptor antagonist (IL-1ra), and transforming growth factor β (TGFβ), IL-6, in contrast to its proinflammatory properties, also exhibits anti-inflammatory effects, illustrating the complexity of cytokine interactions. IL-6 inhibits the production of IL-1 and TNF and stimulates the release of IL-10 and cortisol. Circulating immune cells develop diminished antigen presenting capacity and responsiveness to secondary inflammatory stimulation, which in combination with negative regulators of TLR signaling and inducers of immune cell apoptosis contribute to an anti inflammatory pattern(Thomson & Knolle, *2010).* In addition, there is a neuro-endocrine-immune network in which the cholinergic nervous system responds to efferent signals by the release of acetylcholine, which inhibits proinflammatory cytokine release through ligation to acetylcholine receptors on macrophages (Fig.1.2.). There is mounting evidence that critically ill patients who survive the initial phase of sepsis are subjected to a more immunosuppressive condition, with a propensity to develop secondary

opportunistic infection and reactivation of latent viral infection(Vincent & Beumier, 2013).

The interaction between pathogens and the host is mediated initially via an interaction between pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs). This interaction can result in the release of danger-associated molecular patterns (DAMPs), which have the ability to further amplify the inflammatory response, at least in part, via TLRs. The initial inflammation activates afferent signals that are relayed to the brain; subsequent activation of vagus efferent activity inhibits cytokine synthesis via pathways dependent on acetylcholine receptors on macrophages and other cells through the cholinergic anti-inflammatory pathway (the inflammatory reflex)(Seyedabadi *et al., 2018)*. The resulting innate response of immune cells can result in a balanced reaction leading to pathogen elimination and tissue recovery, or an unbalanced reaction that on the one hand can lead to exaggerated inflammation and tissue injury, and on the other hand to immune suppression(Vincent & Beumier, 2013).

Regulatory T cells (Tregs) play a crucial role in modulating the inflammatory response and participated in sepsis-related immune dysfunctions. However, little is known about the regulatory mechanisms by which Tregs are kept in check during immune responses(Zhao et al., 2021).

Activated CD4 T cells are programmed to secrete cytokines with either of two distinct and antagonistic profiles. They secrete either cytokines with inflammatory (type 1 helper T-cell [Th1]) properties, including TNF-α, interferon-γ, and interleukin-2, or cytokines with anti-inflammatory (type 2 helper T-cell [Th2]) properties for example, interleukin-4 and interleukin-10(Hotchkiss & Karl, 2003).

Sepsis remains the leading cause of death in most intensive care units. Advances in understanding the immune response to sepsis provide the

opportunity to develop more effective therapies. The immune response in sepsis can be characterized by a cytokine-mediated hyper-inflammatory phase, which most patients survive, and a subsequent immune-suppressive phase(Boomer et al., 2014). Patients fail to eradicate invading pathogens and are susceptible to opportunistic organisms in the hypo-inflammatory phase. Many mechanisms are responsible for sepsis-induced immuno-suppression, including apoptotic depletion of immune cells, increased T regulatory and myeloid-derived suppressor cells, and cellular exhaustion(*Boomer et al., 2014*). In past clinical trial for sepsis focus on granulocyte macrophage colony stimulating factor and interferon gamma, immune-therapeutic agents that boost patient immunity. Immuno-adjuvants with promise in clinically relevant animal models of sepsis include anti-programmed cell death-1 and interleukin-7. The future of immune therapy in sepsis will necessitate identification of the immunologic phase using clinical and laboratory parameters as well as biomarkers of innate and adaptive immunity(*Boomer et al., 2014*).

1.2.8 Diagnosis of sepsis

1.2.8.1 Bacterial infection

Clinical differentiation of Gram-positive and Gram-negative sepsis is often attempted but seldom successful, There are situations, however, where it is possible to predict the type of infecting organism, Septic shock may be related to a focal infection, such as necrotizing fasciitis, which is known to be associated with a particular bacterial species, in this case Streptococcus pyogenes. Similarly, there may be clues from pathognomonic rashes, for example, streptococcal and staphylococcal toxic shock syndromes and meningococcemia (Sriskandan & Cohen, 1999).

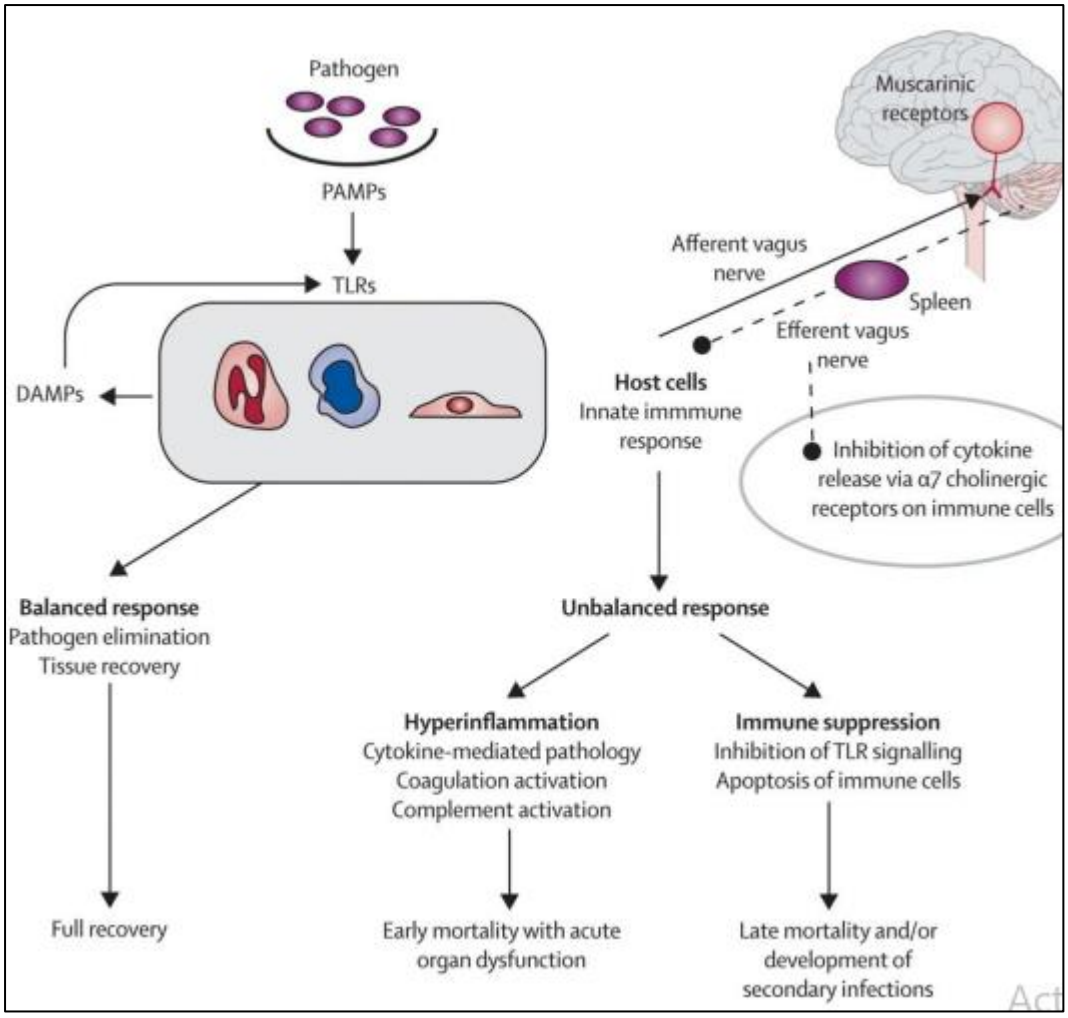

Figure1.2: Neuro-endocrine-immune inflammatory response in sepsis. (Reprinted from van der Poll et al. with permission from Elsevier)*(Vincent & Beumier, 2013)*.

The medical literature that deals with invasion of the bloodstream by microorganisms has been dominated by studies that have emphasized selected aspects of this major clinical problem, Bacteremia and fungemia have been described in many patients with conditions resulting in compromised host defenses(Ross et al., 2021).

Diagnosis of bacterial septicemia is done by culturing blood of patients. Specimens of blood then will be inoculated into various blood culture media depending on which microorganism is suspected.

1.2.8.2 Immunological marker

In this regard, more than 100 distinct molecules have been identified and evaluated for the diagnosis and identification of patients at risk for a poor outcome (Marshall et al., *2003)*. Most of them are still at an experimental state, but some are now being used in daily practice(Pierrakos & Vincent, 2010). For example, C-reactive protein (CRP), produced by hepatocytes upon IL-6 stimulation, is used since many years in detecting patients with infections, but its specificity has been a matter of debate(Clyne & Olshaker, 1999) (Povoa et al., 2005)- (Schmit & Vincent, 2008)..Another marker of infection is procalcitonin (PCT), proposed as a more specific and better prognostic marker than CRP [(Nakamura et al., 2009),(Luzzani et al., 2003) *196]*. Nowadays, PCT is used to direct the duration of antibiotic use (Riedel, 2012),(Schuetz et al., 2012). Below the details of biomarkers that used in current study.

1.2.8.2.1 Presepsin

Presepsin is new biomarkers tested for acute infections with different diagnostic and prognostic value Cluster of differentiation 14 (CD14) is a glycoprotein expressed on the membrane surface of monocytes and macrophages and serves as a receptor for lipopolysaccharides (LPSs) and LPS-binding proteins (LPBs). By activating a proinflammatory signaling cascade on contact with infectious agents, CD14 has a role as a recognition molecule in the innate immune response against microorganisms. During inflammation, plasma protease activity generates soluble CD14 (sCD14) fragments. One of them, called sCD14 subtype (sCD14-ST), or presepsin, is normally present in very low concentrations in the serum of healthy individuals and has been shown to be increased in response to bacterial infections*(*Henriquez-Camacho & Losa, *2014)*.

CD14 is the receptor of lipopolysaccharide-lipopoly-saccharide binding protein (LPS-LBP) complexes(*Zou et al., 2014)*. With the help of thinositol lipid structure, the carboxyl terminus of the molecule anchors in cell membrane and transducts the endotoxin signal through the Toll-like receptor-4, A series of downstream tyrosine protein kinases and mitogen-activated protein kinase are gradually activated including the nuclear transcription factor NF-κB, thus leading to the release of cytokines such as tumor necrosis factor-α, IFN-γ, IL-1β, IL-8 and IL-6.Subsequently, the activation of the secondary inflammatory cascade and acquired immunity stimulate mononuclear macrophages, neutrophils and endothelial cells to release more cytokines and cell adhesion molecules*(Epelman et al., 2015).* This could trigger intense and excessive systemic inflammatory response and activate the coagulation and fibrinolytic systems, resulting in SIRS, sepsis shock, disseminated intravascular coagulation (DIC), and multiple organ dysfunction syndrome (MODS), LPS, and is mainly expressed on the cell surface of monocytes/macrophages or distributed a little bit on the cell surface of neutrophils*(*Thomas *et al., 2020).*

1.2.8.2.2 Calprotectin

Neutrophil granulocytes react to bacterial infections and are one of the first-responders to bacterial infections. When the neutrophil is activated, it releases calprotectin, one of the most abundant proteins in the neutrophil cytosol. Calprotectin consists of two subunits, S100A8 and S100A9. S100A8 has a molecular weight of 10.8 kDa, while S100A9 has a molecular weight of 13.2 kDa. This biomarker increases within hours in response to bacteria or endotoxin. Determination of serum calprotectin has been proposed as a valuable marker of acute appendicitis, rheumatoid arthritis and sepsis. Early release of calprotectin and rapid test turn-around-times suggest that calprotectin can become a useful biomarker with widespread clinical benefits*(*Larsson *et al., 2020).*

Chapter two Materials and Method

2.1. Study design & setting:

This is a case - control study done from period of November (2021) to April (2022) at Al-Hussein teaching hospital / intensive care unit (ICU) in Karbala city /Iraq.

2.2. Subjects:

A forty clinically-diagnosed patients with adult sepsis were included in the study. The patients were divided into 3 subgroups according to severity level of sepsis depending on clinician diagnosis (sepsis, sever sepsis & septic shock). Sofa score for each patient was calculated using on line omni calculator (Aakre *et al., 2017*) .

The patients were (20) males and (20) females with age ranged between (17-70). In addition to 40 healthy control participants with matched age and gender for patient group. Detailed case information sheets involving age, gender and other variables were carried out for each patient by a questionnaire Case No.:

Name, Age, gender. Residency: Rural, Urban. Past surgical history, Duration of ICU stay, Antibiotic Rx, Blood culture result, Before antibiotic, after antibiotic growth results, antibiotic sensitivity test.

2.3. inclusion and exclusion criteria:

Any case with clinical diagnosis of septicemia were included within study. Exclusion criteria include: if a different diagnosis was documented, pulmonary thromboembolism, burns, acute pancreatitis, anaphylaxis, adrenal

insufficiency, thyrotoxicosis & alcohol withdrawal. For control group other causes of inflammation as other type of infection & autoimmune diseases were excluded from study.

2.4. Ethical Approval:

Patients' relatives involved in this study were informed about detailed aim of study and the agreement was obtained verbally from each one before the collection of samples. This study was approved by the Scientific Council of Karbala Medical college the ethical committee for publication ethics at college of medicine in addition to approval pf Karbala Health Directorate.

2.5. Blood sample collection

The specimen was collected from participants after proper disinfection of the skin over the vein and using a fresh sterile syringe for each vein puncture a 10 ml of blood was taken from each patient, 7 ml of blood in culture bottle for identification of bacteria, 3 ml blood in gel tube to obtain serum from patients then centrifugation 1000 x g (or 3000 rpm) for approximately 15 minutes to obtain serum stored in freeze at -20 °C to use it for immunological markers tested by ELIZA.

2.6. Materials

2.6.1. Equipment and Instruments:

In the present study, the following Equipment and Instruments were used (table 2.1 and table 2.2).

Table 2.1 Devices and Instruments with their Manufacturing company and country of origin.

Equipment and Instruments	Manufacturing Company	Country
Autoclave	Hirayama HVE-50	Japan
ELISA Devices (washer & reader)	Bio kit ELx800	U.S.A
Freezer	Panasonic	Korea
Hematology analyzer	Sysmex XN 350	Japan
Incubator	Memmert	Germany
Refrigerator	Panasonic	Korea
Vortex	Clay adams	Germany
Water bath	GFL	Germany
Water distillatory	GFL	Germany
Vitek -2 system Compact Auto analyzer	Biomerieus	France
Bact Alert	Biomerieus	France

Table 2.2: Equipment and Instruments with their country of origin

Equipment and Instruments	Country
Cold medical box	China
Cylinders (250,500 ml)	Germany
EDTA tube	China
Eppendorf tube (0.5 ml & 1.5 ml)	China
Filter paper	China
Flasks (different size)	China

Gel and Clot Activator Tube	China
Gloves	China
Micropipettes (different size)	Japan
Tips (Yellow & Blue)	China

Table 2.3: The Culture Media of this study include:

Medium	Manufacturer	Orgin
Blood Agar Base	Himedia	India
Brain Heart Infusion broth	Himedia	India
MacConkey Agar	Himedia	India
Nutrient Agar	Himedia	India
EMB	Himedia	India
Mannitol salt agar	Himedia	India

2.6.2. ELISA Kit:

Table 2.4 shows ELISA kits used in study.

Table 2.4: ELISA Kits

ELISA Kit	Manufacturing Company	Country
Presepsin	Bioossoy technology laboratory	USA
Calprotectin	Bioossy technology laboratory	USA

2.6.2.1. ELISA Kit Contents of Presepsin:

Presepsin ELISA Kit content of current Study found in table 2.5.

Table 2.5: ELISA kit for detection of human Presepsin

Components	Quantity (96T)	Quantity (48T)
1.Standard solution (1280ng/L)	0.5 ML X 1	0.5MI X 1
2. pre-coated plate	12 *8 Well strips x 1	12 *4 well strips x 1
3. standard diluent	3 ml x 1	3 ml x 1
4.streptavidin -HRP	6 ml x 1	3ml x 1
5. Stop Solution	6 ml x 1	3ml x1
6. Substrate solution A	6ml x1	3mlx 1
7. Substrate solution B	6ml x 1	3ml x1
8.Wash Buffer cocentrate (25)	20ml x 1	20ml x 1
9. Biotinylated human PSPN Antiody	1ml x 1	1ml x1
10.User instruction	1	1

11.Plate sealer	2 pics	2 pics
12.Zipper bag	1pic	1 pic

2.6.2.2. ELISA Kit Content of Human Calprotectin:

Calprotectin ELISA Kit content Of Current Study found in table 2.6

Table 2.6: ELISA kit for detection Human Calprotectin

Components	Quantity (96T)	Quantity (48T)
1.standard solution (640 ng/ ML	0.5ml x1	0.5x1
2.Pre coated ELISA plate	12*8 well strips x1	12*4 well strips x1
3. standard Diluent	3mlx1	3x1
4. streptavidin HRP	6 mlx1	3x 1
5. Stop solution	6 mlx 1	3x1
6. substrate solution A	6mlx1	3x1
7. Substrate solution B	6mlx1	3x1
8. Wash buffer concentrate (25x)	20 mlx1	20 x1
9. biotinylated human CAL antibody	1mlx1ml	1mlx1 ml
10. User instruction	1 ml	1 ml
11. plate Sealer	2 pics	2 pics
Zipper bag	1 pic	1pic

2.7. Methods

2.7.1. Isolation and Identification of Bacteria

Bacterial isolates were detected by inoculating 7ml of blood into a culture bottle, which was then incubated at 37°C for up to 7 days until the final report.

Subcultures were made from all bottles either negative or positive (both bottles which showed signal or not), Brain Heart Infusion broth was utilized for bacterial resuscitation, and blood agar and MacConkey agar were also employed for bacterial identification. They were incubated at 37°C overnight. Each bacterial isolate in the study was subjected to phenotypic bacterial identification, which included colony characterizations, and Gram-stain morphology to distinguish between Gram-positive and Gram-negative bacteria and check the isolate's purity, and bacterial biotyping and identification at the species level using the Vitek-2 system (BioMerieux).

Using a sterile swab, 1-2 colonies from each isolate's pure culture were collected and suspended in a specific transparent plastic polystyrene test tube (a 12 x 75 mm) given by the manufacturer company containing 3 ml sterile saline (aqueous 0.45 percent to 0.50 percent NaCl, pH 4.5 to 7.0) and vortexed

Bacteria cultured in this study *Staphylococcus areus* gram stain show purple cocci found in cluster gram positive cocci. Staphylococcus areaus show hemolysis in blood agar and grow on manitol salt agar which ferment manitol with acid production and change agar color to yellow, also these bacteria give positive result for coagulase and catalase tests. Some patient in this study show staphylococcus epidermises which similar to *staphylococcus areaus* in shape, color and hemolysis on blood agar but differ in some important properties, when grow on manitol salt agar not fermented the manitol and no change the colour of media, this bacteria give negative result for coagulase test, *Salmonella Typhi* show on MaCconkey agar non lactose ferment, Pale yellow .

2.7.2. VITEK 2 compact system was performed following the manufacturer's instructions by several steps as the following:

1-The study isolates that reveal positive result for biochemical tests and identified by API 20E cultured on MacConkey agar and blood agar and incubated at 25°C for 24 hours after the incubation a slide of the isolates were stained by Gram's stain to verify the Gram-negative reaction beside checking the purity of bacteria isolates.

2-By using a sterile swabs 1-2 colony taken from the culture grown on blood agar 24 hours and suspended in special clear plastic polystyrene test tube (a 12 x 75 mm) of manufacturer company containing 3 ml sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) supplied by the company and mixed by vortex.

3-The turbidity of the bacterial suspension was adjusted according to the turbidity of standard table turbidity range via turbidity meter (DensiChek TM) supplied by the company in which the final concentration of the suspension should be prevailed between 0.5-0.63 which is required for Gram negative Identification card.

4-inoculation of Gram negative identification card the card was warmed to room temperature, the test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The filled cassette is placed into filling chamber (a vacuum chamber station). After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced (by negative pressure) through the transfer tube into micro-channels that fill all the test wells after finishing the operator given blue signal.

5-Bar Code Scanner for the ID cards was done for Data Entry.

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the incubator chamber. All card types are incubated on-line at 35.5 + 1.0ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings (measure either turbidity or colored products of substrate metabolism.), and then returned to the incubator until the next read time. Data are collected at 15-minute intervals during the entire incubation period.

Our test data were compared to the respective database in the computer attached to the VITEK 2 operator system.

2.7.3. Preparation of Solutions and Reagent

2.7.3.1. Gram stains solutions

The solutions were prepared according to the required microbiological methods. The solutions included: four solutions crystal violate, iodine, absolute alcohol and safranine.

2.7.3.2 Blood Agar Medium:

Blood agar medium performed according to manufacturer by dissolving 40 gm blood agar base in 1000 ml D.W. The medium was autoclaved at 121ºC for 20 minutes, cold to 45ºC and 5% of fresh human blood was added. It was used as enrichment medium for the bacterial isolates and to determine their ability to hemolysis RBCs .

2.7.3.3 MacConkey Agar Medium:

This medium can be prepared by dissolving 40gm of agar in 1000 ml of D.W and then sterilization in autoclave at 121C°for 20 minutes, this type of media used selective gram-negative media .

2.7.3.4 Brain heart infusion (BHI) broth:

Brain-heart infusion broth was made according to the manufacturing company by dissolving 37 gm in 1 liter of distilled water and autoclaved at 121ºCfor 20 minutes, used for bacterial reactivation if necessary.

2. 7.5. Biomarkers Profile Assay by ELISA

Serum level of Presepsin & Calprotectin was determined by classic sandwich-ELISA using ELISA research kits.

2. 7.5.1 Principle of Sandwich ELISA technique

Antibody-sandwich ELISA may be the most useful of the immune sorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid.To detect antigen, the wells of microtiter plates are coated with specific(capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and a different antigenspecific antibody conjugated to enzyme (developing reagent) is added, followed by another incubation. Unbound conjugate is washed out and substrate is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

2.7.5.2. Assay principle (Human calprotectin & Presepsin ELISA)

This test is used the Sandwich-ELISA principle, in which the plate had been pre-coated with human Presepsin, calprotectin antibody. Presepsin or calprotectin present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human Presepsin, calprotectin antibody is added and binds to Presepsin or Calprotectin in the sample. Then streptavidin -HRP is

added and binds to the biotinylated Presepsin or Calprotectin Antibody. After incubation unbound streptavidin – HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human Presepsin or Calprotectin. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.7. 5.3. Procedure of The Test:

1. Preparation of all reagents, standard solution and samples as instructed. The assay is performed at room temperature.
2. Determination of the number of strips required for the assay. Insertion the strips in the frames for use. The unused strips would be stored at 2-8 c.
3. Addition of 50 ul standard well.
4. Addition of 40 ul sample to sample wells and then addition of 10ul anti-PSPN or anti calprotectin antibody to sample wells, then addition of 50ul streptavidin -HRP to sample wells and standard wells (Note blank control well). Mixing well. Covering the plate with sealer. Incubation for 60 minutes at 37C.
5. Removal of the sealer and washing the plate for 5 times with wash buffer. Soaking wells with 300ul wash buffer for 30 seconds 1 minute for each wash. for automated washing, aspiration of each well and wash 5 times with wash buffer. Blotting the plate onto paper towels or other absorbent material
6. Addition of 50 ul substrate solution A to each well and then addition of 50ul substrate solution B to each well incubation of plate covered with a new sealer for 10 minutes at 37 C in the dark.
7. Addition of 50ul stop solution to each well, the blue color will change into yellow immediately.

8. Determination of the optical density (OD value) of each well immediately using a microplate reader set to 450 nm with 10 minutes after added the stop solution.

2.8. Statistical analysis:

Information from the questionnaire and all test results from study groups samples were entered a data sheet. The data analysis for this work was generated using The Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA) and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020).

Descriptive statistics was performed on the data of each group. Values were illustrated by n (%) for categorical, Scale variables were presented by mean ± standard deviation for normal data while non-normal data, The distribution of the data was checked using Shapiro-Wilk test as numerical means of assessing normality. To compare between groups, we used the $\chi 2$-test, T test. ANOVA test used to compare more than 2 groups.

Biomarkers were compared using Pearson's r correlation test to evaluate the relationship within the case study.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p- values <0.05 (two-side) were considered to be statistically significant.

The optimal threshold with high specificity and sensitivity for sepsis cases was detected using receiver operating characteristic (ROC) analysis. It was found out that all the values of P were two-sided, and a $P < 0.05$ was considered to be statistically significant.

Chapter Three

Result

3.Result

3.1 Demographic & Clinical Data of The Studied Groups:

Age group distribution and gender for disease group are clarified in table 3.1. In addition, temperature mean is with in normal range, respiratory rate mean increases from reference value (26.94±7.65), systolic blood pressure (SBP) means with in reference value, Glasgow coma scale mean decrease from normal range (10.65±2.19), partial pressure of oxygen (PaO2) means slightly below normal reference value, partial pressure of carbon dioxide (PaCO2) mean increased compared to reference value (46.75±20.55). Figure 3.1 revealed the distribution of patients according to sepsis severity which shows that 50% (20) of patients presented as sepsis.

Table 3.1 Demographic & clinical characters of patient group (n= 40)

Parameter		**Mean +SD**	**Reference value**
Age (mean ± SD)		40.67±14.24	-
	Age groups	N (%)	
	17-27	9 (22.5)	
	28-38	9 (22.5)	
	39-49	10 (25)	
	50-60	8 (20)	
	61-72	4 (10)	
Gender	Male	20 (50)	
	Female	20 (50)	
Temperature		37.11±0.20	36.5-37.2 C
Respiratory rates/min.		26.94±7.65	12-16
HR (heart rate)/min		68.07±25.83	60-100
Systolic blood pressure (SBP) mmHg		118.15±31.69	<120
Urine output cc/day		1440±588.69	800-2000
Glasgow coma scale		10.65±2.19	13-15

Partial pressure of oxygen (PaO2)	74.41±28.56	75-100(mmHg)
Partial pressure of carbon dioxide (PaCO2)	46.75±20.55	38-42(mmHg)

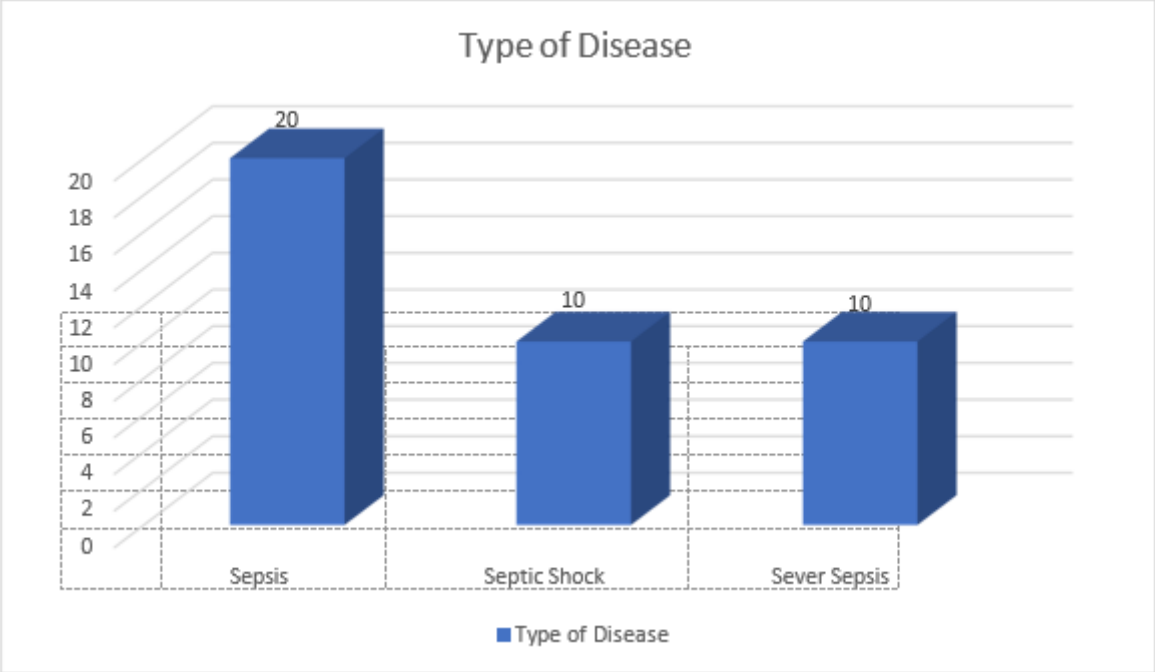

Figure (3.1): Type of disease

3.2 Laboratory Investigations for Inflammatory Parameters of Patients with Sepsis:

Table 3.2 showed that Serum level of patients showed elevation in C. reactive protein levels also Random blood sugar test of patients revealed elevation in glucose level while the mean of hemoglobin and hematocrit were within normal range and not increased.

Normal plasma level of (PT, PTT &INR) were recorded. Total white blood cell counts were high in contrast lymphocytes were near lower limit in all patients mean, while serum level of both (serum creatinine &blood urea tests) which performed to estimate renal function were found high. Additionally,

Serum level of liver enzyme (ALT, AST, total bilirubin) were within normal range and no increased in their mean levels.

Table 3.2 Laboratory Parameters of Patients of The Study:

Parameter	Mean +SD	Normal reference range
C.reactive protein (mg\l)	13.44±53.69	8-10
Random blood sugar (mg\dl)	157.55±76.84	80-140
Hemoglobin (g\l)	11.49±1.47	13.8-17.2 for male 12.1-15.1 for female
Hematocrit %	38.35±48.4	36-50
Platelet count $\times 10^9$ /L	368.17±122.2	150-450
Partial thromboplastin time PTT	12.03±3.52	25-35
INR (international normalized ratio) %	0.348±0.46	<1.5
White blood cells Counts $\times 10^9$/L	24.56±10.54	4.5-11.0
Lymphocyte count$\times 10^9$/L	1.89±1.65	1-4
serum creatinine (mg/dl)	4.63±7.4	0.5-1.2
B. urea (mg/dl)	55.10±27.4	5-20
Total bilirubin(mg/dl)	0.502±1.002	0.1-1.2
Aspartate Aminotransferase (AST) U/L	17.31±5.28	8-48
Alanine transaminase(ALT) U/L	16.5±10.37	7-55

-

3.3 Calprotectin & Presepsin levels in Study Group:

Table 3.3 showed that calprotectin and presepsin levels in patient group were elevated compared to control group which is significant for calprotectin (P=0.019) but did not reach statistical significance for presepsin.

Table 3.3 Calprotectin & Presepsin levels in study group:

Parameter	**Mean +SD Patient group)(**	**Mean+ SD (Control group)**	**P-value**
Calprotectin (ng)	69.33±45.59	49.03±28.45	0.019*
Presepsin (ng)	255.71±357.3	137.53±180.2	0.066

T-student test, Results Are Presented As Mean ± Sd, P<0.05 Considered -Significantly Different.

3.4. Diagnostic Utility for Calprotectin & Presepsin

ROC curve analysis revealed that both biomarkers had good specificity percent for diagnosis of sepsis 77.5%, 80% for presepsin and calprotectin respectively. Areas under curve were 75.8%, 65% for presepsin and calprotectin respectively Presepsin had highly significant diagnostic utility with p-value<0.001, while calprotectin had p-value of 0.02 which is also significant as shown in table 3.4 & figures 3.2.,3.3.

Table 3. 4: Area Under the Curve, optimal threshold, Sensitivity and specificity of Presepsin1 & Calprotectin levels obtained by the ROC curves Patient

Test Variable	AUC	P Value	Sensitivity %	Specificity %	Cut-Off Points	Youden Index	CI (95%)
Presepsin	75.8 %	<0.001 *	67.5%	77.5%	116.70	0.45	0.651 - 0.866
Calprotectin	65%	0.021*	55%	80%	64.98	0.35	0.528 -0.772

- significant

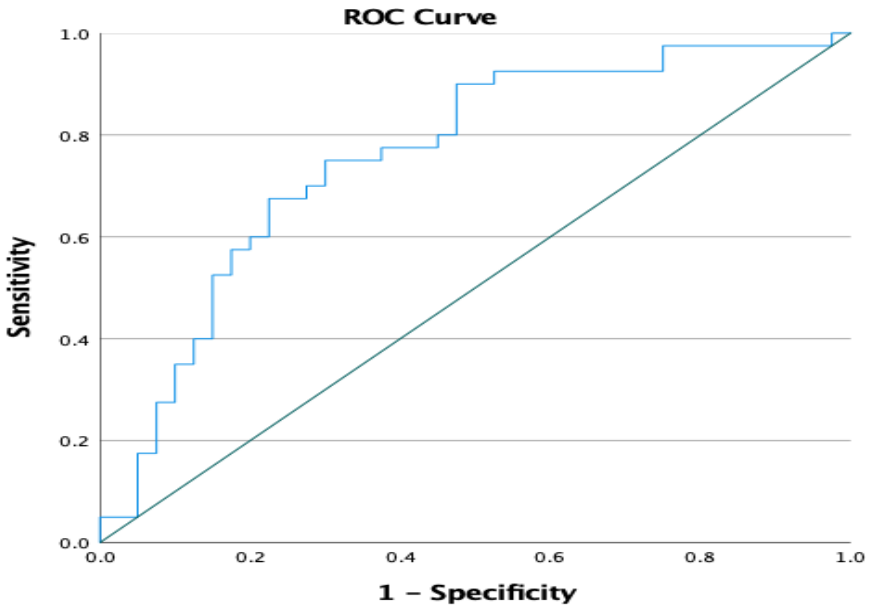

Figure 3.2: ROC curves for Presepsin1 in patients group to analyse the optimal diagnostic points for predicting of sepsis cases compared to control group.

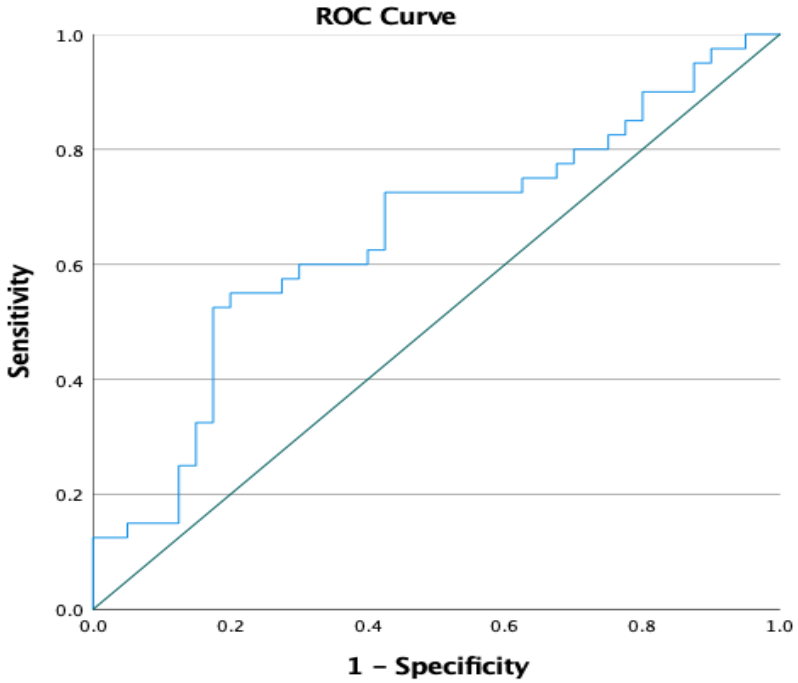

Figure 3.3: ROC curves for Calprotectin in patients group to analyse the optimal diagnostic points for predicting of sepsis cases compared to control group.

3.5 Calprotectin & Presepsin serum levels in subgroups of sepsis:

Table 3.5 showed no significant differences in Calprotectin & Presepsin levels between subgroups of sepsis. Although presepsin increases in sever sepsis, (511.88±161.87) whereas calprotectin levels were more in septic shock group (59.53±18.82).

Table 3.5 Calprotectin & Presepsin serum levels in subgroups of sepsis:

variables	(N=40)			P Value
	Sepsis N= 20	Septic Shock N= 10	Sever Sepsis N= 10	
Presepsin	137.96±30.85	476.62±150.72	511.88±161.87	0.44
Calprotectin	36.40±8.14	59.53±18.82	49.75±15.73	0.67
ANOVA test, Results Are Presented As Mean ± Sd, P<0.05 Considered Significantly Different,				

3.6 Calprotectin & Presepsin serum levels among sofa score in patients' groups:

Table 3.6 clarified association between Calprotectin & Presepsin serum levels and sofa score in patient's groups, which revealed that calprotectin serum levels means were (67.99±47.9, 69.23±41.1, 77.69±55.6) from the lowest to highest score (no significant , P =0.929). Whereas mean Presepsin levels were (312.8±448.8, 153.3±89.9, 220.02±125.7) respectively and again non statically significance is obtained (P=0.452).

Table 3.6 Calprotectin & Presepsin serum levels among sofa score in

Sofa score	N (%)	Calprotectin mean± SD	P value	Presepsin Mean± SD	P value
0-6	24(60)	67.99±47.9	0.929*	312.8±448.8	0.452
7-9	12(30)	69.23±41.1		153.3±89.9	
10-12	4(10)	77.69±55.6		220.02±125.7	

ANOVA test, No significant difference at P<0.05 *

3.7 Etiological pathogens distribution among patients' group:

As shown in table 3.7 etiological pathogen distribution for patient group after blood culturing were as follows: *Staph aureus* 3(7.5%), *Staphylococcus epidermidis* 4(10%), *Salmonella typhi* 16(40%) & Candida 1(2.5%).

Table 3.7 Etiological pathogens distribution for patients' group

Pathogen	N (%)
Staph aureus	3(7.5)
Staphylococcus epidermidis	4(10)
Salmonella typhi	16(40)
Candida	1(2.5)
No growth	16(40)

3.8 Blood Culture Results According to The Sepsis Clinical Course of Patients:

In Sepsis group blood culture result was positive growth in 12 (30%) and 8(20%) sample was negative, while in the sever sepsis blood culture result was positive growth in 3(7.5) and 7 (17.5%) was negative .The blood culture result of the septic shock was positive in 9 (22.5) and only one patient (2.5%) was growth negative. These results showed a significant statistical significance (P=0.04). as illustrated in table (3.8) the significance is between sever sepsis and septic shock group where significant most of patients had positive bacterial growth.

Table 3.8 Blood culture results according to the sepsis clinical course of patients of the study:

Sepsis clinical course	Blood culture result		P-value
	Growth positive N= 24	Growth negative N=16	
Sepsis	12(30%)	8(20%)	0.040* -Sepsis Vs sever septic =0.121 -Sepsis Vs septic shock=0.90 -Sever sepsis Vs septic shock= **0.006***
Severe sepsis	3(7.5)	7(17.5%)	
Septic shock	9(22.5)	1(2.5%)	

Chi-square test, Significant difference at P<0.05

3.9Type of bacterial isolates from patients according to the sepsis severity:

The frequency and distribution of bacterial species according to subgroups of sepsis, severe sepsis and septic shock were as follows: for *Staphylococcus epidermidis* (n = 4), isolated from one (20%) blood samples in a patient with severe sepsis course and three (37.5%) isolates from blood samples in patients with septic shock, *Staphylococcus aureus*(n = 3), three (30%) isolates of *Staphylococcus aureus* isolated from blood samples in a patient with sepsis course and no isolates from the two other subgroups. *Salmonella typhi* (n = 16), isolated in 7 blood Samples (70%) isolates from sepsis,4(80%) isolates in severe sepsis and 5 (62.5%) isolates from septic shock patients as illustrated in table (3.9). There is no significant variation between the percentages of isolates between the sepsis clinical course subgroups.

Table3.9: Type of bacterial isolates from patients according to the sepsis clinical course of the patients of the study:

Type of bacterial isolates	Total	Sepsis	Severe sepsis N. (%)	Septic shock N. (%)	P-value
Staphylococcus epidermidis	4	0(0%)	1(20%)	3(37.5%%)	0.105
Staphylococcus aureus	3	3(30%)	0(0%)	0(0%)	
Salmonella typhi	16	7(70%)	4(80%)	5(62.5%)	

3.10 Distribution of Gender and Age Groups Based on Results of Bacterial Growth:

The present study's findings showed that both sexes (men and females) were liable to bacterial infection with males having the higher percent of infection (66.7%). When the data of bacterial growth analyzed compared to the age groups there was no significant variation between the age intervals of study patients, but the age range of (28 to 53) years were the most associated with infection as recorded in table (3.10).

Table (3.10) Distribution of gender and age groups based on results of bacterial growth

Variable	Category	Bacterial Growth		P-Value
		Positive Growth N= 24	Negative Growth N=16	
Gender	Male	16(66.7 %)	4(25%)	0.022*
	Female	8(33.3%)	12(75%)	
Age	15 - 27 Years	5(20.9%)	4(25%)	0.956
	28 - 40 Years	8(33.3%)	5(31.25%)	
	41 - 53 Years	8(33.3%)	4(25%)	
	More Than 54 Years	3(12.5%)	3(18.75%)	

Chi-square test, Significant difference at P<0.05 *

3.11 Association of Calprotectin & Presepsin Serum levels with Bacterial Growth:

A non-significant association was found between bacterial growth and serum levels of both biomarkers. Although higher levels of presepsin were present in patients' group (245.73±347.46) as in table 3.11. table 3.12 compared the specific type of pathogen with levels of

markers and again non-significant result obtained, but with higher level of presepsin & calprotectin in *S. typhi* group (225.56±137.10, 91.98±51.04) respectively.

Table 3.11: Mean differences of Presepsin & Calprotectin in patients group according to bacterial growth

Biochemical Parameters	(N=40)		P Value
	No Growth N= 16	Growth N= 24	
Presepsin	269.21±380.58	245.73±347.46	0.84
Calprotectin	62.83±45.40	74.15±46.15	0.45
t-student test, Results Are Presented As Mean ± Sd, P<0.05 Considered Significantly Different,			

Table 3.12: Mean differences of Presepsin & Calprotectin among Patients Group with Different types of Pathogen

Biochemical Parameters	(N=23)			P Value
	Coagulase Negative Staph N= 4	Candida Albicans +Staph Aureus N= 4	Salmonella Typhi N= 16	
Presepsin	167.45±155.82	114.05±28.41	225.56±137.10	0.37
Calprotectin	78.07±70.11	52.64±19.18	91.98±51.04	0.48
ANOVA test, Results Are Presented As Mean ± Sd, P<0.05 Considered Significantly Different,				

3.12 Mortality Rate & Outcome Among Patients' Group:

A non-significant result for association of biomarkers levels and outcome were obtained as shown in table 3.13. in addition, Table 3.14 showed the Mortality rate association with sofa which is non-significant also (P=1.0), although the higher percent of death is associated with the lowest score.

Table 3.13: Mean differences of Presepsin & Calprotectin among patients group with different outcomes

Biochemical Parameters	(N=40)			P Value
	Dead N= 7	Healing N= 20	Hospital Care N= 13	
Presepsin	220.58±180.24	310.83±478.54	189.82±159.98	0.62
Calprotectin	49.53±19.99	76.96±57.76	68.27±30.77	0.39
ANOVA test, Results Are Presented As Mean ± Sd, P<0.05 Considered Significantly Different,				

Table 3.14 Mortality rate:

Sofa score	**N (%)**	**Outcome Mortality N (%)**	**P value**
0-6	24(60)	5(20.83)	1.03
7-9	12(30)	1(8.33)	
10-12	4(10)	1(25)	
Total	40	7(17.5)	

Chi-square test, No significant difference at P<0.05

3.13 Pearson's correlation analysis of biomarkers & clinical characteristics in patients' group:

Using pearson correlation to find any association between markers revealed positive significant correlation between calprotectin and CRP, also between presepsin and urine output p-value were = 0.006,0.019 respectively. All other correlation were non-significant as demonstrated in table 3.13.

Table 3.15: Pearson's rcorrelation analysis of biomarkers in patients with sepsis disease.

Paramrters	Calprotectin	Presepsin
Pao2	r=0.1 P=0.81	r=0.3 P=0.10
Paco2	r=0.1 P=0.64	r=0.1 P=0.60
CRP	r=0.4 **P=0.006**	r= -0.1 P=0.77
RBS	R= -0.1 P=0.87	R=0.1 P=0.81
HB	R= -0.1 P=0.06	R= -0.2 P=0.30
Hematocrit	R=0.2 P=0.92	R=0.2 P=0.31
WBC	R= -0.1 P=0.98	R=0.1 P=0.74
Lymph	R= -0.1 P=0.69	R=-0.1 P=0.86
S .Creatinine	R=0.1 P=0.73	R= -0.2 P=0.31
B.Urea	R=0.1 P=0.72	R=0.3 P=0.10
Total Bilirubin	R=0.1 P=0.98	R=0.2 P=0.26
Temp	R= -0.2 P=0.36	R= -0.1 P=0.78
GCS	R= -0.1 P=0.52	R=0.1 P=0.73
Urine Out Put	R= -0.1 P=0.88	R=0.4 **P=0.019**
SBP	R= -0.2 P=0.25	R= -0.1 P=0.87
HR	R=0.1 P=0.98	R=0.2 P=0.20
RR	R= -0.1 P=0.59	R= -0.1 P=0.88
PLT	R= -0.1 P=0.88	R= -0.3 P=0.12

AST	R=0.3 P=0.08	R= -0.1 P=0.82
ALT	R= -0.1 P=0.60	R= -0.2 P=0.21
PTT	R=0.2 P=0.21	R=0.1 P=0.74
INR	R=-0.1 P=0.43	R=0.1 P=0.46

Chapter Four
Discussion, Conclusion& Recommendations

4.1 Discussion:

4.1.1. Demographic, clinical data and Laboratory parameters of the studied groups:

Present study data include: temperature mean is with in normal range, respiratory rate mean increase from reference value which is against what was found by previous study (Gadomski *et al., 1994).* This could be explained on bases of time of recording readings which can affect means. systolic blood pressure (SBP) mean with in reference value, Glasgow coma scale mean decrease from normal range (10.65±2.19) which is similar to other past studies (Seymour et al., 2010). Partial pressure of oxygen (PaO2) mean slightly below normal reference value, partial pressure of carbon dioxide (PaCO2) an increased compared to reference value (46.75±20.55) this result is expected as saturation of O2 is affected in sepsis and resul is in agreement of past studies (Gadrey et al., 2019).

Laboratory investigation of patients in current study founded increment in C.reactive protein levels also random blood sugar test of patients. This result similar to past studies(Rhee *et al.,* 2019). Stress is well known to cause high blood sugar values and CRP increase as non-specific inflammatory marker which increase in any inflammatory process. While the mean of hemoglobin and hematocrit were within normal range and not increased. This result is in disagreement with other study that showed low means for Hb and hematocrit (Obaid et al., 2020). This difference is attributed to many causes as sample size, cause of sepsis whether surgical or medical with long course of chronicity which can lead to anemia of chronic diseases.

Normal plasma level of (PT, PTT &INR) were recorded, this is similar to what was found by Rosenqvist (2021). Total white blood cell counts were high, which in agreement with Mayr et al. study (Mayr et al., 2014). Elevated WBC count is indicator of inflammation and infection that occur in sepsis. In Contrast

lymphocytes were lower in all patients; that is similar to Kolesnichenko *et al*. (2021). This result could indicate the etiology of underlying microorganism as bacterial infection or in some cases of viral infection. Serum level of both serum creatinine &blood urea tests which performed to estimate renal function were found high in the mean of the patients. This increment reflects the state of renal failure as part of multiple organ failure in sepsis. The result consistent to other study (Pepic et al., 2021).

Additionally Serum level of liver enzyme (ALT, AST, total bilirubin) were within normal range and no increased in their mean levels, which is in contrast to past studies results (Kumalo et al., 2016*).* This could be due to small sample size of present study.

4.1.2.Calprotectin & Presepsin serum levels in study group:

The current study founded that levels of calprotectin in patient group were significant (P=0.019) compared to control group while non-significant result obtained for presepsin (P=0.066). this could point the role of calprotectin in inflammatory process of sepsis. Calprotectin is one of the earliest biomarkers for detection of the inflammatory response to infections especially in sepsis (Jonsson *et al., 2017).*

This result is consistent with past studies for measurement of serum levels of calprotectin in neonates and adult (Decembrino *et al, 2015),* (Wirtz et al., 2020).

Current finding also declares that both markers were found to have good diagnostic utilities for differentiating sepsis patients from control group. this result is similar to study of Larsson et al, 2019 which found that the AUC for calprotectin was 0.79. Other study showed that calprotectin had a sensitivity of 62.5% and a specificity of 69.7% in neonate with sepsis which is near results of

current study taking in consideration differences in age groups of sepsis (Decembrino *et al., 2015).*

Other studies also showed that presepsin levels were significantly elevated in sepsis with good diagnostic utility(Aliu-Bejta et al., 2020), while our study fail to achieve the statistically significant level although levels were also elevated in patients' group. this could be due to small sample size.

This study also showed that diagnostic utility of presepsin was significant for differentiating patients from control group. this result went with past studies which conclude the importance of Presepsin as a new biomarker and play a crucial role as a supplemental method in the early diagnosis of sepsis(Zou et al., 2014), *(*Lee *et al., 2022*),(Velissaris *et al., 2021*).

Zhang et al.2015 revealed that The overall diagnostic sensitivity of presepsin for sepsis was 0.83 (95% CI: 0.77–0.88), and specificity was 0.78 (95% CI: 0.72–0.83). The AUC was 0.88 (95% CI: 0.84–0.90). These results when compared to current data which are specificity of 77.5%, sensitivity of 67.5% and AUC= 75.8 showed a clear similar finding.

When comparing levels of Calprotectin & Presepsin in subgroups of sepsis, no significant differences were found. Although presepsin increases in sever sepsis, (511.88±161.87) whereas calprotectin levels were more in septic shock group (59.53±18.82). The study of Wirtz et al., 20202 revealed that high concentration of calprotectin at admission to ICU is associated with more severity of sepsis (Siljan *et al., 2019).*

Regarding studies for presepsin which found that study conclude higher levels in patients with septic shock than in those with sepsis ($p = 0.002$) (Lee *et al., 2022*). Also, similar findings found by Aliu-Bejta et al,2020, study with a conclusion that Presepsin had a good diagnostic ability to differentiate septic shock from sepsis in the study groups.

Regarding Calprotectin & Presepsin serum levels among sofa score subgroups of patient's groups, a non-significant result obtained. Although higher levels of presepsin were within the lowest score i,e the worst sepsis state. Calprotectin levels in contrast is higher in the highest score this could be explained as mentioned above depending on admission date when it is high at admission this lead to bad prognosis and low sofa score and vice versa (Siljan et al., 2019). Past study showed that Higher values of presepsin were observed in septic patients at presentation (time 0)(*Ulla et al., 2013)*. This is one of the drawback on current study, it doesn't correlate serum markers levels with duration of admission. Aliu-Bejta et al ,2020 study found a strong correlation of presepsin with SOFA score ($p < 0.0001$) .

4.1.3. Etiological Pathogens Distribution Among Patients' Group:

An important role in the etiology of sepsis in adults among present study was played by *Salmonella typhi (n*=16) which was the prominence bacterial pathogen followed by and *Staphylococcus epidermidis*, *Staphylococcus aureus* (n=3) n=4 and *candida* (n=1)

Ramachandran reported that some of the most frequently isolated bacteria in sepsis are *Staphylococcus aureus ,Streptococcus pyogenes*, *Klebsiella spp*., *Escherichia coli ,salmonella* and *Pseudomonas aeruginosa*.(Ramachandran, 2014) . Also several bacterial etiological surveys studies for sepsis revealed that various species of *Klebsiella, Enterobacter, Acinetobacter*, and *Pseudomonas, E. coli*, *salmonella* and *S. aureus* are major sepsis etiologies with regional variation(Legese et al., 2022). These microorganisms are more prevalent among all causative pathogens, present study percents are similar to the rates in previous reports in developing and under developing countries.(Rosenqvist, 2021), (Kolesnichenko et al., 2021), (Legese et al., 2022).

In a research published in 2016, Almuhayawi reported that the proportions of gram-positive and G ram-negative bacteria that cause sepsis are often comparable. The most prevalent Gram-negative bacteria are *Escherichia coli*, *Klebsiella species*, *Pseudomonas aeruginosa*, *Enterobacter species*, and *Serratia species*, whereas the most prevalent Gram-positive bacteria are *Staphylococcus aureus*, *Enterococcus species*, *viridans streptococci*, and *Streptococcus pneumoniae*.(Almuhayawi, 2016).

In this study *salmonellae* was the highest bacterial pathogen that recorded in positive patients blood culture samples and this was in accordance with several researchers showed that *salmonella* was the main bacterial pathogen between the bacterial etiology of sepsis patients .(Gordon et al., 2008),(Shahunja et al., 2015), ,(Thompson et al., 2013), (Mishra et al., 2022)

The study found one case of candidemia. which differs markedly from other reports of fungal infections ranging from 5·9–28·3% . (Finfer et al., 2004), (Vincent et al., 2006) , (Cheng et al., 2007)

Previous research has found that Candida fungus have a considerably larger role in the development of BSI in infants and children in their first year of life than in adults , Sepsis caused by yeast is becoming more common. Currently, candidemia is thought to be the fourth most prevalent BSI in ICUs. The main pathogen causing candidemia has been identified as *Candida* . However, throughout time, the epidemiology of candidemia has altered quickly, increasing the likelihood of finding different Candida species. *(Almuhayawi, 2016; Pfaller & Diekema, 2007).* This difference in results could be due to different sample size .

The present study's findings showed that the age range of 39 to 49 years was the most impacted, and that both sexes (males and females) were receptive to

bacterial infection, with males being more responsive to infection. (Abed Mosa & Mohammed Ali, 2021)

In this investigation, *salmonella typhi* isolates, *Staphylococcus aureus*, and *Staphylococcus epidermidis* were all correctly identified using the automated system VITEK2 compact system. The ability to recognize multiple Staphylococcus species that the traditional approach is unable to distinguish is another benefit of the VITEK 2 technology. Numerous commercially available automated systems have been looked at for routine laboratory usage; VITEK 2 displays a range of coagulase-negative Staphylococci genera and species. Several peer-reviewed studies have demonstrated that automated VITEK 2 technology and VITEK 2 ID cards provide reliable and accurate findings for Gram-positive cocci and Gram-negative bacilli that are clinically significant .

Variations in microbiological patterns of sepsis, severe sepsis and septic shock may be due to patient demographic differences, comorbidities, less widespread use of broad-spectrum antibiotics, or less use of invasive therapies or procedures. Nonetheless, in our investigation, microorganisms, in sepsis, sever sepsis and septic shock were not revealed significant difference.

A non-significant association was found between bacterial growth and serum levels of both biomarkers. Although higher levels of presepsin were present in patients' group (245.73±347.46) compared the specific type of pathogen with levels of markers and again non-significant result obtained, but with higher level of presepsin & calprotectin in S. typhi group (225.56±137.10, 91.98±51.04) respectively. These results are similar to past study (Djordjevic & Dragasevic),but disagree with another study (Endo *et al., 2012*).

Different findings may be caused by a variety of variables, such as the degree of antibiotic resistance, the patient's profile, treatment guidelines, and the clinical staff members who use the information from the microbiology laboratory in patient care.

The parameters in the clinical samples might be different from those in the simulated cultures. These may include the varied blood cell composition that is often seen in clinical blood cultures, antimicrobial drugs, and the transport time of blood culture bottles.

However, it would be extremely challenging to conduct the necessary research for the detection of uncommon microorganisms like anaerobic bacteria and uncommon clinical situations like polymicrobial sepsis in prospective clinical investigations. The results from the simulated samples may likely represent how well the techniques function analytically in everyday clinical practice.

This research has certain drawbacks. First, because there was just one center, there could have been bias in regards to ICU admission, the caliber of treatment, and administration. Second, there was no attempt to assess ICU acquired infections, and third, we did not assess the treatment packages for Surviving Sepsis. Iraq should host a multicenter trial on the incidence and prognosis of severe sepsis treated with the sepsis bundle.

4.1.4. Mortality rate & outcome among patients' group:

A non-significant result for association of biomarkers levels and outcome was obtained, in addition the Mortality rate association with sofa is non-significant also, although the higher percent of death is associated with the lowest score. The overall survival was significantly impaired in septic patients with high baseline calprotectin concentrations ($p = 0.036$). Also, patients with increasing calprotectin serum concentrations within the first week of ICU admission showed an improved overall survival ($p = 0.009$) & ends with a conclusion that high calprotectin concentrations at ICU admission predict long-term mortality risk, whereas increasing calprotectin concentrations are

associated with a favorable long-term outcome*(T. Wirtz et al., 2020)*. Larsson et al study suggest similar conclusion that calprotectin concentrations at admission were higher in non-survivors than in survivors at day 30 *(Larsson et al., 2020).* Another recent study showed that patients with higher presepsin levels (≥ 821 pg/mL) had significantly higher mortality rates than those with lower presepsin levels (< 821 pg/mL) (log-rank test; p = 0.004) and could help clinicians identify patients with sepsis with poor prognosis, but conclude that Presepsin was an independent risk factor for 30-day mortality among patients with sepsis and septic shock *(Lee et al., 2022)*.

The explanation for none achieving statistical significance for association between current study biomarkers or sofa score and mortality rate could be due to difference in sample size or lack of follow up for patients as it is time consuming.

4.1.5. correlation analysis of biomarkers & clinical characteristics in patients' group:

Using pearson correlation to find any association between markers revealed positive significant correlation between calprotectin and CRP. This result is similar to study which conclude that Calprotectin concentrations strongly correlated with the C-reactive protein (p < 0.001)(*Wirtz et al., 2020*) . another study confirmed the positive relation between calprotectin and CRP (*Bartáková et al., 2019).*

Present findings showed that a positive correlation between presepsin and urine output also present. This result is consistent to what is concluded by recent study that presepsin may be a good marker for diagnosis of septic shock based on the data of admission (as urine output is one indicator of renal failure in septic shock)(Sekine *et al., 2021).*

All other correlation were non-significant, this results is in agreement with past study which conclude that most variables commonly affecting C-reactive protein and procalcitonin values do not affect presepsin levels, which suggests that presepsin could be an effective sepsis marker. *(Pugni et al., 2015).* The differences in correlation could be affected by sample size, study circumstances and follow up in addition to change in ethnic groups and genetic background.

4.2 Conclusions:

1. Calprotectin and presepsin levels elevated in adult sepsis.

2. Calprotectin & presepsin could be regarded as good biomarkers for diagnosis of sepsis. In addition, both study markers have relevant specificity for diagnosis of sepsis.

3. No association between severity of sepsis and levels of study markers.

4. Direct positive correlation between calprotectin and CRP.

5. Gram negative bacteria *Salmonella typhi* is the most bacteria resulted from blood culture of sepsis and it is associated with more severe form of sepsis which is septic shock.

6. No association between positive bacterial growth and severity of sepsis.

4.3 Recommendation:

1. Further studies with larger sample size are required for better understanding of the effect and association of Presepsin, Calpotectin with severity of sepsis, sofa score and causative pathogens.

2. comparing other inflammatory markers such as procalcitonin with study markers is highly recommended.

3. Follow up study with another second readings of inflammatory markers is recommended to detect association with clinical course of disease and prognosis.

4. Non-aerobic blood culture is recommended for wide range detection of causative pathogens.

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الخلاصة

يُعرَّف الإنتان بأنه اختلال وظيفي يهدد الحياة ناجم عن استجابة غير منظمة للعدوى. لديها معدلات عالية من الامراضية والوفيات وهي مشكلة صحية عامة في جميع أنحاء العالم .

تم وصف الإنتان بأنه متلازمة الاستجابة الالتهابية الجهازية SIRS بثلاثة مستويات من الشدة: الإنتان ، الإنتان الشديد ، والصدمة الإنتانية. تم فحص العديد من علامات الالتهاب في الإنتان باستخدام أدوات تشخيصية مختلفة مثل CRP و Procalcitonin. يعد بريسبسين وكالبروتكتين علامات جديدة لم يتم دراستها في العراق .

يمكن أن ترتبط مسببات الأمراض المختلفة بتطور الإنتان مثل الفطريات والفيروسات والطفيليات والبكتيريا. إن معرفة العامل الممرض المسؤول للإنتان أمر أساسي لنهج الإدارة اللاحق.

الهدف من الدراسة هو تقييم مستويات المصل من Presepsin و Calprotectin في المرضى الذين يعانون من تسمم الدم وكذلك مقارنة الفائدة التشخيصية لهذه المؤشرات الحيوية مع طرق التشخيص التقليدية المستخدمة في تسمم الدم. أيضا تقييم مسببات الأمراض المسببة لتسمم الدم.

تم تضمين أربعين مريضا تم تشخيصهم سريريا مع تعفن الدم لدى البالغين في الدراسة. كان المرضى (20) ذكر و (20) انثى تراوحت اعمارهم بين (17-70). بالإضافة إلى 40 مشاركًا يتمتعون بصحة جيدة من ذوي العمر والجنس المتطابقين لمجموعة المرضى. تم قياس مستويات المصل من Presepsin و Calprotectin باستخدام كتات ELISA

ارتفعت مستويات الكالبروتكتين والبريسيبسين في المصل في مجموعة المرضى مقارنة بمجموعة السيطرة وكانت النتيجة معنوية لكالبروتكتين (P = 0.019، P = 0.066) على التوالي. يُظهر Presepsin فائدة تشخيصية مهمة. نتيجة غير مهمة لكل من مستويات المؤشرات الحيوية في الفئات الفرعية للإنتان: تعفن الدم الحاد والصدمة الإنتانية والإنتان (49.75 ± 15.7 ، 59.53 ± 18.8 ، 36.40 ± 8.14) على التوالي بالنسبة لكالبروتكتين ، ومستويات بريسيبسين في الصدمات الإنتانية ، والإنتان الشديد والإنتان (476.62 ± 150.7 ، 551.88 ± 161.87 ، 137.96 ± 30.85) على التوالي.

من بين 40 شخصًا مسجلين ، كان لدى 24 مريضًا مسببات جرثومية مؤكدة للإنتان وعدوى المبيضات في مريض واحد وإيجابية الجرام (7) وسالبة الجرام (16) مع ارتباط غير مهم بمستويات علامات الدراسة. في الختام ، يمكن أن يكون كل من الكالبروتكتين والبريسيبسين المرتفعين في مرضى الإنتان والبريسيبسين علامة بيولوجية جيدة ذات خصوصية عالية لتشخيص الإنتان. بالإضافة إلى ذلك ،

كانت السالمونيلا التيفية سالبة الجرام هي الكائنات الحية الدقيقة الأكثر انتشارًا التي تم الحصول عليها من مزرعة الدم لهؤلاء المرضى.

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء / كلية الطب

فرع الاحياء المجهرية

تقييم البريسبسين , الكالبروتكتين والمسببات البكتيرية للمرضى البالغين في الحالات الحرجة من تسمم الدم

رسالة مقدمة الى

مجلس كلية الطب وهي جزء من متطلبات نيل شهادة الماجستير في الاحياء المجهرية

من قبل الطالبة

هجران توفيق عادل

بكالوريوس علوم/ جامعة كربلاء (2016)

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