UNIVERSITY OF BELGRADE

FACULTY OF BIOLOGY

Mr Atia Ramadan M. Elkilany

THE SIGNIFICANCE OF POLYMORPHISMS FOR Toll-LIKE RECEPTORS (TLR) 2, 3, AND 4 GENES, AND GENE FOR VITAMIN D RECEPTOR FOR THE COURSE AND OUTCOME OF THE DISEASE IN PATIENTS WITH TRAUMA AND/OR SEPSIS

Doctoral dissertation

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UNIVERZITET U BEOGRADU

BIOLOŠKI FAKULTET

Mr Atia Ramadan M. Elkilany

ZNAČAJ POLIMORFIZAMA GENA ZA Toll-u SLIČNE RECEPTORE (TLR) 2, 3, I 4, I GENA ZA RECEPTOR ZA VITAMIN D ZA TOK I ISHOD BOLESTI KOD PACIJENATA SA TRAUMOM I/ILI SEPSOM

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Final Examininig Commitee

_____

Dr Biljana Božić, full professor of Faculty of biology, University of Belgrade, Thesis Supervisor

_____

Dr Zvonko Magić, full professor of Medical Faculty of Military Medical Academy, University of defense in Belgrade

_____

Dr Maja Šurbatović, full professor of Medical Faculty of Military Medical Academy, University of defense in Belgrade

Date: _____

Candidate:

_____

Mr Atia Ramadan M. Elkilany

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At last but not the least I would like to dedicate this thesis to my family, my wife Mofeda and children Heim, Suinds, Mohamed, and Sahd because they were my biggest support during PhD study.

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The significance of polymorphisms for Toll-like receptors (TLR) 2, 3, and 4 genes, and gene for vitamin D receptor for the course and outcome of the disease in patients with trauma and/or sepsis

ABSTRACT

Considering that TLR2, TLR3, TLR4, and VDR play very important role in inflammatory processes, the question arises whether presence of polymorphisms in these genes is associated with susceptibility to sepsis. The aim of this study was to examine the association of *TLR2*, *TLR3*, *TLR4*, and *VDR* genes polymorphisms with clinical characteristics and outcome of Serbian critically ill patients. A follow-up study was conducted on 121 Caucasian Serbian critically ill patients. Nine polymorphisms in *TLR*s and VDR genes: *TLR2* (rs5743708), *TLR3* (rs3775291, rs5743312), *TLR4* gene (rs4986790, rs4986791), *VDR FokI* (rs2228570), *VDR TaqI* (rs731236), *VDR ApaI* (rs7975232), and *VDR EcoRV* (rs4516035) were genotyped by Real Time PCR method. Investigated polymorphisms in *TLR2* and *TLR4* genes were not associated with clinical characteristics and outcome of critically ill patients. The *TLR3* rs3775291 polymorphism was associated with patient's outcome (p=0.018). Patients with sepsis and *TLR3* rs3775291 mutated genotype had four times higher mortality rate compared to the *wild type* and heterozygous carriers. Multivariate regression analysis showed that age, sex and *TLR3* rs3775291 polymorphism are independent variables of outcome of critically ill patients. For the first time, our preliminary findings indicate role of TLR3 with MyD88 independent signaling and its polymorphism (*TLR3* rs3775291) in sepsis and survival in Serbian critically ill patients. Further, in this study statistically significant difference in the frequency of *VDR FokI* (rs2228570) between the critically ill patients and healthy subjects is shown. However, in patients with trauma, who did not develop sepsis, did not detected VDR *FokI* mutated genotype. Multivariate regression analysis which was used to asses the independent variables that may affect mortality of critically ill patients, showed that age, and *VDR FokI* rs2228570 polymorphism are statistically

significant variables. Significant association between *VDR FokI* rs2228570 polymorphism and cause of sepsis and critically ill status was noticed. For the first time, the significant tendency toward negative association between *TLR4* rs4986791 polymorphism and *VDR FokI* rs2228570 was noticed in the Caucasian Serbian critically ill population. Importance of this research is in simultaneously determining the polymorphisms of two receptor groups which are important to immune response.

Keywords: *tlr genes, vdr genes, polymorphisms, sepsis, trauma, inflammation*

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Značaj polimorfizama gena za Toll-u slične receptore (TLR) 2, 3, i 4, i gena za receptor za vitamin D za tok i ishod bolesti kod pacijenata sa traumom i/ili sepsom

SAŽETAK

Imajući u vidu da TLR2, TLR3, TLR4 i VDR igraju veoma važnu ulogu u procesu inflamacije, posatvlja se pitanje da li je prisustvo polimorfizama ovih gena pitanje u vezi sa mogućnođću za razvoj sepse. Cilj ovog istraživanja je ispitivanje asocijacije polimorfizama *TLR2, TLR3, TLR4* i *VDR* gena sa kliničkim karakteristikama i ishodom vitalno ugroženih pacijenata sa intenzivnom negom u Srbiji. Studija je uključila 121 vitalno ugroženog pacijenata sa intenzivnom negom bele rase u Srbiji. Izvršena uje genotipizacija devet polimorfizama *TLR* i VDR gena: *TLR2* (rs5743708), *TLR3* (rs3775291, rs5743312), *TLR4* gene (rs4986790, rs4986791), *VDR FokI* (rs2228570), *VDR TaqI* (rs731236), *VDR ApaI* (rs7975232), i *VDR EcoRV* (rs4516035) primenom Real Time PCR metode. Ispitiani polimorfizmi u *TLR2* i *TLR4* genima nisu povezani sa kliničkim karakteristikama i ishodom vitalno ugroženih pacijenata sa intenzivnom negom. *TLR3* rs3775291 polimorfzam je povezan sa ishodom vitalno ugroženih pacijenata sa intenzivnom negom (p=0.018). Pacijenti sa sepsom i *TLR3* rs3775291 mutiranim genotipom imaju četiri puta veći stepen mortaliteta u poređenju sa nosiocima *wild type* genotipa i heterozigotima. Multivarijantna regresiona analiza je pokazala da su godine, pol i *TLR3* rs3775291 polimorfizam nezavisne varijable ishoda vitalno ugroženih pacijenata sa intenzivnom negom. Po prvi put, nađi preliminarni nalazi ukazuju na ulogu TLR3 sa MyD88 nezavisnom signalizacijom i njegovog polimorfizma (*TLR3* rs3775291) u sepsi i preživljavanju vitalno ugroženih pacijenata sa intenzivnom negom u Srbiji. Dalje, u ovoj studiji je pokazana statistički zmačajna razlika u zastupljenosti *VDR FokI* (rs2228570) polimorfizma izmežu vitalno ugroženih pacijenata sa intenzivnom negom i zdravih osoba. Međutim, kod pacijenata sa traumom koji nisu razviji sepsu nije detektovano prisustvo VDR *FokI* mutatiranog genotipa. Multivarijantna regresiona

analiza koja se koristi za procenu nezavisnih varijabli koje mogu da utiču na mortalitet vitalno ugroženih pacijenata sa intenzivnom negom pokazala je da su godine i *VDR FokI* rs2228570 polimorfizam statistički značajne varijable. Zabeležena je i značajna povezanost *VDR FokI* rs2228570 polimorfizma i uzroka sepse kod vitalno ugroženih pacijenata sa intenzivnom negom. Po prvi put je pokazana tendencija ka značajnoj povezanosti između *TLR4* rs4986791 i *VDR FokI* rs2228570 polimorfizama kod vitalno ugroženih pacijenata sa intenzivnom negom u Srbiji. Značaj ovog istraživanja je u istovremenom određivanu polimorfizama ove dve grupe receptora koji su važni za imunski odgovor.

Ključne reči: *tlr genes, vdr genes, polymorphisms, sepsis, trauma, inflammation*

Naučna oblast: Fiziologija životinja i čoveka
Uža naučna oblast: Imunologija
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1. INTRODUCTION

1.1. The basic facts about sepsis

Word "sepsis" presented for the first time by *Hippocrates* (ca. 460-370 BC) and is derived from the Greek word sipsi ("make rotten"). In addition, the Persian 'father of modern medicine', Bin Sina (also known as Avicenna, ad 980–1037), note that the poisoning usually associated with fever. However, it was not until the 18th century that Louis Pasteur linked the evanescence of Organic materials to the presence of bacteria and microorganisms, and *Ignaz Semmelweis* observe the significant effect of hygienic measures on decreasing the mortality rate of women during birth. In 1914, *Hugo Schottmuller* laid the foundations for the modern definition of sepsis and was the first to describe the presence of inflammation was an essential in the disease. Decades later, the ideas of Lewis Thomas led to a shift in concept of sepsis by popularizing the theory that "…it is the [host] response … that makes the disease" [1]. Sepsis affects about 700,000 people annually and accounts for about 210,000 deaths that occur annually in the United States. According to recent reports and a rise in the rates between 1.5% and 8% per annum [2, 3]. Although technical developments in intensive care units (ICUs) and advanced supportive treatment. Septic patients are usually hospitalized for long periods, seldom leaving the ICU before 2–3 weeks. Accordingly, sepsis represents a major burden to the US health care system, with appreciated costs to be nearly $16.7 billion per year [3].

Sepsis originally meant "putrefaction," a decomposition of organic matter by bacteria and fungi. Since then time, a wide range of definitions have been applied to sepsis, including sepsis syndrome, severe sepsis, septicemia, and septic shock (*Bone, 1991 and Bone, 1991*). In 1991, the American College of Chest Physicians/Society of Critical Care Medicine developed a new set of terms and definitions to define "sepsis" in a more precise manner (*Society of Critical Care Medicine Consensus Conference Committee, 1992 and Bone, 1996*). The definitions take into account the findings that

sepsis may result from a many of infectious agents and microbial mediators and may not be linked with actual blood stream infection. Despite the use of these standards has been criticized, they continues provide a useful framework to approach patients with infectious diseases [4]. The term “systemic inflammatory response syndrome” was coined to describe the common systemic response to a wide variety of insults. It is characterized by 2 or more of the following clinical manifestations: (1) a body temperature of more than 38°C or less than 36°C; (2) a heart rate greater than 90 beats/min; (3) tachypnea, as manifested by a respiratory rate of more than 20 breaths/min; and (4) an alteration of the white blood cell count of greater than 12,000 cells/mm3, less than 4000 cells/mm3, or the presence of more than 10% immature neutrophils. When SIRS is the result of make sure that infectious process, it is termed sepsis.

Severe sepsis is defined as sepsis plus either organ dysfunction or evidence of hypoperfusion or blood pressure. Septic shock is a subset of severe sepsis and is defined as sepsis-induced hypotension, despite continuing adequate fluid resuscitation, with the presence of hypoperfusion abnormalities or organ dysfunction.

The patient populations most affected to sepsis include those with the following specifications: (1) age younger than 1 year or older than Five and a half decades almost (65 years), (2) chronic diseases, (3) immunosuppression, (4) broad-spectrum antibiotic use, and (5) exposure to infection linked with surgical and invasive procedures [5]. Aging is related to a decline in immune function [6]. The expression used to describe this phenomenon is immunosenescence [7]. Studies have shown that age-related changes in the immune system lead to T cell and B cell weakness among others outside of this debate [8]. Physical disability that contribute to the significant risks of sepsis in the elderly include (1) dementia (senile), (2) decreased gag and cough reflex, (3) immobility, (4) skin collapse, (5) poor urinary bladder emptying, and (6) obstruction leading to infection (urolithiasis, neoplasm, and cholelithiasis) [6].

1.2. Sepsis diagnosis and diagnostic testing

The diagnosis of sepsis is difficult, particularly in the intensive care setting, wherein early signs and symptoms are often superimposed by the patient's underlying diseases. Early organ dysfunction may be the first symptom noted by doctors [9]. Other diagnostic criteria may include (1) circulatory instability, (2) arterial hypoxia (3) oliguria (less than 0.5 mL/kg/h), (4) clot (coagulopathy), (5) change liver function tests. The list of standards was set by the International Sepsis Definitions Conference. Serum lactate level is a diagnostic sign in septic patients [9]. Lactate is generated by anaerobic cellular metabolism and perhaps reflect the degree of cellular defect in sepsis. Arterial lactate levels are commonly used as a global indicator of oxygen deficits. Serial lactate levels can reflect adequacy of hemodynamic resuscitation efforts. Many serological markers may aid in the early diagnosis of sepsis. Because of the complexity of the sepsis syndrome, several studies have been conducted to identify lab tests that may shorten the time to diagnosis sepsis. Genetic factors are also a focus of studies in relation to why some patients succumb to severe sepsis and septic shock faster than others do [10]. Studies have been conducted to make sure why patients with similar properties (such as age and gender), when exposed to the same pathogen, have mixed reactions. For example, some patients have been identified with a specific genotype related to decrease in fibrinolysis [11]. This reduction in fibrinolysis leads to altered clotting paths, which, in turn, places these patients at a greater risk for MODS or septic shock.

1.2.1. Epidemiological data

1.2.1.1. Age

There is a direct relationship among old age and the incidence of severe sepsis y septic shock, with a considerable increase in incidence in elderly people [3, 12]. The

incidence of severe sepsis among infants is also elevated, with an annual rate of 5.3 cases per 1,000 populations [3]. The average age of patients with severe sepsis in the majority studies is between 60 to 65 years, and when the patients are stratified at the age of 65, the relative risk for sepsis was 13 times higher for patients aged 65 and above. Gram-negative microorganisms are more common in elderly patients than in younger patients. Escherichia coli has found to be the most common microorganism in patients older than 65 years, while Staphylococcus aurous was the most common microorganism in younger patients with community acquired bacteremia [13]. Also, the source of infection has also been different among older patients with sepsis than among younger patients. Inflammatory urinary system is more frequently the source of sepsis in elderly patients than in younger patients.

1.2.1.2. Ethnic background

Epidemiologic studies have shown a large number of cases of severe sepsis and septic shock in black people, which refers to the possible genetic predisposition. Instead, a higher spread rate of renal disease and diabetes in the black population might explain the higher incidence of these syndromes [14, 15]. In addition, a high number of cases of severe sepsis and septic shock in black people could be related to the higher percentage of black people living in Life of poverty. Other, the average age of black people has been found to be decline in black people than in white people. Higher infection rate and increases the risk of acute organ dysfunction in black people as compared to white people individuals could explain racial differences in severe sepsis. Finally, race specific genetic polymorphisms in the host response to infection may predispose some ethnic groups to increased incidence or worse outcomes with sepsis [14].

1.2.1.3. Sex

Women are less likely than Men to develop sepsis, [15]. However, it is unclear whether this difference could be the result of a higher prevalence of comorbidity in men, or whether women are protected against the inflammatory changes that occur in severe sepsis and septic shock [3]. Respiratory infections are the main source of severe sepsis and septic shock, which is most common in men than in women, followed by intra-abdominal infections, urinary tract infections and primary bloodstream infections. Respiratory infections and abdominal infections appear to have a worse prognosis than other foci.

1.2.1.4. Mortality

Estimated Centers for Disease Control and Prevention (CDC) that sepsis is the main reason tenth of death in the United States [16]. Severe sepsis is considered to be the most common cause of death in non-coronary in intensive care units. Deaths resulting to severe sepsis overrun the numbers of people with other diseases that attract higher public awareness, such as breast cancer and AIDS [17]. The mortality ratio of severe sepsis and septic shock are 25 to 30% and 40 to 70%, respectively.

1.3. Pathophysiology of sepsis

Starting the pathophysiology of sepsis with the entry of organisms into the blood through the skin or the respiratory, genitourinary, or gastrointestinal tract [18]. Micro-organisms that enter the body may include bacteria, yeast, viruses, and/ or parasites. This complex syndrome is characterized by concurrent activation of inflammation and coagulation in response to microbial insult [19]. Microorganisms that lead to sepsis, Gram negative bacilli (mainly *Escherichia coli*, *Klebsiella species*, and *Pseudomonas aeruginosa*) and Gram positive cocci (mainly *Staphylococci* and *Streptococci*) is the most common microbes isolated from patients with severe sepsis

and septic shock [20]. Representing fungi, Candida only about 5% of all cases of severe sepsis.

1.3.1. Gram negative sepsis

Most cases are caused Gram-negative sepsis by *Enterobacteriaceae* such as *E. coli* and *Klebsiella* species. *Pseudomonas aeruginosa* is the third most common cause. Usually occur Gram-negative infections in the lung, abdomen, bloodstream, or urinary tract. Lipopolysaccharide is an important component of the outer membrane of Gram negative bacteria and has a pivotal role in Gram-negative sepsis incentive [21]. Lipopolysaccharide binding protein in host cells binds to lipopolysaccharide in the bacteria and transfers it to CD14 [22]. CD14 is a protein anchored in the outer leaflet of the plasma membrane, although it also known exists as a melting plasma protein that attaches lipopolysaccharide to CD14 negative cells, such as endothelial cells. CD14 in the extracellular space and therefore cannot induce cellular activation without a transmembrane signal transducer coreceptor.

The first source of infection is the lung both in severe sepsis and in septic shock, followed by the abdomen, the urinary tract, soft tissues and primary blood stream infection [23, 24]. Intra-abdominal and respiratory sources of sepsis have been considered as risk foci of infection, because these foci were linked with increased mortality than other sources of sepsis. These foci have also been related do not be enough empirical antimicrobial treatment [25].

1.3.2. Gram positive sepsis

The most common reasons that cause sepsis of gram positive bacteria are Staphylococci (mainly *Staph aureus* and *coagulase-negative staphylococci*) and streptococci (*Strep pyogenes, viridans streptococci, Strep pneumoniae*). They are usually in charge of for infections of skin and soft tissue, infections associated with intravascular devices, primary bloodstream infections, or respiratory infections. Gram

positive organisms can cause sepsis by at least two mechanisms: by producing egzotoxins that act as superantigens and by components of their cell walls stimulating immune cells [26]. Superantigens are molecules that bind to MHC class II molecules of antigen presenting cells and to Vβ chains of T cell receptors. In doing so, they activate large num-bers of T cells to produce large amounts of pro-inflammatory cytokines. Staphylococcal enterotoxins, toxic shock syndrome toxin-1, and streptococcal pyrogenic egzotoxins are examples of bacterial superantigens. Gram positive bacteria without egzotoxins can also induce shock, maybe by stimulating innate immune responses through similar mechanisms to those in Gram negative sepsis. In fact, Toll-like receptor (TLR) 2 has been shown to mediate cellular responses to heat killed Gram positive bacteria and their cell wall structures (peptidoglycan, lipoproteins, lipoteichoic acid, and phenol soluble modulin) [27].

1.3.3. Fungi as cause of sepsis

The disseminated form of fungal infection can initiate the cytokine-releasing series, leading to a state of shock. Like LPS, fungal proteins can activate the macrophages, the endothelial cells, and the complements. Fungal molecules from *Cryptococcus neoformans* and *Aspergillus fumigatus* interact with TLR4 and induce the production of proinflammatory cytokines via the NF-κB pathway. Toxins have not been found to contribute to the state of shock in fungemia. Immunocompromised patients are particularly exposed to complications resulting from of fungal infections [28, 29].

1.3.4. Virus as cause of sepsis

Infections inflicted to the tissues by viruses can be categorized into 3 various mechanisms: (1) the direct lytic impacts on the host cells as a result of the viral replication, (2) lysis to distant organ cells from the activated complement cascade due to virus-antibody reaction, and (3) the action of circulating proinflammatory cytokines

released as a provocative response to the viral injury [30]. Viral particles also bind to membrane CD14 and can evoke the activation of NF-kB through signaling TLR. TLR3 responds to viral double-stranded RNA, while TLR4 acts as a receptor for respiratory syncytial virus protein F [28]. Viral toxins did not appear to cause damage to cells directly, and did not prove its role in the genesis of shock.

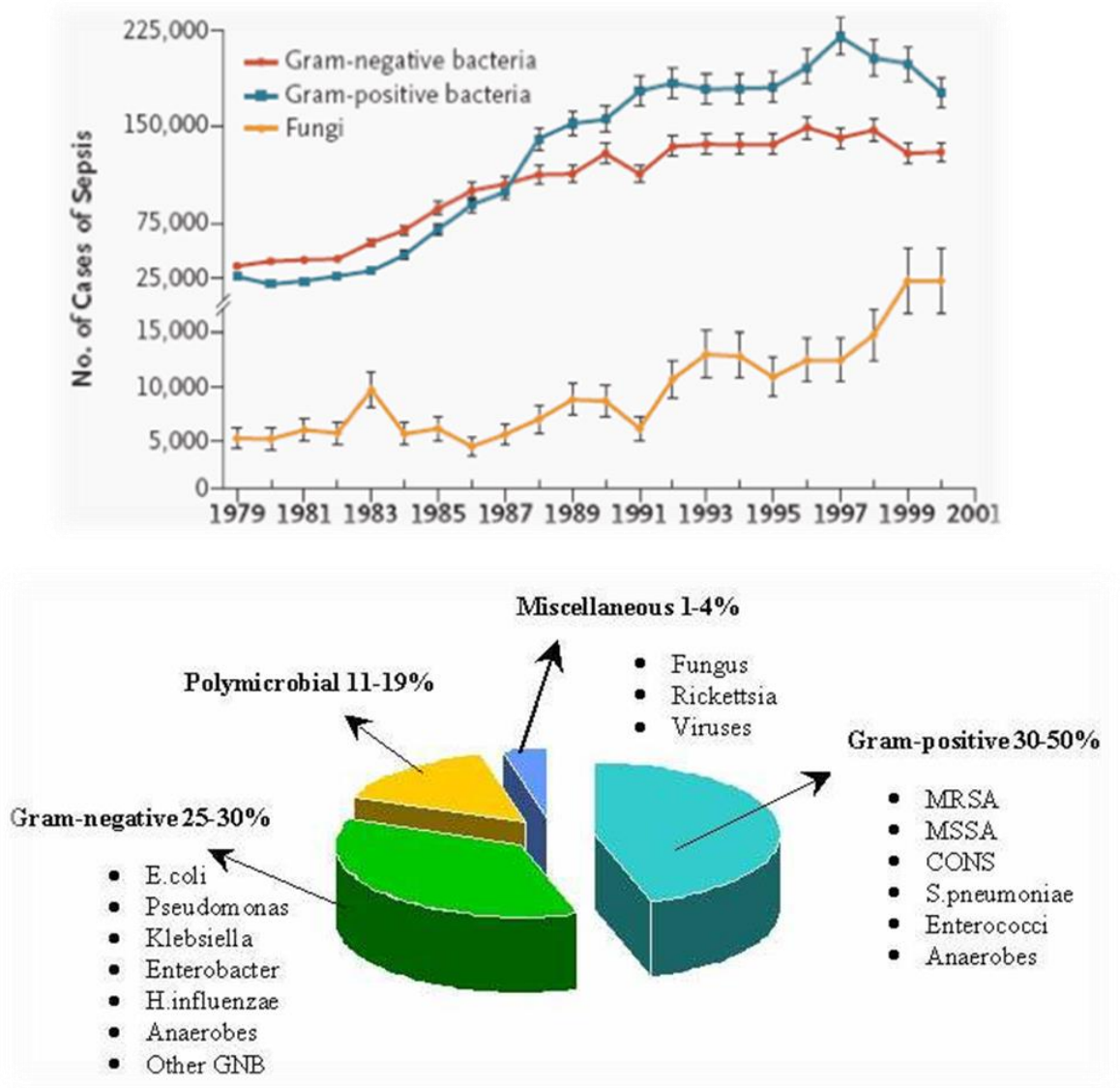

Figure 1. Number of cases of sepsis in the United States by the causative organism, 1979 to 2000 [15]

The incidence of gram-negative sepsis has diminished throughout the years to 25-30% in 2000. Gram-positives account for most of cases up to 30-50%. In 11-19% cases, the etiology is polymicrobial in nature. Fungi, viruses and parasites account for 1-4%, but their recurrence could be reduced [31, 32]. Finally cultures can be negative in 30% of cases, fundamentally in patients with community-acquired sepsis who are treated with antibiotics before admission.

1.4. Treatment of sepsis

The treatment of sepsis is usually focused to provide supporting failing organ systems with interventions including (1) fluid replacement, (2) airway management, (3) antibiotic therapy, and (4) use of vasopressors. The goals of fluid resuscitation in septic shock are restoration of tissue perfusion and normalization of oxidative metabolism [33]. Rising cardiac output and oxygen delivery is dependent on expansion of blood and plasma volume [34]. Invasive hemodynamic monitoring via a pulmonary artery catheter should be used in patients who do not respond quickly to initial fluid or those with poor physiologic reserve [35]. Antimicrobial therapy should be initiated the moment that blood cultures are taken and other relevant sites have been cultured. The choice of initial therapy is based on knowledge probably pathogens at specific sites of local infection. Experimental antibiotics that are effective against both Gram-positive and Gram-negative bacteria and should be managed intravenously. Once the organism is identified, then the antibiotic regimen may be simplified [36]. Significant other treatment methods for sepsis include (1) tight blood sugar control, (2) nutritional support, and (3) corticosteroid management [37].

1.5. Immune response in sepsis

A normal response to an infection includes a series of complex immunologic reactions inclusive cytokine activation of cells against infection with pathogens by production of cytokines like TNF-α, interferons, interleukins and chemokines. The systemic response to infection which leads to sepsis and septic shock is mediated by a complex cytokine network. Cecal bind and puncture (CLP) in mice causes symptoms like to those found in septic patients and serves as a model for peritonitis and polymicrobial sepsis. The model has been a mainstay of basic scientific sepsis research [38, 39]. The animal models for hemorrhagic shock and sepsis surly have helped in getting a better understanding of the immune response of the patients. In alignment with this idea comparable changes in the immune system have been reduced in trauma and septic patients, addition mice and rats after hemorrhagic shock and sepsis. Hemorrhagic shock leads to decreased LPS-dependent proinflammatory cytokine response of various macrophages which is associated with anincrease portability to bacterial infections. Hemorrhagic shock in mice and rats has been found to limit LPS-induced TNF-α and IL-1β by splenic macrophages [40, 41]. Decrease in TNF-α producing ability of whole blood or peripheral white blood cells after hemorrhagic shock has been shown by several other groups [42]. An early pro-inflammatory response described by the release of pro-inflammatory mediators, including TNF-α, IL-1β, IL-6 and chemokines (referred to as the systemic inflammatory response syndrome, SIRS). Pro-inflammatory cytokine expression is counter-regulated by the release of anti-inflammatory cytokines such as IL-10 and IL-4. The latter response is usually termed as recompense anti-inflammatory response syndrome (CARS) (Figure 2). As depicted in the chart, sepsis is characterized initially by an exuberant production of pro-inflammatory cytokines, leukocyte activation and tissue injury which is then followed by a release of anti-inflammatory cytokines, leukocyte deactivation and immunosuppression.

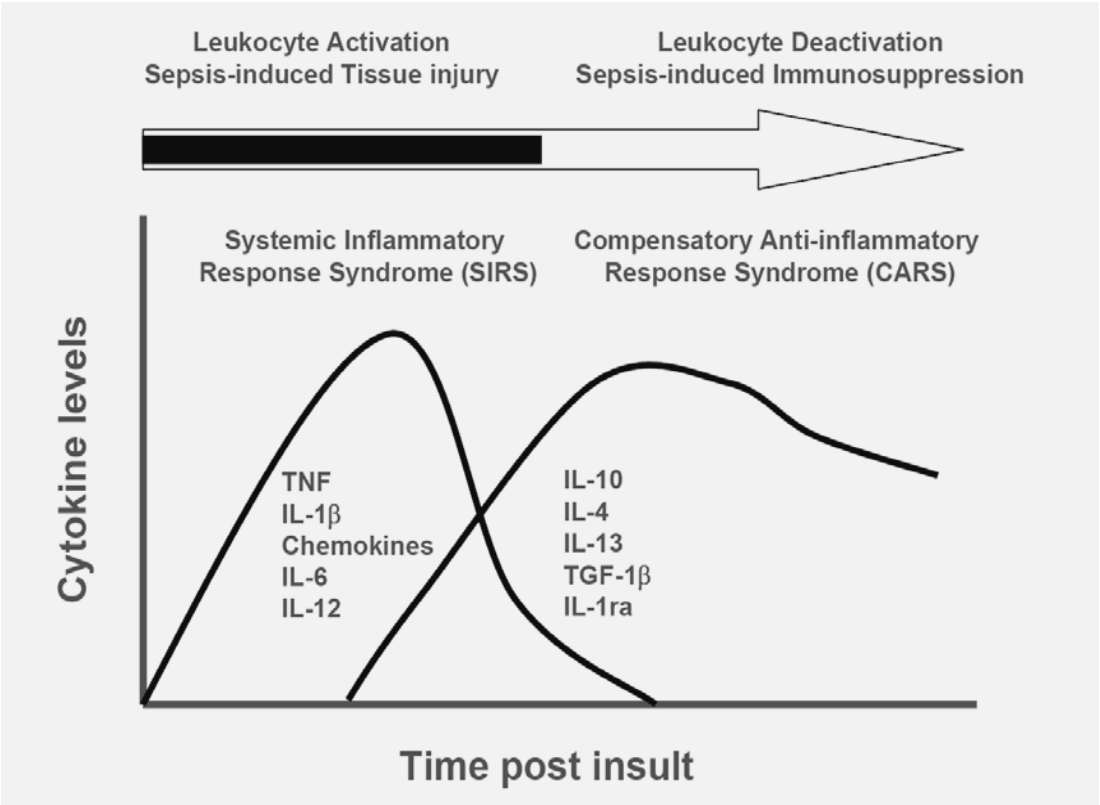

Figure 2. Expression of cytokines through sepsis (modified from [43])

The latter phase is thinks to mediate the profound state of immunosuppression which occurs in connected with substantial impairment in immune functions (sepsis-induced leukocyte "deactivation" or "immunoparalysis" [44]

The antigen presentation is considered to be a main function in hemorrhage and sepsis-induced inflammatory response. Monocytes', macrophages' and dendritic cells' function as antigen presenting cells are well thoughtful in regard to results of the disease [45, 46, 47].

The earliest studies of sepsis focused on infection, but clinical studies found that immune response in sepsis plays a critical role, and hence sepsis is defined as a systemic inflammatory response syndrome (SIRS) after serious microbial infection. Despite, a simple anti-inflammatory treatment does not diminish the mortality of

sepsis. Some sepsis patients die at early stage of inflammatory response, but most patients die in the later stage of sepsis with a longer period of immune suppression. Thus researchers and scientists began to question whether the mortality of sepsis is only linked to uncontrolled pro-inflammatory response. It was remarked that immune paralysis and acquired immune system dysfunction together play important roles in the course of immunosuppression [48]. The immune pathogenesis of sepsis is very complex.

1.5.1. Receptors of immune cells in sepsis

In the early state of sepsis, the excessive stimulation of the antigen recognition system and the release of pro-inflammatory mediators lead to dangerous multi-system dysfunction in the body. Immune cells express a chain of receptors on membrane surface called pattern recognition receptors (PRRs); these receptors can lead the body's defense reaction quickly after tissue injury or bacterial infection. Bacterial infection can be detected by pathogen-associated molecular patterns (PAMPs), and necrotic tissue immune recognition molecules are intracellular proteins or the media resulting from dead cells. PRRs subtypes or Toll-like receptors (TLRs) are important receptors which can recognize pathogens and stimulate inflammatory response. During sepsis, microbial infection or necrotic tissue released high levels of detrimental substances, resulting in the activation of systemic immune response and excessive activation of immune cells. Too much release of cytokines plays a destructive impact. The excessive release of cytokines plays a destructive impact. TLR-4 activation is important in inflammatory response triggering because of TLR-4 expressed in G-bacteria outer membrane, and TLR-4 is able to form a receptor complex with CD14 and MD2 to mediate lipopolysaccharide (LPS) recognition, subsequently triggering an inflammatory response [49, 50].

1.5.1.1. Sepsis-induced Innate Immune Suppression

Innate immune cells are the first cellular responders to invading organisms and are therefore vital to host defense. These cells including monocytes (and their mature form, tissue macrophages), dendritic cells, and neutrophils recognize pathogens through constitutively expressed receptors including TLR and receptors for the Fc fragment of immunoglobulin. Once stimulated, these cells should quickly and strongly produce pro-inflammatory cytokines. In addition, innate immune cells most be able to recognize and ingest pathogens as well as process and present antigens to members of the adaptive immune system. Monocytes, such as, present antigens on cell-surface major histocompatibility complex (MHC) class II molecules such as human white blood cells antigen (HLA)-DR.

1.5.1.2. Sepsis-induced Adaptive Immune Suppression

The adaptive immune system is consisting of lymphocytes. Unlike innate immunity, the adaptive immune system encouraged the development of highly pathogen-specific effectors cells that respond to separate antigens. Lymphocytes generally dependent on members of the innate immune system for antigen processing and presentation, without which cells of the adaptive immune system typically cannot become activated. Once activated, B lymphocytes mature into antibody secreting plasma cells. In mean time, T lymphocytes are grouped by cell surface marker expression into families that lead to a variety of biological functions. Whilst CD8+ (cytotoxic) T cells are responsible for directly killing infected cells, CD4+ (helper) T cells produce cytokines to direct the development and activate another branch of the immune system. These stages are characterized by the development of immunologic memory, such that the response to the first exposure to a particular antigen is slower and less robust than subsequent exposures. In the setting of sepsis, therefore, believed that the adaptive immune system important for the modulation of the subacute phase of disease, exerting its effects on the order of days after sepsis onset, rather than

hours. CD4+ T cells are notable for their immunomodulatory role and they usually divided into subgroups of T-helper (Th) cells based on their cytokine release profiles. Th1 cells secrete pro-inflammatory cytokines such as IFNγ while Th2 cells produce anti-inflammatory cytokines such as IL-10 and IL-4 which deviate the immune response toward antibody production and away from cell mediated killing. Th17 cells are a recently elucidated pro-inflammatory subset of helper T cells. They are identified by the production of IL-17 and IL-22, is believed to have an important role in autoimmune pathology, and may participate to the overall pro-inflammatory adaptive immune phenotype in other disease states as well [51].

1.5.1.3. T cells in sepsis

Despite CD4+CD25+ T cells include only a small part of the T lymphocyte population in the immune system, these cells seems to be possess potent regulatory properties on cellular activation which make them an important participant in the inhibition of immune responsiveness through sepsis. Can be summarized proposed interactions between Treg cells and effector cells in sepsis are in Fig. 3.

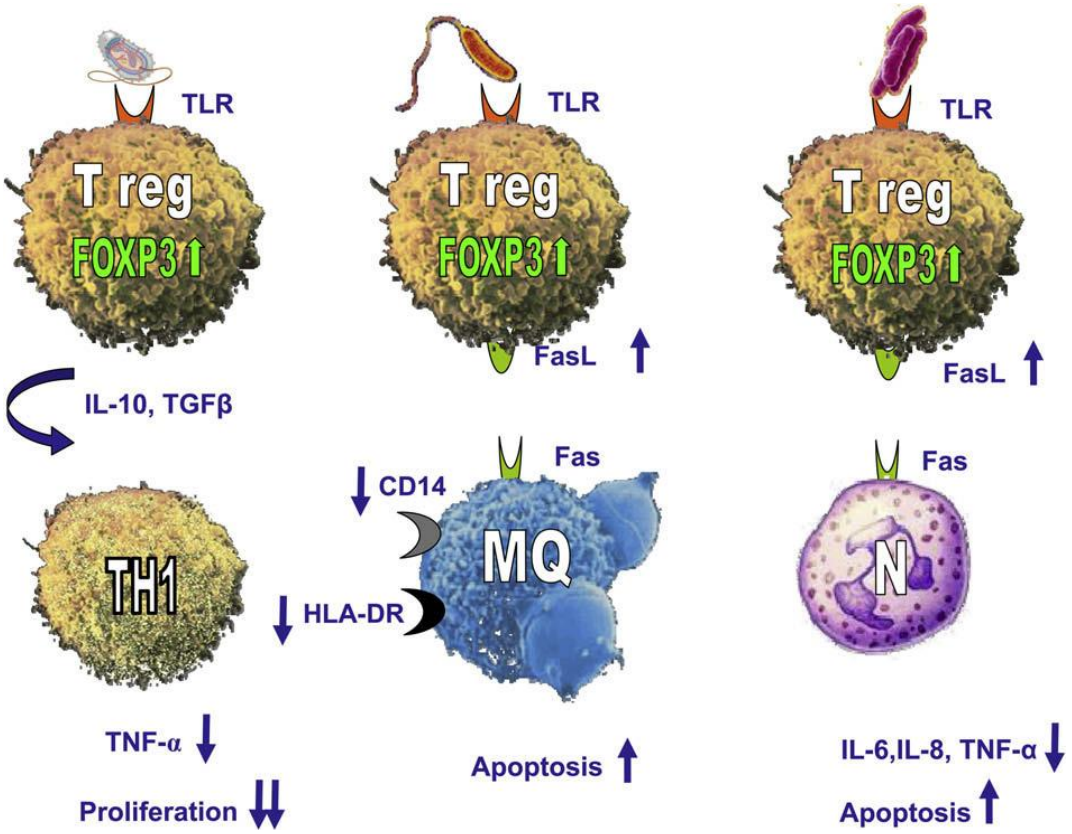

Figure 3. The interactions between Treg cells and effector cells in sepsis: following sepsis, the number and suppressive function of Treg cells increase. Treg cells decrease Th1 proliferation and secretion of the inflammatory cytokine TNF-a. Treg cells also increase the rate of apoptosis of monocytes and neutrophils. Finally, they decrease TNF-a, IL-6, and IL-8 secretion by neutrophils [52].

1.5.1.4. The Role of the Complement System in Sepsis

Plasma C3a, C4a and C5a concentrations and the survival rate of patients with sepsis are too negatively linked [53]. Remarkable, C3a has together pro-inflamatory and anti-inflammatory effects. Mice that have a lack of C3aR are more sensitive to septic shock, and their intracellular levels of pro-inflammatory media is a marked increase C3a and C3aR in combination may promote the pituitary gland to secrete anti-inflammatory hormones, indicating the anti-inflammatory properties of C3a. We

found that the excessive generation of C5a would lead to the injury of the body. C5a is capable to cause immune paralysis, multiple organ failure, apoptosis of thymus cells and adrenal cells, defect blood clotting system and septic myocardial injury [54]. In sepsis patients, C5a has an important role through several different ways (Fig. 4) [55]. In addition to C5aR, C5a can combine with C5L2 specifically. C5L2 was thought to be a competitive antagonist of C5aR, but new studies discovered C5L2 is a functional receptor [55, 56]. Decrease of C5L2 expression on the neutrophil surface is associated to the incidence of multiple organ failure, suggesting that C5L2 is involved in the pathogenesis of sepsis [57]. Evidence suggests that the synergic effects of C5aR and C5L2 increase the inflammatory response to sepsis despite the two receptors have various functions [55]. For instance, C5a-induced MIF release depends on C5aR signaling, and C5L2 mediates the C5a-dependent release of HMGB1 [54, 58]. It is worth mentioning blockade of one of C5a receptors can reduce the mortality of sepsis patients moderately, but the two receptors can play a protective role in severe sepsis if they are blocked. Through new clinical trials have been getting excellent results by dual blocking of C5aR and C5L2 compared with a single blockade. As a result, C5a can be relied upon as an effective treatment of sepsis. And this treatment has the advantage of non-intervention on the formation of membrane attack complex (MAC), which prevents from microbial invasion. However, in the very early complement activation occurs in sepsis, so a reliable, sensitive bedside monitoring system is necessary to monitor the degree of complement activation.

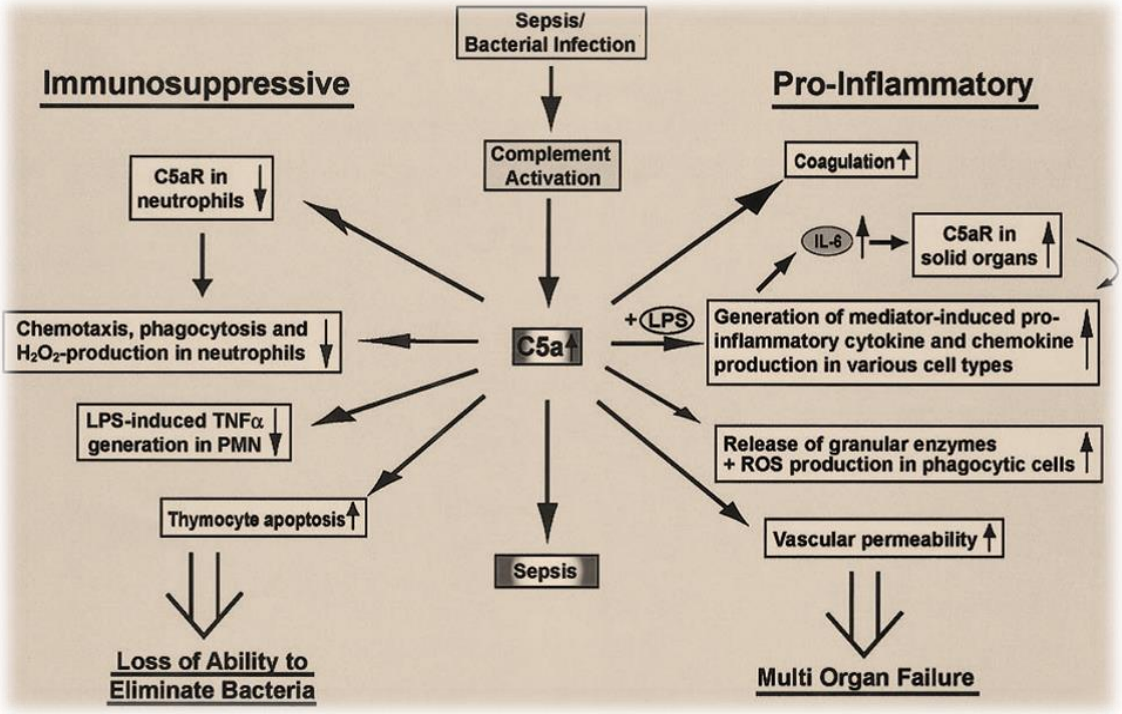

Figure 4. The key role of C5a/C5aR activation for the development of sepsis [59]

1.5.1.5. Phagocytosis and TLRs

Play the process of phagocytosis an important role in defending the body against microbial pathogens, since it triggers both degradation of pathogens and subsequent presentation of pathogen-derived peptide antigen. TLR recognition of pathogens cause to expression of genes like inflammatory cytokines and costimulatory molecules. Phagocytosis-mediated antigen display together with TLR-dependent gene expression of inflammatory cytokines and co-stimulatory molecules, instruct development of antigen-specific acquired immunity (Fig. 5). For this, it is important to describe the relationship between phagocytosis and TLRs. In the loss of TLR2/TLR4 or MyD88, a common adaptor in TLR signaling, phagocytosis of bacteria inclusive Escherichia coli, Salmonella typhimurium and *Staphylococcus aureus* has been shown to be impaired cause to impaired phagosome maturation [60].

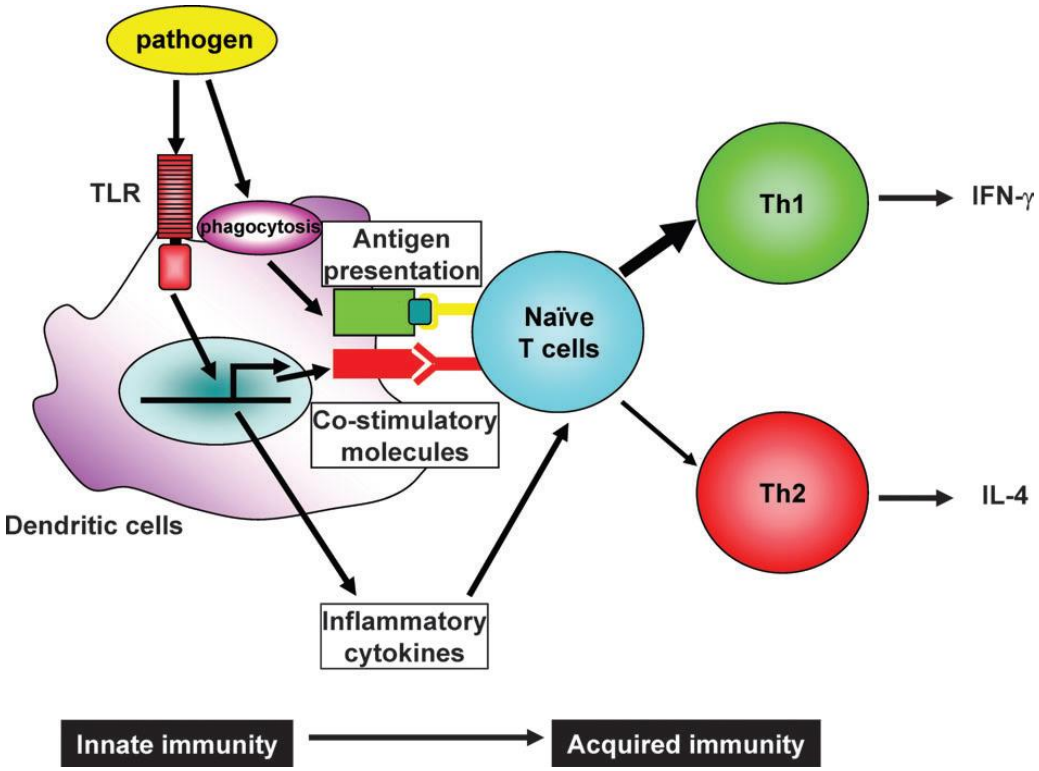

Figure 5. Innate and adaptive immunity. Innate immune cells, like dendritic cells and macrophages, engulf pathogens by phagocytosis, and present pathogen-derived peptide antigens to naıve T cells. furthermore, TLRs recognize pathogen-derived components and induce expression of genes, such as co-stimulatory molecules and inflammatory cytokines. Phagocytosis-mediated antigen presentation, with each other with TLR-mediated expression of co-stimulatory molecules and inflammatory cytokines, instruct development of antigen-specific adaptive immunity, particularly Th1 cells [60].

1.5.2. Macrophages and sepsis

Infections are fought in the body together cellular defenses, including monocytes, macrophages, and neutrophils, and humoral defenses merging antibodies and the complement pathways. Recognition of pathogens by extracellular CD14, TLR2, and TLR4 on the membranes of monocytes and macrophages cause of the

release of cytokines to activate cellular defenses [61-64]. Differentiation of T cells into type 1 helper cells (Th1) Because of cellular activation leads to differentiation of T cells into type 1 helper cells (Th1), which secrete proinflammatory cytokines such as interferon-γ (IFN- γ), interleukin-1β (IL-1β), IL-2, and IL-12, and type 2 helper cells (Th2), which secrete anti-inflammatory cytokines such as IL-4, IL-10, and IL-13. The degree to which these cytokines are released is a function of a lot of variables, including infectious circumstance, genetic susceptibility, and coexisting conditions. Trends in recent findings lead to the consensus that sepsis results from an imbalance in the host regulation of pro-inflammatory SIRS and the compensatory anti-inflammatory response syndrome.

1.5.2.1. Macrophage migration inhibitory factor

Macrophage migration inhibitory factor is a cytokine it has been newly discovered to be important in innate immunity and sepsis [65]. It is constitutively expressed in large quantity by by immune, endocrine, and epithelial cells and is quickly released after exposure to microbial products and pro-inflammatory cytokines. Macrophage migration inhibitory factor regulates innate immune responses to endotoxin and Gram negative bacteria by modulating the expression of TLR4, enable macrophages and other cells in the first line of defense for fast response [66]. Patients suffering from inflammatory and infectious diseases, including severe sepsis and septic shock was discovered to have high levels of macrophage migration inhibitory factor [67]. Immunoneutralization of macrophage migration inhibitory factor or cancellation of the Mif gene protects mice against lethal endotoxemia, Gram positive toxic shock syndromes, and experimental bacterial peritonitis. Conversely, mice injected with macrophage migration inhibitory factor together with live bacteria or microbial toxins have increased death rates [68, 69]. This factor thus has the possibility to Life-threatening when expressed in excess during sepsis. Development of remedy to prevent the production of macrophage migration inhibitory factor or inhibit its function may help treat severe sepsis and other inflammatory diseases. MIF

is a cytokine that which was described at the outset as a T-cell product and was later found to be produced by other cells such as pituitary cells, macrophages and monocytes (Figure 11). MIF is able of stimulation T cells and inducing pro-inflammatory cytokine production in macrophage [70, 71]. Administration of MIF induces lethality after infusion of LPS. MIF production by macrophages is induced by low levels of glucocorticoids, and MIF has the ability to overcome glucocorticoid-induced inhibition of pro-inflammatory cytokine production by monocytes [70].

1.5.3. TLR polymorphisms in sepsis

Sepsis is the leading cause of mortality and morbidity in intensive care units [2]. Repair of severe tissue injury (traumatic or non-traumatic) is highly complex, dynamic and interactive physiological, cellular, biochemical, and molecular process. This process involves coordinated recruitment, proliferation, and intracellular communications amongst multiple cell types including inflammatory cells, local and mobilized distant stem cell/progenitor cell populations, and vascular endothelial cells. The most serious complication of major injury is the sequential dysfunction of vital organs (Multiple Organ Dysfunction Syndrome, MODS), which is usually associated with severe sepsis [72]. Therefore, the prevention of sepsis is fundamental in the treatment of trauma patients. Genetic variants, in particular single nucleotide polymorphisms (Single Nucleotide Polymorphism, SNP) could be critical determinants of inter-individual differences of inflammatory response and clinical recovery of critically ill patients [73, 74]. Variations in the genes and their associated differences in response to injury may participate in the development of new genetic diagnostic and therapeutic interventions that can improve outcomes and recovery of patients with severe trauma.

Any cause that leads to infection, stress and tissue damage with delivery of danger signals is recognized by the immune system according to accepted "danger model" of the immune response. Cells of the immune system are activated by Pathogen-Associated Molecular Patterns (PAMPs) and Danger-Associated Molecular

Patterns (DAMPs). Inflammation occurs as the primary response to the presence of PAMPS and DAMPs that are recognized by the Pattern Recognition Receptors (PRRs). PRRs begin production of inflammatory mediators that can alter the function of many tissues and organs [75-77]. Toll-like receptors (TLRs) represent the most important PRR group. TLRs ligation can trigger intracellular signals that lead to phenotypic and functional changes in cells that are recognized appropriate ligands by these receptors [78]. The existence of 10 TLRs types is confirmed in humans. TLRs recognize and bind wide spectrum of PAMPs [79].

TLR2, TLR3 and TLR4 play the important role in inflammatory processes in different pathological conditions, including severe sepsis [80]. TLR2 is involved in the recognition of a wide spectrum of Gram-positive and Gram-negative bacterial molecules, fungi, parasites and viruses [81]. TLR4 is essential for recognition of bacterial lipopolisaharide (LPS). TLR4 interacts with three different extracellular proteins: LPS binding protein (LBP), CD14 and myeloid differentiation protein 2 (MD-2) [78]. Due to its activation, MyD88 and TRIF dependant pathways are activated which leads to the inflammatory cytokines induction [82]. TLR3 is the only TLR which does not use the MyD88 dependent pathway and TLR3 recognizes double-stranded RNA [79]. Although activation of innate immune response by PAMPs was reported to contribute to hyper-inflammation and organ injury during sepsis, many aspects of sepsis immunopathogenesis need further elucidation. It could be assumed that presence of SNPs in TLRs genes could compromise its function and contribute to complex phenotypes including severe sepsis. In previous paper from our group, we showed significant association of $CD14_{159}$ polymorphism with the type of infecting microorganism in critically ill patients [83]. Considering that TLR2, TLR3, and TLR4 play very important role in inflammatory processes by different signaling pathways, the question arises whether, presence of SNPs in these genes is associated with underlying cause of sepsis, the type of infecting microorganisms and outcome of critically ill patients. In addition, associations of these SNPs with development of secondary sepsis in trauma patients were analyzed. Analysis of the SNPs of the above

genes could be important in defining the pathogenetic mechanisms and potential therapeutic targets in these patients.

1.6. The role of vitamin D in sepsis

The prevalence of vitamin D insufficiency across the globe is high and rising. The reported prevalence varies depending on definitions but a recent review estimated that around 1 billion people worldwide have insufficient vitamin D levels [84]. Humans get vitamin D through synthesis in the skin and to a lesser extent from limited dietary sources (Figure 6).

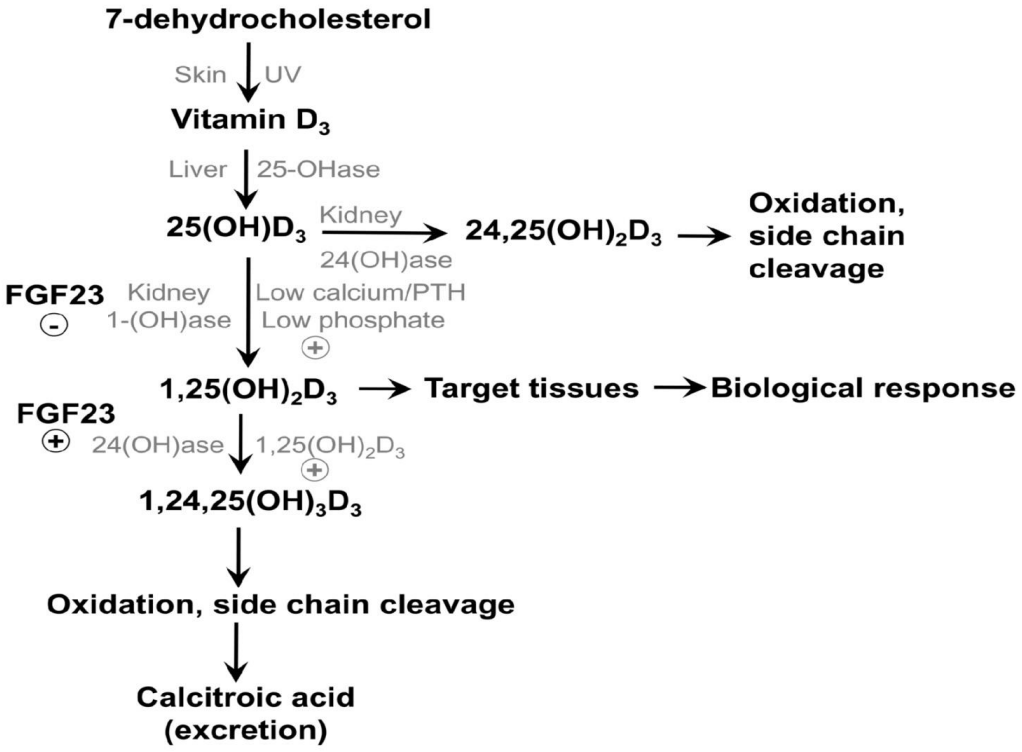

Figure 6. Vitamin D metabolism

Solar ultraviolet-B (UVB) radiation converts 7-dehydroxycholesterol in the skin to previtamin D. Skin synthesis of vitamin D is dependent upon the amount of UVB radiation and on individual characteristics like age and skin color. The intensity of UVB radiation varies according to latitude, season and time of the day. The further away from the equator people live, the more months of the year solar exposure is insufficient for vitamin D production in the skin. Other factors that influence vitamin D synthesis in the skin include age and skin pigmentation. Skin synthesis of vitamin D decreases with age and increased skin pigmentation reduces production because melanin in the epidermis absorbs UVB radiation. Lastly, sunscreen and clothing prevent vitamin D production [85]. Oily fish is the best dietary source of vitamin D and traditional western diets are generally low in vitamin D. Vitamin D from the skin or diet is metabolized primarily in the liver to 25D by the enzyme 25-hydroxylase (25(OH) ase). 25D is the "storage form" of vitamin D and is used to determine the vitamin D status of individuals [86].

1.6.1. Immunomodulatory role of vitamin D

The influence of VD_3 metabolites in the immune system, particularly of 1, 25(OH) $_2$ VD_3, has been known for more than 20 years. *In vitro*, 1, 25$(OH)_2$ vitamin D3 exerts a marked inhibitory effect on adaptive immune cells **(FIG. 2).** It inhibits T-cell proliferation [87, 88], the expression of IL-2 [88, 89] and IFN-γ mRNA and protein in T cells [90, 91]**,** and CD8 T-cell-mediated cytotoxicity [92]. The decrease in the production of IL-2 and IFNγ by 1,25$(OH)_2$ vitamin D_3 is partially mediated by binding of the VDR–RXR complex to the VDRE in the promoters of genes encoding IL-2 [93] and IFN-γ [94]**.** The anti-proliferative effect could be explained, at least in part, by the decrease in IL-2 production, as proliferation is partially rescued by adding exogenous IL-2 [87, 88]**.** These inhibitory effects of 1,25$(OH)_2$ vitamin D3 are most pronounced in the memory T-cell compartment [95, 96]**.** Which is concomitant with the higher expression of VDR in effector and memory T cells compared with naive T cells [97]**.** Moreover, 1,25$(OH)_2$ vitamin D3 enhances nonspecific T-cell suppressor

activity, as measured by the ability of 1,25$(OH)_2$ vitamin D3-treated T cells to suppress primary mixed-lymphocyte reactions and cytotoxic T-cell responses [92].

Overall, the net result of 1,25$(OH)_2$ vitamin D3 action on T cells is to block the induction of T-helper-1 (TH1)-cell cytokines, particularly IFNγ, while promoting TH2-cell responses, an effect mediated both indirectly by decreasing IFNγ production and directly by enhancing IL-4 production [98]. The activity of 1,25$(OH)_2$ vitamin D3 on effector T-cell differentiation is further enhanced by its effect on antigen-presenting DCs, in which it suppresses the synthesis of IL-12, a cytokine that promotes TH1-cell responses [99, 100]. Furthermore, 1,25$(OH)_2$ vitamin D3 also inhibits TH17-cell responses, probably owing in part to its capacity to inhibit IL-6 and IL-23 production [101], and induces the reciprocal differentiation and/or expansion of forehead box protein 3 (FOXP3)+ regulatory T (Treg) cells [102-104]. In addition to its inhibitory effects on T cells, 1,25$(OH)_2$ vitamin D3 decreases B-cell proliferation, plasma-cell differentiation and IgG secretion [87, 105] (Fig. 7). It has been suggested that the effect of 1, 25(OH) 2VD3 on B cells might be indirectly mediated through the effect it has on antigen-presenting-cell (APC) function and/or T-cell help38. Indeed, there are conflicting reports concerning the expression of VDR by B cells [87, 106, 107], leaving it unclear whether 1,25$(OH)_2$ vitamin D3 can act directly on B cells. Cells of the innate immune system can also be inhibited by 1, 25$(OH)_2$ vitamin D3 (FIG. 9), which is known to inhibit the differentiation, maturation and immunostimulatory capacity of DCs by decreasing the expression of MHC class II molecules and of CD40, CD80 and CD86 [98, 100, 108]. Furthermore, VDR-deficient mice have increased numbers of mature DCs in skin-draining lymph nodes. In addition, 1, 25(OH) 2VD3 decreases the synthesis of IL-12, [99, 100] and simultaneously increases the production of IL-10 by DCs. The net result is a decrease in TH1-cell responses and probably an induction of IL-10-producing T regulatory type 1 (TR1) cells [98].

Although 1, 25$(OH)_2$ vitamin D3 primarily has inhibitory effects on the adaptive immune response, some of its effects on innate immune cells are stimulatory. For example, 1, 25$(OH)_2$ vitamin D3 can stimulate human monocyte proliferation *in*

vitro [109] and has been shown to increase the production of both IL-1 and the bactericidal peptide cathelicidin by monocytes and macrophages [85, 89].

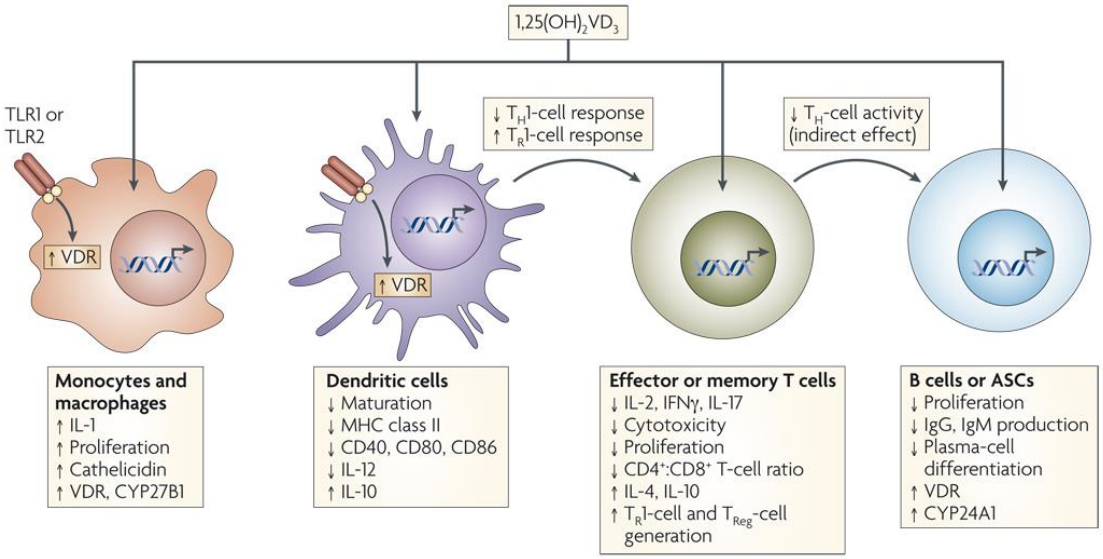

Figure . 7 Mechanisms of vitamin D immunomodulation

Systemic or locally produced $1,25(OH)_2$ vitamin D3 exerts its effects on several immune-cell types, including macrophages, dendritic cells (DCs), T and B cells. Macrophages and DCs constitutively express vitamin D receptor (VDR), whereas VDR expression in T cells is only upregulated following activation. In macrophages and monocytes, $1,25(OH)_2$ vitamin D3 positively influences its own effects by increasing the expression of VDR and the cytochrome P450 protein CYP27B1. Certain Toll-like-receptor (TLR)-mediated signals can also increase the expression of VDR. $1,25(OH)_2$ vitamin D3 also induces monocyte proliferation and the expression of IL-1 and cathelicidin (an antimicrobial peptide) by macrophages, thereby contributing to innate immune responses to some bacteria. $1,25(OH)_2$ vitamin D3 decreases DC maturation, inhibiting upregulation of the expression of MHC class II, CD40, CD80 and CD86. In addition, it decreases IL-12 production by DCs while inducing the production of IL-10. In T cells, $1, 25(OH)_2$ vitamin D3 decreases the

production of IL-2, IL-17 and interferon-γ (IFNγ) and attenuates the cytotoxic activity and proliferation of $CD4^+$ and $CD8^+$ T cells. $1,25(OH)_2$ vitamin D3 might also promote the development of forkhead box protein 3 (FOXP3) $^+$ regulatory T (T_{Reg}) cells and IL-10-producing T regulatory type 1 (T_R1) cells. Finally, $1,25(OH)_2$ vitamin D3 blocks B-cell proliferation, plasma-cell differentiation and immunoglobulin production by antibody-secreting cells.

1.6.1.1. Vitamin D and innate immunity

The innate immune response involves the activation of TLRs on polymorphonuclear cells, monocytes, macrophages and a number of epithelial cells. [110, 111]. Highlights the importance of vitamin D in both innate and adaptive immunity (Fig. 8) [112].

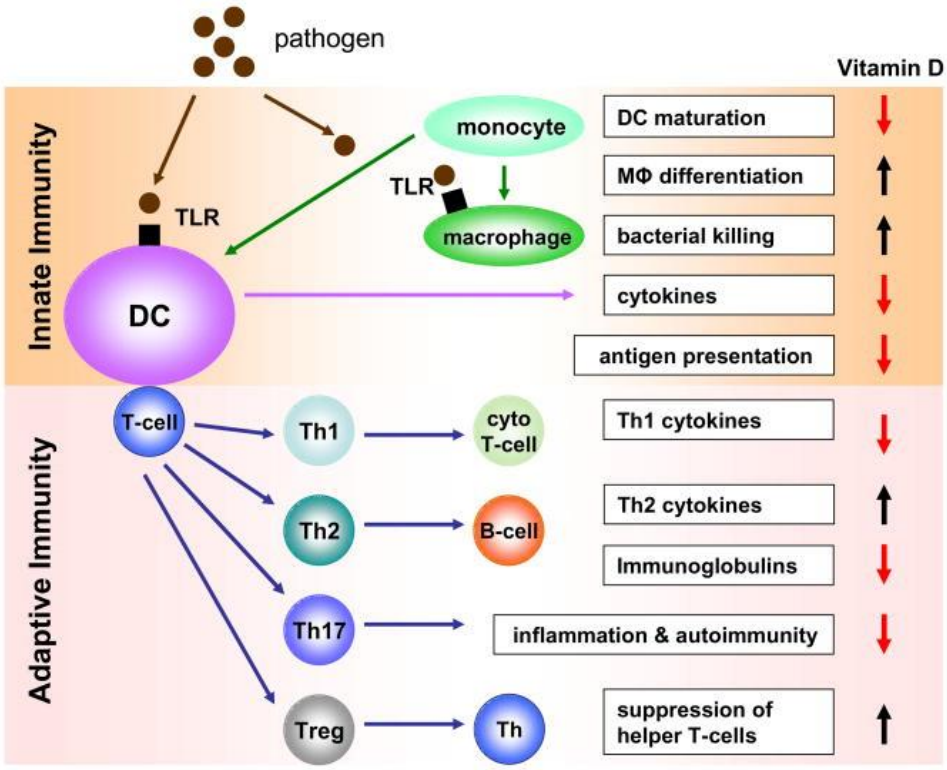

Figure 8. Effects of vitamin D on innate and adaptive immunity. Vitamin D regulates the innate and adaptive immune response to a pathogenic challenge.

Abbreviations: DC, dendritic cell; MØ, macrophage; TH, T-helper cell; TLR, Toll-like receptor; TREG, T regulatory cell. Reprinted from [112]

$1,25(OH)_2$ vitamin D3 primarily influences dendritic cell maturation and macrophage differentiation, and also reduces the release of cytokines [110- 114]. The use of vitamin D for the treatment of infections dates back more than 150 years. Successful use of cod liver oil, an excellent source of cholecalciferol, for the treatment of tuberculosis was reported in 1849 [115]. This result was supported by another study that examined the role of vitamin D in modulating innate immunity and showed a direct association between 25 (OH) vitamin D3 and $1,25(OH)_2$ vitamin D3 supplementation and anti-tuberculosis activity in human monocyte cell lines infected with *Mycobacterium tuberculosis* [116]. CYP27B1 converts 25OHD3 to 1,25(OH)2D3 [117, 118].

The activity of CYP27B1 in certain cells, such as keratinocytes, is regulated primarily by cytokines such as TNF and agonists of TLR pattern recognition receptors. Exposure of human keratinocytes to TNF and IFN-γ stimulates $1,25(OH)_2$ vitamin D3 production [119]. Activation of the TLR 2/1 heterodimer by *M. tuberculosis* has also been shown to upregulate the expression of *CYP27B1* and *VDR* genes in monocytes and macrophages, signifying the important role played by cytokines in producing the active form of vitamin D in these cells [120]. Similarly, stimulation of the complex of TLR4 and CD14 by lipopolysaccharide can increase CYP27B1 expression.

1.6.1.2. Vitamin D and adaptive immunity

The adaptive immune response is initiated by cells specializing in antigen presentation, including dendritic cells and macrophages, which are responsible for presenting antigens for specific recognition by T lymphocytes and B lymphocytes. These cells are capable of a wide repertoire of responses that ultimately determine the nature and duration of the immune response [121]. 1,25(OH) exerts an inhibitory

action on the adaptive immune system by modifying the capacity of APCs to induce T-lymphocyte activation, proliferation and cytokine secretion [87, 89, 121]. $1,25(OH)_2$ vitamin D3 decreases the maturation of dendritic cells, evidenced by the decreased expression of the co-stimulatory molecules HLA-DR, CD40, CD80, and CD86, which are required for antigen presentation and T-cell activation [98, 122]. $1,25(OH)_2$ vitamin D3 also inhibits the release of IL-12 [99] a cytokine responsible for stimulating T-helper 1 (TH1) cell development. The $1,25(OH)_2$ vitamin D3-induced alteration in the balance of TH1 cell subgroups ultimately leads to reduced production of IL-2, IFN-γ, and TNF, which are potent stimulators of inflammation [122, 123]. Collectively, $1,25(OH)_2$ vitamin D3 directly modulates T-cell proliferation and cytokine production, decreases the development of TH1 cells, inhibits TH17 cell development and increases the production of TH2 cells and T regulatory cells (TREG cells**)** (Fig. 9) [84].

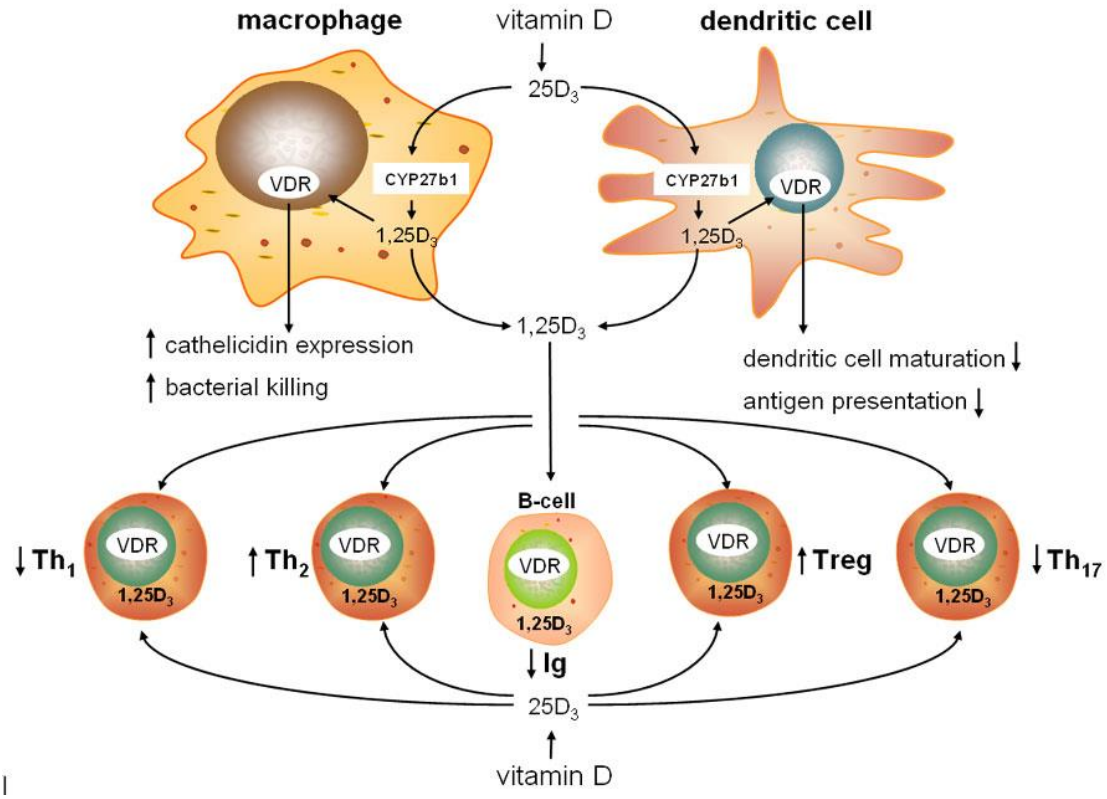

Figure 9. Vitamin D and innate and adaptive immunity [84]

Through these effects, $1,25(OH)_2$ vitamin D3 directly opposes the differentiation of TH cells towards a TH2 phenotype, which is typically observed in renal failure [124]. $1,25(OH)_2$ vitamin D3 induces CD4+/CD25+ TREG cells, [125] which are critical for the induction of immune tolerance [126]. B cells treated with 1,25(OH)2D3 show a reduction in proliferation and maturation to plasma cells, and decreased Ig production [127]. Therefore, $1,25(OH)_2$ vitamin D3 downregulates the adaptive immune response through multiple pathways.

1.6.1.3. Vitamin D and the inflammatory response

Endothelial cells are involved in immune activation during infections, sepsis and transplant rejection. Pretreatment of human microvassels endothelial cells with $1,25(OH)_2$ vitamin D3 inhibits Gram-negative bacterial LPS-induced activation of transcription factor NF-κB, and release of TNF, IL-6 and IL-8, as well as the C-C motif chemokine [127]. Treatment with $1,25(OH)_2$ vitamin D3 also inhibits vasodilatation of the vascular endothelium [127, 128]. Furthermore, $1,25(OH)_2$ vitamin D3 treatment is associated with improved blood coagulation parameters in sepsis-associated disseminated intravascular coagulation [129, 130]. One study examined the association between 25(OH) vitamin D3 levels and levels of the antimicrobial peptide cathelicidin (LL-37) in patients in an intensive care unit setting [131]. The critically ill patients had lower levels of 25(OH) vitamin D3, as well as lower systemic levels of LL-37, than did healthy controls. A couple of potential factors could explain these findings. First, decreased levels of vitamin D-binding protein lead to decreased circulating levels of 25(OH) vitamin D3 [132, 133]. Lower plasma levels of vitamin D-binding protein in early sepsis were shown to be prognostic for disease severity in rat models of the disease [134]. Second, patients with sepsis are likely to be elderly [135] unwell, obese, or have other risk factors for 25(OH) vitamin D3 deficiency [136].

Another important role for $1,25(OH)_2$ vitamin D3 involves regulating feedback control pathways that limit the potential inflammatory damage that could arise from excessive activation of the immune response [112, 114, 121]. $1,25(OH)_2$ vitamin D3 provides feedback regulation of immune activation pathways by downregulating expression of monocyte TLR2 and TLR4 in a dose-dependent and time-dependent manner [112]. Suppression of TLR expression by $1,25(OH)_2$ vitamin D3 activity might limit the extent of innate immune responses, such as heightened inflammatory T-lymphocyte responses that would otherwise promote autoimmunity mediated by Th1 lymphocytes. The inflammatory response usually activated by these receptors is thus suppressed [128]. Other studies confirm that $1,25(OH)_2$ vitamin D3 suppresses LPS-induced TNF production in a dose-dependent manner [137, 138]. This finding is of major importance given that the innate immune response, initiated by microbial sensors, could harm the host by a severely heightened TLR-mediated proinflammatory response [139]. $1,25(OH)_2$ vitamin D3 could therefore have an important role in maintaining the inflammatory response to immune challenge within physiological limits.

1.6.1.4. Vitamin D and the Sepsis Cascade

Monocytes play important roles in the innate immune system as antigen presenting cells as well as in phagocytosis. Human monocytes recognize some PAMPs by a family of transmembrane molecules, the Toll-Like Receptors (TLRs). TLR4 specifically recognizes and binds to LPS, a substance produced by gram-negative bacteria and a potent stimulator of the sepsis inflammatory cascade. Sadeghi et al. [128] demonstrated that human monocytes stimulated with LPS and treated with 1,25 dihydroxyvitamin D ($1,25(OH)_2$ D), showed dose-dependent decreases in TLR2 and TLR4 synthesis, with an increase in CD14, a TLR co-stimulatory molecule. They further found that $1,25(OH)_2$ vitamin D decreased TNFα and tissue factor, both end products of LPS activation and important inflammatory molecules in sepsis. These effects were reversed with the introduction of a VDR antagonist, reinforcing a key

role of vitamin D in this signaling mechanism [128]. Further studies have revealed a role for vitamin D in the endothelial response to LPS. In sepsis, LPS activates endothelial cells to produce transcription factor NF-kB, the pro-inflammatory cytokines IL-6 and IL-8, and the chemokine, RANTES. In the study by Equils et al. [127]**,** human endothelial cells treated with $1,25(OH)_2$ vitamin D then stimulated with LPS, showed significant inhibition of these molecules when compared with cells only exposed to LPS. These findings may suggest that vitamin D acts to modulate the pro-inflammatory endothelial response to LPS. Over the past two decades, these intriguing vitamin D-dependent cellular responses to LPS have also been studied in rat and mouse models of sepsis. Horiuchi et al. [140] exposed mice simultaneously to intraperitoneal LPS and oral $1,25(OH)_2$ vitamin D. Compared with controls, mice that received vitamin D had less expression of the inflammatory molecule, iTXB2, and a decrease in mortality. In 2001, Asakura et al. [129] demonstrated that compared with low-molecular weight heparin, treatment with oral $1,25(OH)_2$ vitamin D had equal or improved effects on hemostatic parameters and markers of organ dysfunction in rats infused with LPS**.** In 2007, Moller et al. performed placebo controlled trials of treatment with $1,25(OH)_2$ vitamin D in three different rat models of sepsis showing varied results [130].

While the different models of sepsis and vitamin D treatments in these experiments make them difficult to compare, when combined with the in vitro data they suggest that vitamin D has important modulatory effects on the innate immune response to LPS-induced sepsis. While LPS is an important molecule in gram-negative sepsis, vitamin D may also have a role in the sepsis cascade induced by fungal organisms. A study by Khoo et al. treated peripheral blood mononuclear cells (PBMC) with $1,25(OH)_2$ vitamin D and exposed them to *C. albicans*. The PBMCs demonstrated significant dose-dependent decreases in production of pro-inflammatory cytokines with a decrease in expression of the PRRs that recognize *C. albicans*. [141]. The multiple functions of vitamin D in the immune system's response to infection suggest it may be an integral component in combating sepsis (Fig. 10) [142]. Venn diagram reflecting the links between vitamin D's roles in innate immune function,

clinical infections and sepsis in the critically ill. The intersections represent the potential increased morbidity and mortality resulting from vitamin D insufficient states predisposing to and exacerbating sepsis.

The basic science data point toward vitamin D's role in the optimal functioning of the innate immune system, in part by producing AMPs such as IL-37; while seeming to temper the inflammatory cascade induced by LPS [142]. The early clinical data on its role in preventing and attenuating infections has suggested a link but intervention trials have produced mixed results, requiring larger randomized controlled trials to help define the relationship. Furthermore, clinical data also point toward a role of vitamin D and critical illness but a direct relationship with sepsis and its severity and outcomes is yet to be determined by further research. Some interesting parallel patterns between vitamin D and seasonal and racial variations in sepsis are currently speculative but interesting questions to be explored. In conclusion, the current picture of vitamin D and sepsis is one of a research field early in its course with many important links that provide fertile ground for further investigation. Such investigation is warranted as vitamin D is inexpensive and safe to administer and even incremental benefits in the outcomes of sepsis may be enacted on a scale to produce a significant public health impact [142].

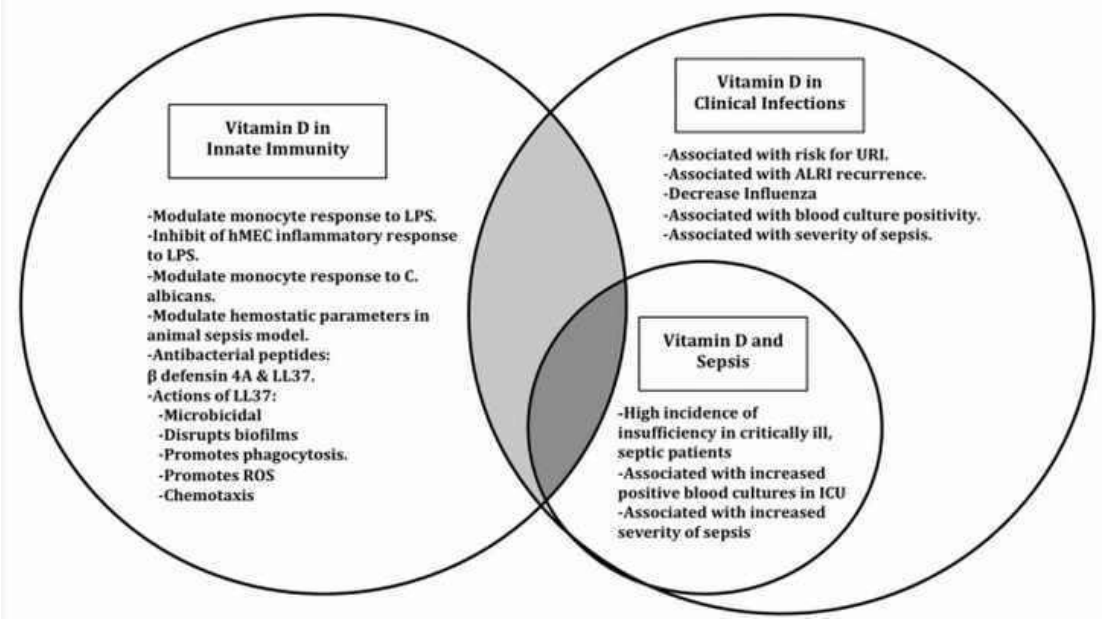

Figure 10. Morbidity and mortality vitamin D insufficiency and sepsis.

2. HYPOTEHSIS AND AIMS

The hypothesis of this thesis is that the TLR and VDR polymorphisms are significant for the course and outcome of the sepsis in critically ill patients in Serbia.

For testing of this hypothesis we studied group of 121 critically-ill patients with severe sepsis and/or trauma on admission to surgical intensive care unit at Military Medical Academy in Belgrade.

1 - The first aim of this study was to examine the polymorphisms of TLRs and VDR in these patients including:

1- *TLR2* polymorphism rs5743708,
2- *TLR3* polymorphism rs3775291,
3- *TLR3* polymorphism rs5743312,
4- *TLR4* polymorphism rs4986790,
5- *TLR4* polymorphism rs4986791,
6- *VDR FokI* polymorphism rs2228570,
7- *VDR TaqI* polymorphism rs731236,
8- *VDR ApaI* polymorphism rs7975232
9- *VDR EcoRV* polymorphism rs4516035

2 – Further, the aim of this study was to examine association of these polymorphisms with outcome, underlying cause of sepsis (pancreatitis, peritonitis) or the type of infecting microorganisms (Gram-positive, Gram-negative, mixed) in Serbian critically ill patients. In addition, in trauma patients associations of these polymorphisms with development of secondary sepsis were analyzed.

3. MATERIAL AND METHODS

3.1. Ethics statements

This study was approved by the Ethics Committee of the Military Medical Academy, according to the Helsinki Declaration from 2008. All participants provided informed consent, and institutional review boards reviewed and approved the procedures at all sites.

3.2. Study group and samples

Study group consisted of 121 critically-ill patients with severe sepsis and/or trauma on admission to surgical intensive care unit (ICU) at Military Medical Academy (Belgrade, Serbia) from July 2010 until May 2012. The Simplified Acute Physiology Score II [143], Acute Physiology and Chronic Health Evolution II score [144], and Sequential Organ Failure Assessment score [145] at 24 hours after ICU admission were calculated and recorded.

Determination of trauma severity was performed using the Injury Severity Score (ISS). ISS was determined using Abbreviated Injury Scale. Great majority of trauma patients were casualties from motor vehicle accidents with blunt and/or penetrating trauma. All patents with trauma with or without development of sepsis had the similar severity of trauma. Sepsis patients entered the study if they had met following criteria (according to 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference): documented or suspected infection plus presence of systemic inflammatory response syndrome and sepsis-associated organ dysfunction, hypotension, hypoperfusion (hyperlactatemia >2mmol/L). Exclusion criteria included age below 18 years, pregnancy, severe chronic respiratory disease, severe chronic

liver disease, malignancy, use of high-dose immunosuppressive therapy, and AIDS. The reduced to potential confounding from ethnic backgrounds, only Caucasians of the Serbian population was enrolled in this study. Informed consent was obtained from subjects or from their legal surrogates before enrollment. The follow-up period was one year.

Critically ill patients were divided in subgroups according to the cause of critically status and present of sepsis and after that were analyzed their demographic and clinical characteristics. The 80 critically ill patients had severe sepsis without trauma. Of 41 trauma patients, 20 developed secondary sepsis.

Peripheral blood samples were collected from the patients and stored at -20^{0}C, until DNA isolation.

3.3. DNA isolation and genotyping

Peripheral blood monocytes (PBMCs) from approximately 30 ml of heparinized peripheral blood were isolated through Ficoll density gradient centrifugation according to standard procedures. Cell preparations were routinely assessed for viability (>95%) by trypan blue dye exclusion.

DNA was isolated using the GeneJet Genomic DNA Purification kit according to the manufacturer's instructions (Fermentas, St. Leon-Rot, Germany). Polymorphisms in TLR2, TLR3, TLR4, and VDR genes were determined by Real Time PCR method, using the TaqMan SNP Genotyping Assays (Applied Biosystems, Foster city, CA, USA).

We analyzed polymorphisms of further genes:

1- *TLR2* polymorphism rs5743708,

2- *TLR3* polymorphism rs3775291,

3- *TLR3* polymorphism rs5743312,

4- *TLR4* polymorphism rs4986790,

5- *TLR4* polymorphism rs4986791,

6- *VDR FokI* polymorphism rs2228570,

7- *VDR TaqI* polymorphism rs731236,

8- *VDR ApaI* polymorphism rs7975232

9- *VDR EcoRV* polymorphism rs4516035

Obtained genotypes were analyzed using the SDS 7500 Software (Applied Biosystems). Details on used TaqMan Assays are available upon request.

3.4. Statistical analysis

Statistical analysis was performed using the SPSS software, version 20.00 (SPSS Ins., Chicago, IL, USA).

Descriptive statistics were presented as mean values with standard deviation or as absolute numbers with percentages for categorical variables. Evaluation of normality was performed with Kolmogorov-Smirnov test.

Student t-test was used to calculate differences between mean values. Mann-Whitney *U*-test was used to determine differences between median values. χ^2 –test was used to test cross tabulation for categorical variables between studied groups. For survival analysis Kaplan-Meier curves was used and compared by the log-rank test. Finally, because variables are inter-related, multivariate regression analysis, stepwise method, was performed to assess the independent variables that may explain outcome of critically ill patients. The Pearson coefficient was used for linear correlation between variables.

The probability of F was used to select the variables to be included in the model, the variables with p-values less than 0.05 were entered and variables with p-values larger than 0.10 were removed from the model. All reported p values were two-sided and considered as significant if were less than 0.05.

4. RESULTS

4.1. Demographic and clinical characteristics of critically ill patients and healthy subjects

From June 2010 until May 2012, 121 critically ill patients were diagnosed at surgical intensive care unit (ICU) at Military Medical Academy (Belgrade, Serbia). Some 101 patients developed sepsis. The general characteristics of 121 critically ill patients and 104 healthy subjects from Serbia are summarized in Table 1. No difference in age, sex, and ethnic background were found between patients and healthy subjects. In 45 patients (37 per cent) no pathogen was identified in blood cultures as the causative microorganism. The source of infection was mainly of abdominal and trauma origin. The mixed infection was the most frequent cause of sepsis (41%), further in 13% patients were detected infection with Gram positive pathogen, infection with Gram negative pathogen, fungi, and combined mixed pathogen and fungi were detected in several per cent (from 2 to 4%). The follow-up period was one year. 57 critically ill patients were death during hospitalization within 90 days, but 70% of these patients death within 28 days.

Table 1. Demographic and clinical characteristics of critically ill patients and healthy subjects

	Critically ill patients n=121	Healthy subjects n = 104
Age (years)	56 ± 19	52 ± 8
Sex, n (%)		
Male	76(63)	90 (86)
Female	45(37)	14(14)
Simplified Acute Physiology Score II, mean ± SD	56.70 ± 9.10	
Acute Physiology and Chronic Health Evolution II score, mean ± SD	23.07 ± 3.20	
Sequential Organ Failure Assessment score, mean ± SD	7.20 ± 2.56	
Reason for ICU admission, n (%)		
Severe sepsis without trauma	80 (74)	
Severe trauma (ISS 27.8 ± 9.8)	41 (26)	
Cause of sepsis, n (%)		
Pancreatitis	17 (17)	
Peritonitis	63 (63)	
Trauma	20 (20)	
Blood culture, n (%)		
Sterile	45 (37)	
Mixed	49 (41)	
Mixed + fungi	4 (3)	
Gram-positive	16 (13)	
Gram-negative	5 (4)	
Fungi	2 (2)	
Outcome, n (%)		
Death	57 (47)	
Survival	64 (53)	
Mortality, n (%)		
< 28 days	39 (68)	
< 90 days	14 (24)	
> 90 days	2 (4)	
> 12 months	2 (4)	

Data are expressed as mean ± SD or absolute number (percentage)

ISS, Injury Severity Score; ICU, Intensive Care Unit

The demographic and clinical characteristics of critically ill patients divided in subgroups according cause of critically status and present of sepsis are showed in Table 2. The 70 critically ill patients had severe sepsis. Differences in age, sex, pathogen in blood and outcome were detected between subgroups of critically ill patients. The most number of patients with sepsis had peritonitis as cause and these patients were the oldest compared to other subgroups of patients. The other major disease associated with sepsis was necrotizing pancreatitis. The third of critically ill patients sustained severe trauma. Determination of trauma severity was performed using the Injury Severity Score (ISS). ISS (mean value, 27.8 ± 9.8) was determined using Abbreviated Injury Scale. Of 41 trauma patients, 20 developed secondary sepsis. The trauma patients with sepsis was younger compared to other subgroups of patients. In the most of patients with sepsis/pancreatitis no pathogen was identified in blood cultures. On the other side, in 51% of patients with sepsis/peritonitis as causative microorganisms was detected mixed pathogen. In the 70% of trauma patients with sepsis no pathogen was identified, as well. Further, the highest mortality range was detected in patients with sepsis/peritonitis. On the other side, in the subgroup of trauma patients without sepsis was detected the highest level of survival.

Table 2. Demographic and clinical characteristics of critically ill patients divided in subgroups according cause of critically ill status

	Patients with sepsis/pancreatitis	Patients with sepsis/peritonitis	Patients with trauma and sepsis	Patients with trauma without sepsis	p value
	n = 17	n = 63	n = 20	n = 21	
Age (years)	52 ± 16	63 ± 17	39 ± 16	54 ± 19	*,#,§§§,††
Sex female/male, n (%)	5(29)/12(71)	34(54)/29(46)	2 (10)/18(90)	4 (19)/17(81)	°°
Pathogen in blood, n (%)					°°
Sterile	11 (64)	13 (21)	13 (65)	21 (100)	
Mixed	4 (24)	32 (51)	0 (0)	0 (0)	
Mixed + fungi	2 (12)	2 (3)	0 (0)	0 (0)	
Gram-positive	0 (0)	11 (17)	5 (25)	0 (0)	
Gram-negative	0 (0)	4 (6)	1 (5)	0 (0)	
Fungi	0 (0)	1 (2)	1 (5)	0 (0)	
Outcome, n (%)					°°
Death	7 (41)	40 (64)	6 (30)	4 (19)	
Survival	10 (59)	23 (36)	14 (70)	17 (81)	
Mortality, n (%)					
< 28 days	4 (57)	28 (70)	3 (50)	4 (100)	
< 90 days	1 (14)	11 (27)	2 (33)	0 (0)	
< 3 > 12 months	2 (29)	0 (0)	0 (0)	0 (0)	
> 12 months	0 (0)	1 (3)	1 (17)	0 (0)	

Data are expressed as mean ± SD or absolute number (percentage)

°° p <0.01 between groups one way ANOVA

* p <0.05, patients with sepsis pancreatitis *vs* patients with sepsis peritonitis

# p <0.05, patients with sepsis pancreatitis *vs* patients with trauma and sepsis

§§§ p <0.0001, patients with sepsis peritonitis *vs* patients with trauma and sepsis

†† p <0.01, patients with trauma and sepsis *vs* patients with trauma without sepsis

4.2. Allele frequencies and genotype distribution in single-nucleotide polymorphisms of several TLR and VDR genes

The polymorphisms prevalence in patients with sepsis/trauma and the control subjects are presented in Table 3. Results of this analysis showed significant allele frequency differences at two SNPs. The statistically significant differences in the frequencies of the TLR and VDR genotypes were observed between the critically ill patients and healthy subjects for the TLR2 (rs3804099) and VDR FokI polymorphisms (rs2228570). For the other TLR and VDR polymorphisms investigated in this study, the differences between the frequencies determined in the critically ill patients and healthy subjects were not observed.

The genotype distribution of the all SNPs was in agreement with the Hardy-Weinberg equilibrium, indicating that the allele and genotype frequencies of these SNPs in the population remained in the equilibrium from generation to generation as a very important genes for immunity.

Table 3. Distribution of TLR and VDR polymorphisms in the group of critically ill patients and healthy subjects

		Cases	Controls			Cases	Controls	
Gene/SNP	**Genotype**	**N**	**N**	**p**	**Allele**	**N**	**N**	**p**
	CC (*wt*)	15	0		C	105	67	
TLR2	CT (*het*)	75	67	**0.001**				**0.015**
rs3804099	TT (*mut*)	31	37		T	137	141	
	CC (*wt*)	91	78		C	210	180	
TLR3	CT (*het*)	28	24	0.988				0.941
rs5743312	TT (*mut*)	2	2		T	32	28	
	GG (*wt*)	54	43		G	160	139	
TLR3	GA (*het*)	52	53	0.347				0.873
rs3775291	AA (*mut*)	15	8		A	82	69	
	CC (*wt*)	108	90		C	229	193	
TLR4	CT (*het*)	13	13	0.507				0.421
rs4986791	TT (*mut*)	0	1		T	13	15	
	AA (*wt*)	108	93		A	229	197	
TLR4	AG (*het*)	13	11	0.968				0.969
rs4986790	GG (*mut*)	0	0		G	13	11	
	TT (*wt*)	19	36		T	97	127	
VDR FokI	TC (*het*)	59	55	**0.000**				**0.000**
rs2228570	CC (*mut*)	43	13		C	145	81	
	CC (*wt*)	23	24		C	101	84	
VDR ApaI	CA (*het*)	53	36	0.367				0.828
rs7975232	AA (*mut*)	45	44		A	143	124	
	TT (*wt*)	43	49		T	148	139	
VDR TaqI	TC (*het*)	62	41	0.170				0.212
rs731236	CC (*mut*)	16	14		C	94	69	
	TT (*wt*)	29	28		T	127	100	
VDR EcoRV	TC (*het*)	69	44	0.056				0.352
rs4516035	CC (*mut*)	23	32		C	115	108	

Data are expressed as absolute number (percentage)

* p <0.05, patients with sepsis pancreatitis *vs* patients with sepsis peritonitis

# p <0.05, patients with sepsis pancreatitis *vs* patients with trauma and sepsis

§§§ p <0.0001, patients with sepsis peritonitis *vs* patients with trauma and sepsis

†† p <0.01, patients with trauma and sepsis *vs* patients with trauma without sepsis

The polymorphisms prevalence in the subgroups of critically ill patients is presented in Table 4. Results of this analysis showed no significant allele frequency differences at all SNPs.

Table 4. Distribution of TLR and VDR polymorphisms in the subgroups of critically ill patients formed according cause of critically ill status

Gene/SNP genotype	Patients with sepsis/pancreatitis N (%)	Patients with sepsis/peritonitis N (%)	Patients with trauma and sepsis N (%)	Patients with trauma without sepsis N (%)	p value
	n = 17	n = 63	n = 20	n = 21	
TLR2 (rs3804099)					
CC (*wt*)	3 (18)	6 (10)	4 (20)	2 (9)	
CT (*het*)	8 (47)	43 (68)	11 (55)	13 (62)	
TT (*mut*)	6 (35)	14 (22)	5 (25)	6 (29)	
TLR3 (rs5743312)					
CC (*wt*)	13 (77)	48 (76)	15 (75)	15 (71)	
CT (*het*)	4 (23)	13 (21)	5 (25)	6 (29)	
TT (*mut*)	0 (0)	2 (3)	0 (0)	0 (0)	
TLR3 (rs3775291)					
GG (*wt*)	10 (59)	26 (41)	9 (45)	9 (43)	
GA (*het*)	3 (18)	28 (45)	9 (45)	12 (57)	
AA (*mut*)	4 (23)	9 (14)	2 (10)	0 (0)	
TLR4 (rs4986791)					
CC (*wt*)	14 (82)	57 (91)	18 (90)	19 (91)	
CT (*het*)	3 (18)	6 (9)	2 (10)	2 (9)	
TT (*mut*)	0 (0)	0 (0)	0 (0)	0 (0)	
TLR4 (rs4986790)					
AA (*wt*)	14 (82)	58 (92)	17 (85)	19 (91)	
AG (*het*)	3 (18)	5 (8)	3 (15)	2 (9)	
GG (*mut*)	0 (0)	0 (0)	0 (0)	0 (0)	
VDR FokI					
TT (*wt*)	2 (12)	12 (19)	13 (65)	6 (29)	
TC (*het*)	10 (59)	32 (51)	7 (35)	6 (29)	
CC (*mut*)	5 (29)	19 (30)	0 (0)	9 (42)	

VDR ApaI				
CC (*wt*)	2 (12)	14 (22)	4 (20)	3 (14)
CA (*het*)	10 (59)	28 (45)	6 (30)	9 (43)
AA (*mut*)	5 (29)	21 (33)	10 (50)	9 (43)
VDR TaqI				
TT (*wt*)	5 (29)	22 (35)	9 (45)	7 (33)
TC (*het*)	11 (65)	34 (54)	8 (40)	9 (43)
CC (*mut*)	1 (6)	7 (11)	3 (15)	5 (14)
VDR EcoRV				
TT (*wt*)	5 (29)	13 (21)	4 (20)	7 (33)
TC (*het*)	7 (42)	40 (64)	13 (65)	9 (43)
CC (*mut*)	5 (29)	10 (16)	3 (15)	5 (14)

Data are expressed as absolute number (percentage)

4.3. Genotype and allele distribution in TLRs genes polymorphisms in critically ill patients

The distribution of analyzed polymorphisms in TLRs genes in the group of Caucasian Serbian critically ill patients is presented in Table 5. Percentage of alleles for investigated *TLR2* gene polymorphism was similar. On the other side, distribution of *wild type* C and G alleles for *TLR3* polymorphisms rs5743312 and rs3775291 were higher compared to mutated T and A alleles, respectively. There were no mutated genotypes of both investigated *TLR4* polymorphisms in our patients that were reflected in the significant difference in the distribution of alleles of this gene.

Table 5. Distribution of TLRs genes polymorphisms in the group of critically ill patients

Gene/SNP	Genotype	N (%)	Allele	N (%)
	CC (*wt*)	15 (12)	C	105 (43)
TLR2	CT (*het*)	75 (62)		
rs3804099	TT (*mut*)	31 (26)	T	137 (57)
	CC (*wt*)	91 (75)	C	210 (87)
TLR3	CT (*het*)	28 (23)		
rs5743312	TT (*mut*)	2 (2)	T	32 (13)
	GG (*wt*)	54 (45)	G	160 (66)
TLR3	GA (*het*)	52 (43)		
rs3775291	AA (*mut*)	15 (12)	A	82 (34)
	CC (*wt*)	108 (89)	C	229 (95)
TLR4	CT (*het*)	13 (11)		
rs4986791	TT (*mut*)	0 (0)	T	13 (5)
	AA (*wt*)	108 (89)	A	229 (95)
TLR4	AG (*het*)	13 (11)		
rs4986790	GG (*mut*)	0 (0)	G	13 (5)

Wt/het/mut- wild type/heterozygote/mutated

The polymorphisms prevalence in the subgroups of critically ill patients is presented in Table 6. There were no significant differences in genotype frequencies for all SNPs studied among analyzed subgroups. It should be noted that only patients with *TLR3* (rs5743312) mutated genotype had sepsis with peritonitis as underlying cause. On the other side, patients with trauma and mutation in *TLR3* rs3775291 polymorphism (10% of trauma patients with sepsis) developed sepsis.

Table 6. Distribution of TLRs genes polymorphisms in the subgroups of critically ill patients

Gene/SNP genotype	Patients with sepsis/pancreatitis	Patients with sepsis/peritonitis	Patients with trauma without sepsis	Patients with trauma and sepsis	p value
	n = 17	n = 63	n = 21	n = 20	
TLR2 (rs3804099), n (%)					
CC (*wt*)	3 (18)	6 (10)	2 (9)	4 (20)	
CT (*het*)	8 (47)	43 (68)	13 (62)	11 (55)	NS
TT (*mut*)	6 (35)	14 (22)	6 (29)	5 (25)	
TLR3 (rs5743312), n (%)					
CC (*wt*)	13 (77)	48 (76)	15 (71)	15 (75)	
CT (*het*)	4 (23)	13 (21)	6 (29)	5 (25)	NS
TT (*mut*)	0 (0)	2 (3)	0 (0)	0 (0)	
TLR3 (rs3775291), n (%)					
GG (*wt*)	10 (59)	26 (41)	9 (43)	9 (45)	
GA (*het*)	3 (18)	28 (45)	12 (57)	9 (45)	NS
AA (*mut*)	4 (23)	9 (14)	0 (0)	2 (10)	
TLR4 (rs4986791), n (%)					
CC (*wt*)	14 (82)	57 (91)	19 (91)	18 (90)	
CT (*het*)	3 (18)	6 (9)	2 (9)	2 (10)	NS
TT (*mut*)	0 (0)	0 (0)	0 (0)	0 (0)	
TLR4 (rs4986790), n (%)					
AA (*wt*)	14 (82)	58 (92)	19 (91)	17 (85)	
AG (*het*)	3 (18)	5 (8)	2 (9)	3 (15)	NS
GG (*mut*)	0 (0)	0 (0)	0 (0)	0 (0)	

Data are expressed as an absolute number (percentage)

NS, non-significant; statistical analysis performed by χ^2 test

Wt/het/mut- wild type/heterozygote/mutated

Further, we analyzed the presence of different TLR mutations. This analysis showed the simultaneous presence of TLR2 rs3804099 and TLR3 rs5743312 mutations only in three patients (table 7). There were no presences of other simultaneous combination of TLR mutations.

Table 7. Distribution of simultaneously TLRs genes polymorphisms in the critically ill patients

Mut_TLR2_TLR3

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	samo TLR2	28	12.4	23.1	23.1
	TLR2 i TLR3.2	3	1.3	2.5	25.6
	samo TLR3.1	2	.9	1.7	27.3
	samo TLR3.2	12	5.3	9.9	37.2
	NIJEDNA	76	33.8	62.8	100.0
	Total	121	53.8	100.0	
Missing	System	104	46.2		
Total		225	100.0		

4.4. Associations of TLRs genes polymorphisms with clinicopathological variables in critically ill patients

Associations of TLRs genes polymorphisms with clinicopathological variables are presented in Table 8. The *TLR3* rs3775291 was associated with patient's outcome (p=0.018). Significant tendency toward association between *TLR3* rs3775291 polymorphism and type of detected pathogen in blood was noticed (p=0.078). No significant associations were observed between the other TLRs genes polymorphisms and analyzed variables characteristics in the critically ill patients (Table 7). Patients with sepsis and *TLR3* rs3775291 mutated genotype had four times higher mortality rate compared to the *wild type* and heterozygous carriers.

Table 8. Association of TLR polymorphisms with sex diagnosis, blood cultures, mortality and outcome of critically ill patients

	N	**TLR2 (rs3804099)** *wt/het/mut*	**TLR3 (rs5743312)** *wt/het/mut*	**TLR3 (rs3775291)** *wt/het/mut*	**TLR4 (rs4986791)** *wt/het/mut*	**TLR4 (rs4986790)** *wt/het/mut*
Sex						
Male	76	9/46/21	56/19/1	34/35/7	70/6/0	69/7/0
Female	45	6/29/10	35/9/1	20/17/8	38/7/0	39/6/0
p^*		NS	NS	NS	NS	NS
Diagnosis						
Sepsis pancreatitis	17	3/8/6	13/4/0	10/3/4	14/3/0	14/3/0
Sepsis peritonitis	63	6/43/14	48/13/2	26/28/9	57/6/0	58/5/0
Trauma with sepsis	20	4/11/5	15/5/0	9/9/2	18/2/0	17/3/0
Trauma	21	2/13/6	15/6/0	9/12/0	9/12/0	9/12/0
p^*		NS	NS	NS	NS	NS
Blood culture						
Sterile	45	5/25/15	30/15/0	21/23/1	39/6/0	39/6/0
Mixed	49	7/36/6	37/10/2	20/21/8	44/5/0	45/4/0
Mixed with fungi	4	1/2/1	4/0/0	2/0/2	4/0/0	4/0/0
Gram positive	16	2/8/6	13/3/0	8/5/3	15/1/0	15/1/0
Gram negative	5	0/2/3	5/0/0	2/3/0	4/1/0	3/2/0
Fungi	2	0/2/0	2/0/0	1/0/1	2/0/0	2/0/0
p^*		NS	NS	0.078	NS	NS
Outcome						
Death	57	8/39/10	41/15/1	21/24/12	49/8/0	49/8/0
Survival	64	7/36/21	50/13/1	33/28/3	59/5/0	59/5/0
p^*		NS	NS	**0.018**	NS	NS
Mortality						
< 28 days	39	6/27/6	25/13/1	15/18/6	35/4/0	35/4/0
< 90 days	14	0/10/4	12/2/0	6/4/4	12/2/0	12/2/0
< 3 > 12 months	2	1/1/0	2/0/0	0/1/1	1/1/0	1/1/0
> 12 months	2	1/1/0	2/0/0	0/1/1	1/1/0	1/1/0
p^*		NS	NS	NS	NS	NS

N total number of patients

NS, non-significant; statistical analysis performed by χ^2 test

[a] Age according to median value of 56 years

*p<0.05 are presented in bold

4.5. Survival analysis according to TLR polymorphism in critically ill patients

The significant decrease of overall survival was observed in critically ill patients with mutated genotype in *TLR3* rs3775291 polymorphism, compared to the patients with *wild type* and heterozygous genotype (p=0.029, log-rank test) (Figure 1). There were no significant differences of overall survival for the other studied TLRs genes polymorphisms (data not shown).

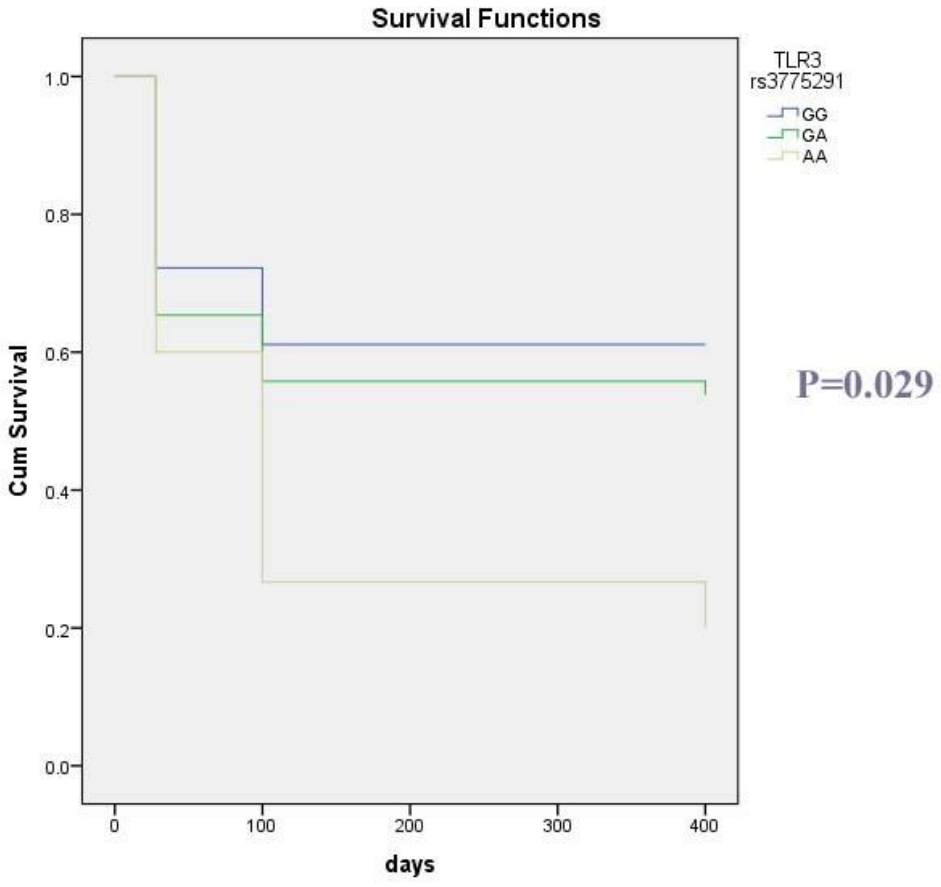

Figure 1. Kaplan-Meier curve of overall survival of critically ill patients' dependent of TLR3 rs3775291 polymorphisms compared by the long-rank test.

4.6. Multivariate regression analysis according to TLR polymorphism in critically ill patients

Multivariate regression analysis with stepwise model was used to asses the independent variables that may affect outcome of sepsis (survival) (Table 9). The variables entered in the model as independent variables were the following: age, sex, and all investigated TLR polymorphisms. In the model outcome of critically ill patients as the dependent variable, the significantly independent variables were age, sex and *TLR3* rs3775291 polymorphism.

Table 9. Multivariate regression analysis (stepwise model) with outcome of sepsis (survival) as dependent variables in critically ill patients

	B	**p**	**F (p)**
Outcome of sepsis (survival)			
(Constant)	2.149		
Age	-0.013	0.000	10.959
Sex	0.218	0.006	(0.000)
TLR3 rs3775291	-0.139	0.012	
$R^2 = 0.353$			

B-parameter estimate, F-Fisher test

4.7. Genotype and allele distribution in VDRs genes polymorphisms in critically ill patients

The distribution of analyzed polymorphisms in VDRs genes in the group of Caucasian Serbian critically ill patients is presented in Table 10. There were mutated genotypes of all investigated *VDR* polymorphisms in our patients. Percentage of alleles for investigated *VDR ApaI* and *VDR EcoRV* genes polymorphism were similar. On the other side, distribution of mutated C allele for *VDR FokI* polymorphisms rs2228570 was higher compared to *wild type* T allele, while distribution of *wild type* T allele for *VDR TaqI* polymorphisms rs731236 was higher compared to mutated C allele.

Table 10. Distribution of VDRs genes polymorphisms in the group of critically ill patients

Gene/SNP	**Genotype**	**N (%)**	**Allele**	**N (%)**
	TT (*wt*)	19 (16)	T	97 (40)
VDR FokI	TC (*het*)	59 (49)		
rs2228570	CC (*mut*)	43 (35)	C	145 (60)
	CC (*wt*)	23 (19)	C	101 (42)
VDR ApaI	CA (*het*)	53 (44)		
rs7975232	AA (*mut*)	45 (37)	T	141 (58)
	TT (*wt*)	43 (36)	C	148 (61)
VDR TaqI	TC (*het*)	62 (51)		
rs731236	CC (*mut*)	16 (13)	T	94 (39)
	TT (*wt*)	29 (24)	A	127 (52)
VDR EcoRV	TC (*het*)	69 (57)		
rs4516035	CC (*mut*)	23 (19)	G	115 (48)

Wt/het/mut- wild type/heterozygote/mutated

The VDRs polymorphisms prevalence in the subgroups of critically ill patients is presented in Table 11. There were no significant differences in genotype frequencies for all SNPs studied among analyzed subgroups. It should be noted that in group of patients with trauma, who did not develop sepsis, did not detected VDR *FokI* mutated genotype. On the other side, 50 percent patients with trauma had mutation in *VDR ApaI* polymorphism but did not develop sepsis.

Table 11. Distribution of VDRs genes polymorphisms in the subgroups of critically ill patients

Gene/SNP genotype	Patients with sepsis/pancreatitis (n = 17)	Patients with sepsis/peritonitis (n = 63)	Patients with trauma and sepsis (n = 20)	Patients with trauma without sepsis (n = 21)
VDR FokI, n (%)				
TT (*wt*)	2 (12)	12 (19)	13 (65)	6 (29)
TC (*het*)	10 (59)	32 (51)	7 (35)	6 (29)
CC (*mut*)	5 (29)	19 (30)	0 (0)	9 (42)
VDR ApaI, n (%)				
CC (*wt*)	2 (12)	14 (22)	4 (20)	3 (14)
CA (*het*)	10 (59)	28 (45)	6 (30)	9 (43)
AA (*mut*)	5 (29)	21 (33)	10 (50)	9 (43)
VDR TaqI, n (%)				
TT (*wt*)	5 (29)	22 (35)	9 (45)	7 (33)
TC (*het*)	11 (65)	34 (54)	8 (40)	9 (43)
CC (*mut*)	1 (6)	7 (11)	3 (15)	5 (14)
VDR EcoRV, n (%)				
TT (*wt*)	5 (29)	13 (21)	4 (20)	7 (33)
TC (*het*)	7 (42)	40 (64)	13 (65)	9 (43)
CC (*mut*)	5 (29)	10 (16)	3 (15)	5 (14)

Data are expressed as an absolute number (percentage)
NS, non-significant; statistical analysis performed by χ^2 test
Wt/het/mut- wild type/heterozygote/mutated

4.8. Associations of VDRs genes polymorphisms with clinicopathological variables in critically ill patients

Associations of VDRs genes polymorphisms with clinicopathological variables are presented in Table 12. Significant association between *VDR FokI* rs2228570 polymorphism and cause of sepsis and critically ill status was noticed (p=0.009). Patients with sepsis with peritonitis as underlying cause had two times higher frequency of *VDR FokI* rs2228570 mutated genotype compared to the presence of mutated genotype in patients with other cause of sepsis. No significant associations were observed between VDR gene polymorphisms and other analyzed variables characteristics in the critically ill patients (Table 12).

Table 12. Association of VDR polymorphisms with clinicopathological variables of critically ill patients

	N	VDR FokI TT/TC/CC	VDR ApaI CC/CA/AA	VDR TaqI TT/TC/CC	VDR EcoRV TT/TC/CC
Sex					
Male	76	10/36/30	14/32/30	29/37/10	20/43/13
Female	45	9/23/13	9/21/15	14/25/6	9/26/10
p		NS	NS	NS	NS
Age					
> 56	52	6/28/18	9/21/22	21/24/7	12/27/13
≤ 56	69	13/31/25	14/32/23	22/38/9	17/42/10
p		NS	NS	NS	NS
Diagnosis					
sepsis pancreatitis	17	1/8/8	2/10/5	5/11/1	5/7/5
sepsis peritonitis	63	12/32/19	14/28/21	22/34/7	13/40/10
trauma + sepsis	20	0/13/7	4/6/10	9/8/3	4/13/3
trauma	21	6/6/9	3/9/9	7/9/5	7/9/5
p		0.009	NS	NS	NS
Hemoculture					
Sterile	45	8/19/18	6/21/18	12/24/9	12/23/10
Mixed	49	10/25/14	12/23/14	21/23/5	11/27/11
Mixed + fungi	4	0/3/1	0/2/2	2/2/0	0/3/1
Gram-positive	16	1/10/5	3/6/7	4/11/1	5/11/0
Gram-negative	5	0/1/4	2/1/2	3/1/1	0/4/1
Fungi	2	0/1/1	0/0/2	1/1/0	1/1/0
p		NS	NS	NS	NS
Outcome					
Death	57	11/27/19	13/22/22	21/29/7	14/33/10
Survival	64	8/32/24	10/31/23	22/33/9	15/36/13
p		NS	NS	NS	NS
Mortality					
< 28 days	39	10/17/12	11/15/13	15/20/4	7/24/8
< 90 days	14	1/7/6	2/6/6	5/7/2	6/7/1
> 90 days	2	0/2/0	0/1/1	1/1/0	1/1/0
> 12 months	2	0/1/1	0/0/2	0/1/1	0/1/1
p		NS	NS	NS	NS

N total number of patients

NS, non-significant; statistical analysis performed by χ^2 test

4.9. Survival analysis according to VDR polymorphisms in critically ill patients

There were no significant differences of overall survival for the all studied VDRs genes polymorphisms (data not shown).

4.10. Multivariate regression analysis according to VDR polymorphism in critically ill patients

Multivariate regression analysis with stepwise model was used to asses the independent variables that may affect mortality (period) (Table 13). The variables entered in the model as independent variables were the following: age, sex, and all investigated VDR polymorphisms. In the model outcome of critically ill patients as the dependent variable, the significantly independent variables were age, and *VDR FokI* rs2228570 polymorphism.

Table 13. Multivariate regression analysis (stepwise model) with mortality of sepsis (survival) as dependent variables in critically ill patients

	B	**p**	**F (p)**
Mortality of sepsis			
(Constant)	5.321		
Age	-0.465	0.000	10.653
VDR FokI rs2228570	0.252	0.033	(0.000)
$R^2 = 0.532$			

B-parameter estimate, F-Fisher test

4.11. Associations between TLR and VDR polymorphisms in critically ill patients

Associations of TLRs genes polymorphisms with VDRs genes polymorphisms are presented in Table 14. The *TLR3* rs3775291 polymorphism was associated with both investigated TLR4 rs4986791 and TLR4 rs4986790 polymorphisms p=0.026 and p=0.008, respectively. In our patients is detected significant association between TLR4 rs4986791 polymorphism and TLR4 rs4986790 polymorphism (p=0.000), as well. Further, significant tendency toward negative association between TLR4 rs4986791 polymorphism and *VDR FokI* rs2228570 was noticed (p=0.051). The VDR *ApaI* rs7975232 polymorphism was associated with VDR TaqI rs731236 polymorphism p=0.000.

Table 14. Association between TLR and VDR polymorphisms in critically ill patients

		TLR2 rs3804099	TLR3 rs5743312	TLR3 rs3775291	TLR4 rs4986791	TLR4 rs4986790	VDR FokI	VDR ApaI	VDR TaqI	VDR EcoRV
TLR2 rs3804099	Pearson Correlation	1	-.035	-.037	.057	.057	.036	.039	-.030	-.046
	Sig. (2-tailed)		.700	.687	.536	.536	.691	.667	.747	.614
	N	121	121	121	121	121	121	121	121	121
TLR3 rs5743312	Pearson Correlation	-.035	1	-.119	.087	-.025	-.084	-.019	.108	-.090
	Sig. (2-tailed)	.700		.194	.341	.790	.358	.832	.237	.324
	N	121	121	121	121	121	121	121	121	121
TLR3 rs3775291	Pearson Correlation	-.037	-.119	1	.203*	.242**	-.057	.002	-.049	-.128
	Sig. (2-tailed)	.687	.194		.026	.008	.531	.987	.590	.161
	N	121	121	121	121	121	121	121	121	121
TLR4 rs4986791	Pearson Correlation	.057	.087	.203*	1	.914**	-.178	-.013	.117	-.055
	Sig. (2-tailed)	.536	.341	.026		.000	.051	.885	.201	.547
	N	121	121	121	121	121	121	121	121	121
TLR4 rs4986790	Pearson Correlation	.057	-.025	.242**	.914**	1	-.139	.023	.077	-.015
	Sig. (2-tailed)	.536	.790	.008	.000		.129	.799	.403	.874
	N	121	121	121	121	121	121	121	121	121
VDR FokI	Pearson Correlation	.036	-.084	-.057	-.178	-.139	1	.110	.025	.040
	Sig. (2-tailed)	.691	.358	.531	.051	.129		.231	.789	.661
	N	121	121	121	121	121	121	121	121	121
VDR ApaI	Pearson Correlation	.039	-.019	.002	-.013	.023	.110	1	.600**	.002
	Sig. (2-tailed)	.667	.832	.987	.885	.799	.231		.000	.986
	N	121	121	121	121	121	121	121	121	121
VDR TaqI	Pearson Correlation	-.030	.108	-.049	.117	.077	.025	.600**	1	.051
	Sig. (2-tailed)	.747	.237	.590	.201	.403	.789	.000		.580
	N	121	121	121	121	121	121	121	121	121
VDR EcoRV	Pearson Correlation	-.046	-.090	-.128	-.055	-.015	.040	.002	.051	1
	Sig. (2-tailed)	.614	.324	.161	.547	.874	.661	.986	.580	
	N	121	121	121	121	121	121	121	121	121

5. DISCUSSION

5.1. The role of TLR polymorphisms in sepsis

To our knowledge, this is the first report of an association between *TLR3* gene polymorphism with survival in critically ill patients that indicates the role of *TLR3* polymorphisms in sepsis. The general major observation in this study is that Serbian critically ill patients with *TLR3* rs3775291 mutated genotype had a significant decrease of overall survival compared to the patients with *wild type* and heterozygous genotype. The obtained results make possible establishing a link between a specific genotype in the *TLR3* gene and factors of tissue with injury and/or infection, which, in future, would be used for implementing molecular-genetic diagnostic procedures for individual treatment of critically ill patients and predicting outcome of the disease.

Sepsis begins with production of mediators that lead to MODS with high mortality rate. The immune pathogenesis of sepsis is very complex [146]. The immune system uses naïve and adaptive immune system to respond to danger signals. Cells of naive immunity recognize PAMPs and DAMPs by PPRs and activate a cascade of preformed plasma proteins and immune cells such as monocytes, macrophages, dendritic cells, neutrophils and lymphocytes. Fast production of pro-inflammatory mediators usually is related to simultaneous production of anti-inflammatory mediators in order to maintain homeostasis in the body. Inflammatory mediators implement mechanisms that will lead to either recovery or death of the organism by mechanisms of sepsis. TLR signaling plays an important role in initiation of the inflammatory response in sepsis [147]. Based on the primary structure, TLRs can be divided into several subfamilies, each of which recognizes a similar PAMP: subfamily TLR1, TLR2 and TLR6 recognizes lipids, while highly related TLR7, TLR8 and TLR9 recognize nucleic acids. However, the main division of TLRs was

performed according to their position in the cell and the corresponding PAMP ligands. The group TLR1, TLR2, TLR4, TLR5, and TLR6 was expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 were located in intracellular compartments such as the endoplasmic reticulum, endosoms, lysosomes, and endolysosomes [148]. Although TLRs are mainly expressed in immune cells, phagocytes and antigen presenting cells, more studies have shown that they are also expressed in the cells of different tissues, including cells of the adrenal glands, gastrointestinal tract, brain, and kidney [149]. Prolonged and excessive activation of TLR and their signaling cascades contributes to the pathogenesis of sepsis. Regarding to all presented data sepsis could be seen as TLR-mediated dysregulation of the immune system following pathogen invasion in which careful balance between inflammatory and anti-inflammatory responses is vital.

Susceptibility and response to infection is, in part, heritable. There are evidences that genetic factors may be relevant and important determinants for interindividual differences in susceptibility to infection [150, 151]. The essential role of TLRs in the inflammation initiation makes them interesting candidates for genetic analysis. We have found that one quarter of our critically ill patients (28 patients) had *TLR2* rs3804099 mutated genotype, 12 patients had *TLR3* rs3775291 mutated genotype, and only two patients had *TLR3* rs5743312 mutated genotype. In three patients, we detected simultaneous presence of *TLR2* rs3804099 and *TLR3* rs3775291 mutated genotypes. Regarding to presence of genotypes in investigated cohort of Serbian critically ill patients, percentage showed that alleles of *TLR2* gene are equally distributed, while *wild type* C and G alleles of investigated *TLR3* gene polymorphisms were more frequent compared to mutated T and A alleles, respectively. The significant differences in the distribution of alleles of both investigated *TLR4* polymorphisms are detected, as well. Song and colleagues (2011) showed that in the Chinese Han population with sepsis lower minor allele frequency (0.32) was detected than in our population (0.57). *TLR4* rs4986791 and rs4986790 mutations are found within approximately 5-10% of total population and were first identified by Arbour

and colleagues [152] in studies describing these SNP's association to a blunted response to inhaled LPS in humans. It has been reported that mutated genotypes of *TLR4* rs4986791 and rs4986790 SNPs were associated with increased susceptibility to Gram-negative bacterial infection [152]. In contrast to these data, in our study there were no mutated genotypes of both investigated *TLR4* polymorphisms.

There are several studies about role of functionally relevant *TLR* polymorphisms in sepsis and trauma. TLR2 and TLR4 are expressed in human adrenals [153] and suggest that both receptors may be involved in the hypothalamic-pituitary-adrenal axis function with significant role in development of immunosupressed conditions in sepsis. Zacharowski and colleagues [154] showed that TLR4 is a major mediator in the crosstalk between the innate immune system and the endocrine stress response in animal model of systemic inflammatory response syndrome. TLR4 is involved in signaling of both exogeneous and endogeneous danger signals and is the prototype of TLR involved in sepsis. Additionally, TLR4 is involved in haemorrhage shock signaling in the interaction with High-Mobility Group Box-1 (HGMB-1), ischemia-reperfusion signaling, toxic challenge signaling, tissue trauma, burn, and wound repair signaling [155]. TIRAP/Mal is an important adaptor molecule for intracellular signaling of both TLR2 and TLR4 [156]. Kumpf and colleagues [157] showed that the presence of *TLR4* gene mutations (rs4986791 and rs4986790) in combination with *TIRAP/Mal* variants resulted in significant increase in the risk of severe infection. Aberrant functioning of the TLR/CD14 pathway of innate immunity changes the risk of infectious complications in patients with severe trauma. It was shown that the *TLR2* T-16934A TA genotype increased the risk of a Gram-positive infection and SIRS trauma patients [158]. The same authors showed that TLR4 variation seemed unrelated to outcome of sepsis. This result about *TLR4* polymorphism is in line with *TLR4* polymorphisms in our cohort of critically ill patients. Additionally, in agreement with literature and our data are results of Jessen and colleagues [159] who showed no correlation exists between severity or outcome of sepsis and TLR4. TLR2 signaling plays a critical role in orchestrating the innate

immune response and the development of sepsis [160, 161]. Up-regulation of *TLR2* gene expression was observed in trauma [161], which might be a mechanism for injury that primes the innate immunity [162]. *TLR2* rs5743704 and rs5743708 polymorphisms could affect transmembrane signaling [163] and be associated with susceptibility to microbial infections, such as Gram-negative sepsis [164]. Results of Chen and colleagues [165] indicate that *TLR2* rs3804099 polymorphism is associated with sepsis morbidity rate in patients with major trauma and might be used as relevant risk estimates for the development of sepsis and MOD. In addition, the same author reported clinical relevance of *TLR2* rs1898830 and rs7656411 polymorphisms for sepsis. The results of *TLR2* rs3804099 polymorphism in our cohort of critically ill patients are not in accordance with presented literature data, since any associations for *TLR2* polymorphism were not observed in our study.

Additionally, gender might be one of factors influencing susceptibility to sepsis in trauma patients. Male gender has been shown to be associated with increased risk for sepsis in some studies [165, 166]. Our results also revealed that 19 of the 28 critically ill patients with variant homozygous genotype of the *TLR2* rs3804099 were males. It is known that TLR2, although regulating the activation of immune cells by a wide range of pathogens, is a PRR mainly responsible from Gram-positive bacteria [167]. However, literature data indicate that predominant pathogens inducing traumatic sepsis are Gram-negative bacteria, although a mixed infection often occurs in patients with trauma [168]. In line with this data, in our patients was detected the mixed infection as the most frequent (41%) infection, while Gram-positive bacteria were detected in 13% of patients and Gram-negative bacteria in 4% of patients. This might be a reason why the *TLR2* polymorphisms are relatively less associated with sepsis and posttraumatic complications in our study.

To our knowledge, this is the first study that investigates *TLR3* polymorphisms in sepsis. We showed decreased overall survival in critically ill patients with *TLR3* rs3775291 mutated genotype. *TLR3* rs3775291 is located in exon 4 and is a missense

mutation (G > A, Leu412Phe) resulting in a functionally impaired receptor. In addition, multivariate regression analysis showed that age, sex and *TLR3* rs3775291 polymorphisms were independently associated with survival in Serbian critically ill patients. Further, potential interesting finding is that only trauma patients with *TLR3* rs3775291 mutated genotype (5% of all trauma patients) developed sepsis. These results could be indicated that functional TLR3 and signaling through them is necessary for adequate response in prevention of sepsis development in trauma patients. On the other side, all critically ill patients with *TLR3* rs5743312 mutated genotype had sepsis with peritonitis as underlying cause. This result could be indicated that normal function of TLR3 with MyD88 independent signaling has a potential role in adequate response to injury and infections in peritoneum in humans concerning to prevent sepsis. In literature, there are no data about role of *TLR3* gene in sepsis and susceptibility to development of sepsis in patients with trauma. Chen and colleagues [165] showed that *TLR9* polymorphisms rs187084 and rs352162 might be used to provide relevant risk estimates for the development of sepsis and MODs in patients with major trauma. These data together with our results about *TLR3* polymorphism indicate that endosomal TLRs that sense different intracellular pathogens and intracellular damages that use different signaling cascade have the potential role in development of severe inflammation and sepsis in patients with trauma and severe surgery. In addition, non-pathogenic factors, such as ischemia and hypoxia from hypoperfusion occurring immediately after trauma, may also lead to the development of sepsis and MODS [169]. Ferreira and colleagues [170] suggested interesting fact that PPARγ activation impaired TLR responses, by inhibiting expression of MyD88 and preventing systemic inflammatory response. Considering this data, the important finding is that PPARγ-2 12Ala allele frequency in the healthy participants of the Serbian population is in the same range as in other Caucasians [171].

The potential limitations of the current study include the relatively small sample size and this study will considered as preliminary results. However, a major limitation

in this approach is the potential for stratification when inappropriate patient-control matching occurs, such as if using healthy blood donors as control group in this group. The only population studied was Serbian, and thus results may not be generalized to other population. Nevertheless, because we enrolled a highly selected and clinically clear cohort of critically ill patients, we could confidently interpret our results. However, since the *TLR3* polymorphisms were negatively associated with survival period, and it indicates the need for further study on the association of the cytokine level and signaling cascade induced by these TLRs in critically ill patients.

In conclusion, the present study has provided evidence that *TLR3* rs3775291 polymorphism might be used as a predictor of outcome in critically ill patients with sepsis, and showed a negative impact of mutated genotype in survival of critically ill patients. It suggests that TLR3, as detector of intracellular signals and changes, with MyD88 independent signaling may exerts an important role in pathogenesis of sepsis. *TLR3* rs3775291 polymorphism may be a useful marker to identify patients with high risk to develop sepsis and high risk for lethal outcome but should be validated in larger prospective studies. Early genotyping may prove to be helpful in the future in identifying critically ill patients at risk for severe sepsis and in identifying, trauma patients at risk for infectious/sepsis. In the end, major steps forward in sepsis are needed to elucidate the potential of TLR in animal TLR3 knockout model and model for TLR3 over expression that supports our conclusion indicate molecular rational. In addition, further step is needed to elucidate the host response pathway and new approaches in sepsis trial design, which take into account patient heterogeneity and the phase of immune response.

5.2. The role of VDR polymorphisms in sepsis

In this study, we have demonstrated clearly that mutated FokI genotype is a very important factor in the homeostasis of immune system and in a development of sepsis in critically ill patients. 1□,25-Dihydroxyvitamin D3 (1,25(OH)2D3), the active metabolite of vitamin D3, exerts its effects by binding to the nuclear vitamin D receptor (VDR) [172], acting as a transcription factor, and influences the transcription of a large and diverse set of genes [173, 174]. 1,25$(OH)_2$D3 has important effects on the growth and function of multiple cell types. The discovery that the VDR is constitutively expressed by antigen-presenting cells such as macrophages and dendritic cells, and is induced in activated T lymphocytes [175, 176], led to the recognition of a central role of 1,25(OH)2D3 in the immune system [177, 178]. In humans, multiple polymorphisms of the VDR gene have also been identified and thoroughly studied [179, 180]. These polymorphisms are distributed throughout the complete VDR gene region: the promoter region exons 1a–1f, the coding exons 2–9, introns and the 30-UTR. The *FokI* polymorphism of the translation start site is the only one that results in VDR proteins with different structures, a long f-VDR or a shorter F-VDR [181].

In our study, in critically ill patients we identified mutated genotypes of all investigated *VDR* polymorphisms (*FokI, ApaI, TaqI* and *EcoRV*). The *FokI* polymorphism is characterized by the presence of either two ATG start codons separated by six nucleotides in the long f-VDR or only one start codon due to a T-to-C substitution in the most 50 ATG codon, resulting in a 3-aa shorter F-VDR protein (424 aa instead of 427 aa). Moreover, it is the only polymorphism that is not linked to any of the other VDR polymorphisms [180], thus giving it a unique role. In our study, the statistically significant differences were observed between the critically ill patients and healthy subjects for the VDR *FokI* polymorphisms (rs2228570). Mutated genotype for *VDR FokI* ff was detected in 13% of control subjects, while approximately 36% of critically ill patients have this mutated genotype (distribution

of mutated C allele for *VDR FokI* polymorphisms rs2228570 was higher compared to *wild type* T allele in critically ill patients). Results for our control subjects are in line with literature data which showed that the population distribution in Caucasians of the different FokI genotypes is approximately 45% Ff, 40% FF and 15% ff [182]. Until now, contradictory reports can be found on a possible functional role of this FokI polymorphism [181, 183-186]. Regarding to the majority of literature data these results indicate important role of signal transduction by VDR receptor in regulation of immune response to infection or trauma, and development of sepsis. We may hypothesized that the mutated *FokI* VDR which present with higher frequencies in critically ill patients can not transduce adequate signal from $1,25(OH)_2D3$ to cells which have import role in immune response. This attitude about possible role of mutated VDR genes is based to next literature facts. We know that $1,25(OH)_2D3$ inhibits cell cycle progression of T lymphocytes and reduces their secretion of cytokines such as IL-2 [187, 188]. Moreover, 1,25(OH)2D3 potently interferes with the maturation and activation of dendritic cells and affects their cytokine secretion [189-191]. As such, $1,25(OH)_2D3$ down-regulates the expression and secretion of IL-12 in a VDR-dependent manner, thereby shifting the ongoing immune reaction away from a T helper 1 towards a T helper 2 profile [192, 193]. In the immune system, as in other $1,25(OH)_2D3$-responsive tissues, the $1,25(OH)_2D3$/VDR complex can directly interact with vitamin D-responsive elements (VDRE) in the promoter region of $1,25(OH)_2D3$ target genes. Additionally, liganded but also unliganded VDR can interfere with signaling by other transcription factors, a mechanism by which $1,25(OH)_2D3$ exerts most of its immunomodulatory effects. The ability of the VDR/$1,25(OH)_2D3$ complex to dose-dependently interfere with the signaling of transcription factors such as NFAT, NF-□B and AP-1 has been shown previously [194-196]. These transcription factors play crucial roles in the regulation of immunomodulatory genes, such as for numerous cytokines, effector enzymes, adhesion molecules and growth factors [18, 19]. As such, $1,25(OH)_2D3$ impedes activation of NF-jB as well as binding of NF-κB to its consensus sequence, which is thought to be responsible for $1,25(OH)_2D3$-induced down-regulation of IL-8 and IL-

12 [192, 197]. Similarly, 1,25$(OH)_2$D3 down-regulates IL-2 expression by interfering with the NFAT- and AP-1-induced signal transduction pathways [187, 188]. Regarding to these data we can presume that mutated VDR *FokI* genotypes can be at least partially responsible for development of sepsis in our critically ill patients.

Our previous results and hypothesis about role of VDR *FokI* polymorphisms may be supported by results which showed that in the group of patients with trauma who did not develop sepsis there was not VDR *FokI* mutated genotype. On the other side, about one half of trauma patients with sepsis had this polymorphism. These results indicate that mutation in VDR *FokI* polymorphism and non-adequate VDR signaling may participate in development of sepsis in patients with trauma. Considering to these results we may presume that early VDR genotyping may prove to be helpful in the future in identifying trauma patients at risk for severe sepsis. That means that further step is needed to elucidate the role of VDR *FokI* polymorphism in sepsis and trauma trial design, which take into infection heterogeneity and the phase of immune response. In addition, regarding to our next result which showed that VDR *ApaI* mutated genotype is presented in approximately equal percentage of trauma patients with sepsis and trauma patients without sepsis we may presume that *VDR ApaI* polymorphism have not effect to development of sepsis in these patients.

Further, we showed that in our critically ill patients there was association of *VDR FokI* rs2228570 polymorphism with cause of sepsis. The mutated genotype of this polymorphism was presented with two times higher frequency in patients with sepsis caused by peritonitis as underlying cause compared to the presence of mutated genotype in patients with other cause of sepsis. The active form of vitamin D (1.25(OH)2D3), is an immune modulator and its interaction with VDR influences both innate and adaptive immunity in response against intracellular pathogens. VDR gene polymorphisms were associated with mycobacteria and viral infections [198]. Additionally, 1.25 $(OH)_2D_3$ reduce the expression of pro inflammatory cytokines such as TNF-α, IFN-γ, IL-2 in immune cells, and can induce the expression of anti-

inflammatory cytokines IL-4 and IL-10 [199]. Considering these results and literature data we may hypothesized that normal signaling during VDR is a very important for adequate immune response to infective microorganism in peritoneum. Problems with mutated VDR may influence normal function of macrophages as resident immune cells in peritoneum. This premise about role of vitamin D as immune modulator in immune response of peritoneal macrophages may be supported by our results about negative association of *VDR FokI* rs2228570 and TLR4 rs4986791 polymorphism in critically ill patients. This association is significant because is TLR4 is one of the most important TLR on macrophages which can sense environment for microbe persistence (Gram-negative bacteria, *Chlamydia pneumonia)* and presence of products of damage cells (heat-shock proteins, fibrinogen).

Multivariate regression analysis which was used to asses the independent variables that may affect mortality of critically ill patients, showed that age, and *VDR FokI* rs2228570 polymorphism are statistically significant variables. This analysis confirms the role of mutated *VDR FokI* genotype for sepsis development and negative impact to survival of critically ill patients.

At the end of analysis of results for VDr polymorphisms, we showed that association between VDR *ApaI* polymorphism and VDR *TaqI* polymorphism, but for these VDR polymorphisms we did not find any significance for status of critically ill patients and sepsis development. Also, *TLR3* rs3775291 polymorphism was associated with both investigated TLR4 rs4986791 and TLR4 rs4986790 polymorphisms.

In conclusion, the present study has provided evidence that *VDR FokI* rs2228570 polymorphism might be used as a predictor of outcome in critically ill patients with sepsis, and showed a negative impact of mutated genotype in survival of critically ill patients. It suggests that VDR, as receptor for important immunomoduloatory molecule, vitamin D, may exerts an important role in

pathogenesis of sepsis. *VDR FokI* rs2228570 polymorphism may be a useful marker to identify patients with high risk (for example with trauma) to develop sepsis and high risk for lethal outcome but should be validated in larger prospective studies. Early genotyping may prove to be helpful in the future in identifying critically ill patients at risk for severe sepsis and in identifying, trauma patients at risk for infectious/sepsis.

Importance of this research is in simultaneously determining the polymorphisms of two receptor groups which are important to immune response. For the first time, in this thesis is showed correlation of TLR and VDR polymorphisms in critically ill patients in Serbia. It is important to emphasize that these results are potential basis for future basic and clinical researches. Basic researches should be focused to detecting of potential cooperation of TLR and VDR signal pathways while clinical researches should be performed considering greater number of patients with sepsis, trauma or other inflammatory diseases. One of the major steps forward in sepsis are needed to elucidate the potential of TLR and VDR modulation in animal TLR3 and VDR knockout models and models for TLR3 and VDR over expression that supports our conclusion indicate molecular rational.

At the end, it is important to emphasize that individual heterogeneity of patients and the phase of immune response is very important facts in sepsis development and it is needed to elucidate the host response pathway and new approaches in sepsis trial design.

6. CONCLUSIONS

1. Individuals carrying the A allele of TLR3 rs3775291 have a higher risk for lethal outcome of sepsis in the Caucasian Serbian critically ill population

2. Patients with trauma without TLR3 rs3775291 mutated genotype gene did not develop sepsis

3. In the Caucasian Serbian critically ill population there no mutated genotypes of TLR4 rs4986790 and rs4986791 polymorphisms.

4. Tag SNPs of TLR2 and TLR4 are not associated with sepsis susceptibility in the Serbian populations

5. The *TLR3* rs3775291 polymorphism was associated with both investigated *TLR4* rs4986791 and *TLR4* rs4986790 polymorphisms. In our patients is detected significant association between *TLR4* rs4986791 polymorphism and *TLR4* rs4986790 polymorphism, as well.

6. In the Caucasian Serbian critically ill population there are mutated genotypes of all investigated VDR polymorphisms

7. Critically ill patients have statistically significant higher frequency of VDR FokI polymorphisms rs2228570 compared to healthy subjects

8. In group of patients with trauma, who did not develop sepsis, did not detected VDR *FokI* mutated genotype

9. In critically ill patients there are significant association between *VDR FokI* rs2228570 polymorphism and cause of sepsis

10. Individuals carrying the C allele of *VDR FokI* rs2228570 have a higher risk for lethal outcome of sepsis in the Caucasian Serbian critically ill population

11. The significant tendency toward negative association between *TLR4* rs4986791 polymorphism and *VDR FokI* rs2228570 was noticed in the Caucasian Serbian critically ill population. Additionally, the *VDR ApaI* rs7975232 polymorphism was associated with *VDR TaqI* rs731236 polymorphism.

12. Genotyping for Toll-like receptors and vitamin D receptor may identify individuals with increased risk for septic complication after trauma and surgical interventions who should be subject to intensified prophylactic measures

7. REFERENCES

1. Thomas L and Germs N (1972). Engl J Med 287: 553–555.

2. Martin GS, Mannino GM, Eaton S and Moss M (2003). The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 348: 1546-1554.

3. Angus D, Linde-Zwirble W, Lidicker J, Clermont G, Carcillo J, Pinsky M (2001) Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med 29(7):1303-1310.

4. Abraham E, Matthay MA, Dinarello CA, Vincent JL, Cohen J, Opal SM, et al. (2000) Consensus conference definitionsactivating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J Immunol 164: 554-557.

5. Kalispell R, Graves B, and Ackerman M (2006) Incidence, pathogenesis and management of sepsis: an overview. AACN Adv Crit Care 17(4): 385-393.

6. Girard T, Opal S, and Wesley E (2005) Insights into severe sepsis in older patients: from epidemiology to evidence-based management. Clin Infect Dis 40: 719-727.

7. Mazzatti D, White A, Rorsey R, Powell J, Pawelec G (2007) Gene expression changes in long-term culture of T-cell clones: genetic effects of chronic antigenic stress in aging and immunosenescence. Aging Cell 6:155-163.

8. Listi F, Candore G, Modica M, Russo M, Di Lorenzo G, Esposito-Pellitteri M, et al. (2006) A study of serum immunoglobulin levels in elderly persons that provides new insights into B cell immunosenescence. Ann NY Acad Sci 1089: 487-495.

9. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, et al. (2003) 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Critical Care Medicine 31 (4): 1-13.

10. Gogos C, Lekkou A, Papagerorgiou O, Siagris D, Skoutelis A, Bassaris H (2003) Clinical prognostic markers in patients with severe sepsis: a prospective analysis of 139 consecutive cases. J Infect 47: 300-306.

11. Bennermo M, Held C, Stemme S, Ericsson CG, Silveira A, Green F, et al. (2004) Genetic predisposition of the interleukin 6 response to inflammation: implications for a variety of major diseases? Clin Chem 50(11): 2136- 2140.

12. Wang HE, Shapiro NI, Angus DC, Yealy DM (2007) National estimates of severe sepsis in United States emergency departments. Crit Care Med 35 (8): 1928-36.

13. Diekema DJ, Pfaller MA, Jones RN, & SENTRY Participants Group (2002) Age-related trends in pathogen frequency and antimicrobial susceptibility of bloodstream isolates in North America: SENTRY Antimicrobial Surveillance Program, 1997–2000. Int J Antimicrob Agents 20 (6): 412–418.

14. Mayr FB, Yende S, Linde-Zwirble WT, Peck-Palmer OM, Barnato AE, et al. (2010) Infection Rate and Acute Organ Dysfunction Risk as Explanations for Racial Differences in Severe Sepsis. JAMA 303 (24): 2495-503.

15. Martin G, Mannino DM, Eaton S, Moss M (2003) The epidemiology of sepsis in the United Sates from 1979 through 2000. N Engl J Med 348(16): 1546-1554.

16. Hoyert DL, Arias E, Smith BL, Murphy SL, Kochanek KD (2001) Deaths: final data for 1999. Natl Vital Stat Rep 49 (8): 1–113.

17. Moss M (2005) Epidemiology of Sepsis: Race, Sex, and Chronic Alcohol Abuse. Clin Infect Dis 41 (7): S490-S497.

18. Rangel-Frausto M, Pittet D, Costigan M, Hwang T, Davis C, Wenzel R (1995) The natural history of the systemic inflammatory response syndrome (SIRS): a prospective study. JAMA 273: 117-123.

19. Kellum JA, Kong L, Fink MP, Weissfeld LA, Yealy DM, Pinsky MR, et al. (2007) Understanding the inflammatory cytokine response in pneumonia and sepsis. Arch Intern Med 167(15): 1655-1663.

20. Bochud PY, Glauser MP, Calandra T (2001) Antibiotics in sepsis. Intensive Care Med 27 (1): S3348.

21. Alexander C and Rietschel ET (2001) Bacterial lipopolysaccharides and innate immunity. J Endotoxin Res 7: 167-202.

22. Ulevitch RJ and Tobias PS (1999) Recognition of gram-negative bacteria and endotoxin by the innate immune system. Curr Opin Immunol 11: 19-22.

23. Annane D, Aegerter P, Jars-Guincestre MC, and Guidet B (2003) Current epidemiology of septic shock: the CUB-Rea Network. Am J Respir Crit Care Med 168 (2): 165-72.

24. Kumar A, Zarychanski R, Light B, Parrillo J, Maki D, Simon D, Laporta D, Lapinsky S, Ellis P, Mirzanejad Y, Martinka G, Keenan S, Wood G, Arabi Y, Feinstein D, Kumar A, Dodek P, Kravetsky L, Doucette S,& Cooperative Antimicrobial Therapy of Septic Shock (CATSS) Database Research Group (2010) Early combination antibiotic therapy yields improved survival compared with monotherapy in septic shock: a propensity-matched analysis. Crit Care Med 38 (9): 1773-1785.

25. Zaragoza R, Artero A, Camarena JJ, Sancho S, González R, Nogueira JM (2003) The influence of inadequate empirical antimicrobial treatment on patients with bloodstream infections in an intensive care unit. Clin Microbiol Infect 9 (5): 1-7.

26. Calandra T (2001) Pathogenesis of septic shock: implications for prevention and treatment. J Chemother 13: 173-180.

27. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11: 443-451.

28. Lien E and Ingalls RR (2002) Toll-like receptors. Crit Care Med 30(1):S1-S11.

29. Hadley S, Lee WW, Ruthazer R, Nasraway SA (2002) Candidemia as a cause of sepsis shock and multiple organ failure in immunocompromised patients. Crit Care Med 30: 1808-1814.

30. Tyler UL and Fields BN (1995) Introduction to virus and viral diseases: In: Mandell GL, Bennett JE, Dolin R, eds. Principles and Practice of Infectious Diseases. 4th ed. New York, NY: John Wiley & Sons.

31. Annane D, Aegerter P, Jars-Guincestre MC, and Guidet B (2003) Current epidemiology of septic shock: the CUB-Rea Network. Am J Respir Crit Care Med 168 (2): 165-72.

32. Alberti C, Burn-Buisson C, Buchardi H, Brun-Buisson C, Burchardi H, Martin C, et al. (2002) Epidemiology of sepsis and infection in ICU patients from an international multicentric cohort study. Intensive Care Med 28: 108-121.

33. Lin S, Huang C, Lin H, Liu C, Wang C, Kuo H (2006) A modified goal-directed protocol for improved clinical outcomes in intensive care unit patients with septic shock: a randomized controlled trial. Shock 26(6): 551-557.

34. Piccinni P, Dan M, Barbacini S, Carraro R, Lieta E, Marafon S, et al. (2006) Early isovolaemic haemofiltration in oliguric patients with septic shock. Intensive Care Med 32: 80-86.

35. Porth C. (2005) Pathophysiology: Concepts of Altered Health Status.Philadelphia, PA: Lippincott Williams & Wilkins.

36. Garnacho-Montero J, Garcia-Garmendia J, Barrero-Almodovar A, Jiminez-Jimenez F, Perez-Paredes C, Ortiz-Leyba C (2003) Impact of adequate empirical antibiotic therapy on the outcome of patients admitted to the intensive care unit with sepsis. Crit Care Med 31(12): 2742-2751.

37. Dellinger R, Carlet J, Masur H, Gerlach H, Calandra T, Cohen J, et al. (2004) Surviving sepsis campaign guidelines for management of severe sepsis and septic shock. Crit Care Med 32(3): 858-873.

38. Deitch EA (1998) Animal models of sepsis and shock: a review and lessons learned. Shock 9: 111.

39. Wichterman KA, Baue AE, Chaudry IH (1980) Sepsis and septic shock-a review of laboratory models and a proposal. J Surg Res 29: 189-201.

40. Ertel W, Morrison MH, Ayala A, Dean RE, Chaudry IH (1992) Interferon-gamma attenuates hemorrhage-induced suppression of macrophage and splenocyte functions and decreases susceptibility to sepsis. Surgery 111: 177-187.

41. Flohe S, Ackermann M, Reuter M, Nast-Kolb D, Schade FU (2000) Sublethal hemorrhagic shock reduces tumor necrosis factor-alpha-producing capacity in different cell compartments. Eur Cytokine Netw 11: 420-426.

42. Schroder J, Kahlke V, Fandrich F, Zabel P, Kremer B (1998) Tumor necrosis factor-alpha hyporesponsiveness of rat intestinal mononuclear cells and whole portal venous blood after hemorrhagic shock. Crit Care Med 26: 526-532.

43. Polk HC, Wellhausen SR, Regan M (1986) A systematic study of host defense processes in badly injured patients. Ann Surg 204: 282-297.

44. Zanotti S and Kumar A. Cytokine modulation in sepsis and septic shock. Expert Opin Investig Drugs 11: 1061-1075.

45. Laudanski K and Wyczechowska D (2005) Monocyte-related immunopathologies in trauma patients. Arch Immunol Ther Exp (Warsz) 53: 321-328.

46. Cavaillon JM and Adib-Conquy M (2005) Monocytes/macrophages and sepsis. Crit Care Med 33: S50-69

47. Boontham P, Chandran P, Rowlands B, anf Eremin O (2003) Surgical sepsis: dysregulation of immune function and therapeutic implications. Surgeon 1: 187-206.

48. Flierl MA, Rittirsch D, Nadeau BA, Chen AJ, Sarma JV, Zetoune FS et al. (2007) Phagocyte-derived catecholamines enhance acute infl ammatory injury. Nature 449: 721-725.

49. Ohto U, Fukase K, and Miyake K (2007) Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science 316: 1632-1634.

50. Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, et al. (2007) Crystal structure of the TLR4–MD-2 complex with bound endotoxin antagonist Eritoran. Cell 130: 906-917.

51. Ouyang W, Kolls JK, and Zheng Y (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 28: 454-67.

52. Kessel A, Bamberger E, Masalha M, and Toubi E (2009) The role of T regulatory cells in human sepsis. J Autoimmunity 32: 211-215.

53. Gerard C (2003) Complement C5a in the sepsis syndrome - too much of a good thing? N Engl J Med 348: 167-169.

54. Flierl MA, Rittirsch D, Nadeau BA, Day DE, Zetoune FS, Sarma JV, et al. (2008) Functions of the complement components C3 and C5 during sepsis. FASEB J 22: 3483-3490.

55. Rittirsch D, Flierl MA, Nadeau BA, Day DE, Huber-Lang M,Mackay CR, et al. (2008) Functional roles for C5a receptors in sepsis. Nature Med 14: 551-557.

56. Chen NJ, Mirtsos C, Suh D, Lu YC, Lin WJ, McKerlie C, et al. (2007) C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a. Nature 446: 203-207.

57. Huber-Lang M, Sarma JV, Rittirsch D, Schreiber H, Weiss M, Flierl M, et al. (2005) Changes in the novel orphan, C5a receptor (C5L2), during experimental sepsis and sepsis in humans. J Immunol 174: 1104-1110.

58. Rossaint R, Cerny V, Coats TJ, Duranteau J, Fernández-Mondéjar E, Gordini G, et al. (2006) Key issues in advanced bleeding care in trauma. Shock 26: 322-331.

59. Abe K, Miyazaki M, Koji T, Furusu A, Nakamura-Kurashige T, Nishino T, et al. (2001) Enhanced expression of complement C5a receptor mRNA in human diseased kidney assessed by in situ hybridization. Kidney Int 60: 137–146.

60. Blander JM and Medzhitov R (2004) Regulation of phagosome maturation by signals from toll-like receptors. Science 304: 1014.

61. Marshall JC, Vincent JL, Fink MP, Cook DJ, Rubenfeld G, Foster D, et al. (2003) Measures, markers, and mediators: toward a staging system for clinical sepsis. A report of the Fifth Toronto Sepsis Roundtable, Toronto, Ontario, Canada, October 25–26, 2000. Crit Care Med 31: 1560–1567.

62. Van Amersfoort ES, Van Berkel TJ, and Kuiper J (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin Microbiol Rev 16: 379–414.

63. Hotchkiss RS, Tinsley KW, and Karl IE (2003) Role of apoptotic cell death in sepsis. Scand J Infect Dis 35: 585-592.

64. Peters K, Unger RE, Brunner J, and Kirkpatrick CJ (2003) Molecular basis of endothelial dysfunction in sepsis. Cardiovasc Res 60: 49–57.

65. Froidevaux C, Roger T, Martin C, Glauser MP, and Calandra T (2001) Macrophage migration inhibitory factor and innate immune responses to bacterial infections. Crit Care Med 29: S13–S15.

66. Roger T, David J, Glauser MP, and Calandra T (2001) MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature 414: 920–924.

67. Calandra T, Echtenacher B, Roy DL, Pugin J, Metz CN, Hultner L, et al. (2000) Protection from septic shock by neutralization of macrophage migration inhibitory factor. Nat Med 6: 164–170.

68. Calandra T and Roger T (2003) Macrophage migration inhibitory factor: a regulator of innate immunity. Nature Rev Immunol 3: 791-800.

69. Bozza M, Satoskar AR, Lin G, Lu B, Humbles AA, Gerard C, et al. (1999) Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J Exp Med 189: 341–346.

70. Calandra T, Bernhagen J, Mitchell RA, and Bucala R (1994) The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. J Exp Med 179: 1895-1902.

71. Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, et al. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365: 756–759.

72. Wang ZG (2005) Advances in basically scientific research on trauma. Chin J Traumatol 21: 6-10.

73. Schroder NW and Schumann RR (2005) Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis 5: 156-164.

74. Jiang JX (2005) Genomic polymorphisms and sepsis. Chin J Traumatol 21: 45-49.

75. Medzhitov R and Janeway CA (2002) Decoding the patterns of self and non-self by the innate immune system. Science 296: 298-300.

76. Figdor CG, van Kooyk Y and Adema GJ (2002) C-type lectin receptors on dendritic cells and Langerhans cells. Nat Rev Immunol 2: 77-84.

77. Underhill DM (2007) Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. Immunol Rev 219: 75-87.

78. Kawai T and Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373-384.

79. Barbalat R, Ewald SE, Mouchess ML, and Barton GM (2011) Nucleic acid recognition by the innate immune system. Ann Rev Immunol 29: 185-214.

80. Verstak B, Hertzog P and Mansell A (2007) Toll-like receptor signaling and the clinical benefits that lie within. Inflamm Res 56: 1-10.

81. Akira S, Uematsu S and Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783-801.

82. Kawai T and Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 21: 317-337.

83. Surbatovic M, Grujic K, Cikota B, Jevtic M, Filipovic N, Romic N, et al. (2010) Polymorphisms of genes encoding tumor necrosis factor-alpha, interleukin-10, cluster of differentiation-14 and interleukin-1ra in critically ill patients. J Crit Care 25: 542.e1-542.e8.

84. Bikle DD (2009) Vitamin D and immune function: understanding common pathways. Curr Osteoporos Rep 7: 58–63.

85. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357: 266–281.

86. Ponchon G, Kennan A, and DeLuca HF (1969) "Activation" of vitamin D by the liver. J Clin Invest 48(11): 2032-2037.

87. Lemire JM, Adams JS, Sakai R, and Jordan SC (1984) 1α, 25-dihydroxyvitamin D_3 suppresses proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells. J Clin Invest 74: 657–661

88. Rigby WF, Stacy T, and Fanger MW (1984) Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D_3 (calcitriol) J Clin Invest 74: 1451–1455.

89. Bhalla AK, Amento EP, and Krane SM (1986) Differential effects of 1,25-dihydroxyvitamin D3 on human lymphocytes and monocyte/macrophages: inhibition of interleukin-2 and augmentation of interleukin-1 production. Cell Immunol 98(2): 311-322.

90. Reichel H, Koeffler HP, and Norman AW (1987) Synthesis *in vitro* of 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 by interferon-gamma-stimulated normal human bone marrow and alveolar macrophages. J Biol Chem 262(23): 10931-10937.

91. Rigby WF, Denome S, and Fanger MW (1987) Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA. J Clin Invest 79(6): 1659-1664.

92. Meehan MA, Kerman RH, and Lemire JM (1992) 1,25-Dihydroxyvitamin D3 enhances the generation of nonspecific suppressor cells while inhibiting the induction of cytotoxic cells in a human MLR. Cell Immunol 140(2): 400-409.

93. Alroy I, Towers TL, and Freedman LP (1995) Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. Mol Cell Biol 15(10): 5789-5799.

94. Cippitelli M and Santoni A (1998) Vitamin D3: a transcriptional modulator of the interferon-gamma gene. Eur J Immunol 28(10): 3017-3030.

95. Müller K, Rieneck K, Hansen MB, and Bendtzen K (1992) 1,25-Dihydroxyvitamin D3-mediated suppression of T lymphocyte functions and failure of T cell-activating cytokines to restore proliferation. Immunol Lett 34(1): 37-44.

96. Müller K and Bendtzen K (1992) Inhibition of human T lymphocyte proliferation and cytokine production by 1,25-dihydroxyvitamin D3. Differential effects on CD45RA+ and CD45R0+ cells. Autoimmunity 14(1): 37-43.

97. Veldman CM, Cantorna MT, and DeLuca HF (2000) Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. Arch Biochem Biophys 374(2): 334-338.

98. van Etten E and Mathieu C (2005) Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. J Steroid Biochem Mol Biol 97(1-2): 93-101.

99. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. (1998) Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. J Clin Invest 101(1): 252-262.

100. Penna G and Adorini L (2000) 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol 164(5): 2405-2411.

101. Penna G, Amuchastegui S, Cossetti C, Aquilano F, Mariani R, Sanvito F, et al. (2006) Treatment of experimental autoimmune prostatitis in nonobese diabetic mice by the vitamin D receptor agonist elocalcitol. J Immunol 177(12): 8504-8511.

102. Daniel C, Sartory NA, Zahn N, Radeke HH, and Stein JM (2008) Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. J Pharmacol Exp Ther 324(1): 23-33.

103. Gorman S, Kuritzky LA, Judge MA, Dixon KM, McGlade JP, Mason RS, et al. (2007) Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes. J Immunol 179(9): 6273-6283.

104. Penna G, Roncari A, Amuchastegui S, Daniel KC, Berti E, Colonna M, et al. (2005) Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+ regulatory T cells by 1,25-dihydroxyvitamin D3. Blood 106(10): 3490-3497.

105. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE (2007) Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. J Immunol 179(3): 1634-1647.

106. Veldman CM, Cantorna MT, and DeLuca HF (2000) Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. Arch Biochem Biophys 374(2): 334-338.

107. Shirakawa AK, Nagakubo D, Hieshima K, Nakayama T, Jin Z, Yoshie O (2008) 1,25-dihydroxyvitamin D3 induces CCR10 expression in terminally differentiating human B cells. J Immunol 180(5): 2786-2795.

108. Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R (2001) Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs:

a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. Proc Natl Acad Sci USA 98(12): 6800-6805.

109. Ohta M, Okabe T, Ozawa K, Urabe A, and Takaku F (1985) 1 alpha,25-Dihydroxyvitamin D3 (calcitriol) stimulates proliferation of human circulating monocytes in vitro. FEBS Lett 185(1): 9-13.

110. Bikle DD (2008) Vitamin D and the immune system: role in protection against bacterial infection. Curr Opin Nephrol Hypertens 17(4): 348-352.

111. Abdelsadik A and Trad A (2001) Toll-like receptors on the fork roads between innate and adaptive immunity. Hum Immunol 72(12): 1188-1193.

112. Hewison M (2010) Vitamin D and the immune system: new perspectives on an old theme. Endocrinol Metab Clin North Am 39(2): 365-79.

113. Maalouf NM (2008) The noncalciotropic actions of vitamin D: recent clinical developments. Curr Opin Nephrol Hypertens 17: 408–415.

114. Andress D (2007) Nonclassical aspects of differential vitamin D receptor activation: implications for survival in patients with chronic kidney disease. Drugs 67(14): 1999-2012.

115. Williams C (1849) On the use and administration of cod-liver oil in pulmonary consumption. Lond J Med 1: 1–18.

116. Rook GA, Steele J, Fraher L, Barker S, Karmali R, O'Riordan J, et al. (1986) Vitamin D3, gamma interferon, and control of proliferation of Mycobacterium tuberculosis by human monocytes. Immunology 57: 159–163.

117. Crowle AJ, Ross EJ, and May MH (1987) Inhibition by 1,25(OH)2-vitamin D3 of the multiplication of virulent tubercle bacilli in cultured human macrophages. Infect. Immun 55: 2945–2950.

118. Krutzik SR, Hewison M, Liu PT, Robles JA, Stenger S, Adams JS, et al. (2008) IL-15 links TLR2/1-induced macrophage differentiation to the vitamin D-dependent antimicrobial pathway. J Immunol 181: 7115–7120.

119. Bikle DD, Pillai S, Gee E, and Hincenbergs M (1991) Tumor necrosis factor-alpha regulation of 1,25-dihydroxyvitamin D production by human keratinocytes. Endocrinology 129(1): 33-38.

120. Liu S, Tang W, Zhou J, Stubbs JR, Luo Q, Pi M, et al. (2006) Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. J Am Soc Nephrol 17(5): 1305-1315.

121. Bikle DD (2009) Vitamin D and immune function: understanding common pathways. Curr Osteoporos Rep 7(2): 58-63.

122. Seibert E, Levin NW, and Kuhlmann MK (2005) Immunomodulating effects of vitamin D analogs in hemodialysis patients. Hemodial Int 9 (1): S25-9.

123. Xu H, Soruri A, Gieseler RK, and Peters JH (1993) 1,25-Dihydroxyvitamin D3 exerts opposing effects to IL-4 on MHC class-II antigen expression, accessory activity, and phagocytosis of human monocytes. Scand J Immunol 38(6): 535-540.

124. Sester U, Sester M, Hauk M, Kaul H, Köhler H, Girndt M (2000) T-cell activation follows Th1 rather than Th2 pattern in haemodialysis patients. Nephrol Dial Transplant 15(8): 1217-1223.

125. Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L (2001) Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. J Immunol 167(4): 1945-1953.

126. Sakaguchi S, Yamaguchi T, Nomura T, and Ono M. Regulatory T cells and immune tolerance. Cell 133(5): 775-787.

127. Equils O, Naiki Y, Shapiro AM, Michelsen K, Lu D, Adams J, et al. (2006) 1,25-Dihydroxyvitamin D inhibits lipopolysaccharide-induced immune activation in human endothelial cells. Clin Exp Immunol 143(1): 58-64.

128. Sadeghi K, Wessner B, Laggner U, Ploder M, Tamandl D, Friedl J, et al. (2006) Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. Eur J Immunol 36(2): 361-370.

129. Asakura H, Aoshima K, Suga Y, Yamazaki M, Morishita E, Saito M, et al. (2001) Beneficial effect of the active form of vitamin D3 against LPS-induced

DIC but not against tissue-factor-induced DIC in rat models. Thromb Haemost 85(2): 287-290.

130. Møller S, Laigaard F, Olgaard K, and Hemmingsen C (2007) Effect of 1,25-dihydroxy-vitamin D3 in experimental sepsis. Int J Med Sci 4(4): 190-195.

131. Jeng L, Yamshchikov AV, Judd SE, Blumberg HM, Martin GS, Ziegler TR, et al. (2009) Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. J Transl Med 23; 7-28.

132. Dahl B, Schiødt FV, Ott P, Wians F, Lee WM, Balko J, et al. (2003) Plasma concentration of Gc-globulin is associated with organ dysfunction and sepsis after injury. Crit Care Med 31: 152–156.

133. Watkins RR, Yamshchikov AV, Lemonovich TL, and Salata RA (2011) The role of vitamin D deficiency in sepsis and potential therapeutic implications. J Infect 63(5): 321-326.

134. Watt GH, Ashton SH, Cook JA, Wise WC, Halushka PV, Galbraith RM (1989) Alterations in plasma levels and complexing of Gc (vitamin D-binding protein) in rats with endotoxic shock. Circ Shock 28(3): 279-291.

135. Engel C, Brunkhorst FM, Bone HG, Brunkhorst R, Gerlach H, Grond S, et al. (2007) Epidemiology of sepsis in Germany: results from a national prospective multicenter study. Intensive Care Med 33(4): 606-618.

136. Forrest KY and Stuhldreher WL (2011) Prevalence and correlates of vitamin D deficiency in US adults. Nutr Res 31(1): 48-54.

137. Giovannini L, Panichi V, Migliori M, De Pietro S, Bertelli AA, Fulgenzi A, et al. (2001) 1,25-dihydroxyvitamin D(3) dose-dependently inhibits LPS-induced cytokines production in PBMC modulating intracellular calcium. Transplant Proc 33(3): 2366-2368.

138. Takahashi K, Horiuchi H, Ohta T, Komoriya K, Ohmori H, Kamimura T (2002) 1α,25-dihydroxyvitamin D3 suppresses interleukin-1β-induced interleukin-8 production in human whole blood: an involvement of erythrocytes in the inhibition. Immunopharmacol. Immunotoxicol 24: 1–15.

139. Anders HJ, Banas B, and Schlöndorff D (2004) Signaling danger: toll-like receptors and their potential roles in kidney disease. J Am Soc Nephrol 15(4): 854-867.

140. Horiuchi H, Nagata I, and Komoriya K (1991) Protective effect of vitamin D3 analogues on endotoxin shock in mice. Agents Actions 33(3-4): 343-348

141. Khoo AL, Chai LY, Koenen HJ, Kullberg BJ, Joosten I, van der Ven AJ, et al. (2011) 1,25-dihydroxyvitamin D3 modulates cytokine production induced by Candida albicans: impact of seasonal variation of immune responses. J Infect Dis 203(1): 122-130.

142. Kempker JA, Han JE, Tangpricha V, Ziegler TR, and Martin GS (2012) Vitamin D and sepsis. Division of Pulmonary and Critical Care Medicine; Emory University School of Medicine; Atlanta, GA USA; 2 Division of Endocrinology, Metabolism and Lipids; Emory University School of Medicine; Atlanta, GA USA,

143. Le Gall JR, Lemeshow S, and Saulnier F (1993) A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA 270: 2957-2963.

144. Knaus WA, Draper EA, Wagner DP, and Zimmerman JE (1985) APACHE II: a severity of disease classification system. Crit Care Med 13: 818-829.

145. Moreno RP, Vincent JL, Matos R, Mendonça A, Cantraine F, Thijs L, et al. (1999) The use of maximum SOFA score to quantify organ dysfunction/failure in intensive care. Results of prospective multicentre study. Intensive Care Med 25: 686-696.

146. Hotchkiss RS and Karl IE (2003) The pathophysiology and treatment of sepsis. N Engl J Med 348: 138-150.

147. Akira S, Takeda K, and Kaisho K (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2: 675-680.

148. Underhill DM (2007) Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. Immunol Rev 219: 75-87.

149. Doi K, Leelahavanichkul A, Yuen PST, and Star RA (2009) Animal models of sepsis and sepsis-induced kidney injury. The Journal of Clinical Investigation 119 (10): 2868-2878.

150. Arcaroli J, Fesseler MB, and Abraham E (2005) Genetic polymorphisms and sepsis. Shock 24: 300-312.

151. Kumpf O and Schumann RR (2008) Genetic influence on bloodstream infections and sepsis. Int J Antimicrob Agents 32 (1): S44-S50.

152. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones, M, et al. (2000) TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 25 (2): 187-191.

153. Lee HK, Lee J and Tobias PS (2002) Two lipoproteins extracted from Escherichia coli K-12 LCD25 lipopolysaccharide are the major components responsible for Toll-like receptor 2-mediated signaling. J Immunol 168: 4012-4017.

154. Zacharowski K, Zacharowski PA, Koch A, Baban A, Tran N, Berkels R, et al (2006) Toll-like receptor 4 plays a crucial role in the immune-adrenal response to systemic inflammatory response syndrome. PNAS 103 (16): 6392-6397.

155. Castellheim A, Brekke OL, Espeviks T, Harboe M, and Mollnes TE (2009) Innate immune responses to danger signals in systemic inflammatory response syndrome and sepsis. Scand J Immunol 69: 479-491.

156. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. (2001) Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 413 (6851): 78-83.

157. Kumpf O, Giamarellos-Bourboulis EJ, Koch A, Hamann L, Mouktaroudi M, Oh DY, et al. (2010) Influence of genetic variations in TLR4 and TIRAP/Mal on the course of sepsis and pneumonia and cytokine release: an observational study in three cohorts. Crit Care14 (3): R103.

158. Bronkhorst MW, Boyé ND, Lomax MA, Vossen RH, Bakker J, et al. (2013) Single-nucleotide polymorphisms in the Toll-like receptor pathway increase susceptibility to infections in severely injured trauma patients. J Trauma Acute Care Surg 74 (3): 862-870.

159. Jessen KM, Lindboe SB, Petersen AL, Eugen-Olsen J, and Benfield T (2007) Common TNF-alpha, Eugen-Olsen JIL-1 beta, PAI-1, uPA, CD14 and TLR4 polymorphisms are not associated with disease severity or outcome from Gram negative sepsis. BMC Infect Dis 7: 108.

160. Arcaroli J, Fesseler MB, and Abraham E (2005) Genetic polymorphisms and sepsis. Shock 24: 300-312.

161. Hoth JJ, Hudson WP, Brownlee NA, Yoza BK, Hiltbolf EM, Meredith JW, et al. (2007) Toll-like receptor 2 participates in the response to lung injury in a murine model of pulmonary contusion. Shock 28: 447-452.

162. Peterson HM, Murphy TJ, Purcell EJ, Shelley O, Kriynovich SJ, Lien E, et al. (2007) Injury primes the innate immune system for enhanced Toll-like receptor reactivity. J Immunol 171: 1473-1483.

163. Bochud PY, Hawn TR, and Aderem A (2003) Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepramatous leprosy is unable to mediate mycobacterial signaling. J Immunol 170: 3451-3454.

164. Woehrle T, Du W, Goetz A, Hsu HY, Joos TO, Weiss M, et al (2008) Pathogen-specific cytokine release reveals an effect of TLR2 Arg753Gln during Candida sepsis in humans. Cytokines 41: 322-329.

165. Chen K, Gu W, Zeng L, Jiang D, Zhang L, Zhou J, et al (2011) Identification of haplotype TAG SNPs within the entire TLR2 gene and their clinical relevance in patients with major trauma. Shock 35: 35-41.

166. Eachempati SR, Hydo L, and Barie PS (1999) Gender-based differences in patients with sepsis. Arch Surg 134: 935-940.

167. Jiang JX (2005) Genomic polymorphisms and sepsis. Chin J Traumatol 21: 45-49.

168. Jiang JX (2009) Nosocomial infection in trauma patients: risk factors and strategies. Acta Acad Med Milt Tert 31: 13-14.

169. Soreide K (2009) Epidemiology of major trauma. Br J Surg 96: 697-698.

170. Ferreira A, Sisti F, Filgueiras L, Wang S, Wang J, Cunha F, et al. (2013) *In vivo* PPAR-gamma activation inhibits MyD88 expression to protect mice from polymicrobial sepsis. The Journal of Immunology 190: 206.1.

171. Lukic N, Stankovic A, Dincic E, Bundalo M, Krsmanovic Z, Alavantic D, et al. (2013) The Ala/Ala genotype of PPARY Pro12 Ala polymorphism is associated with late onset of multiple sclerosis. Archives of Biological Sciences 65(2): 447-453.

172. Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, et al (1998) The nuclear vitamin D receptor: Biological and molecular regulatory properties revealed. J Bone Miner Res 13: 325–349.

173. Carlberg C and Polly P (1998) Gene regulation by vitamin D3. Crit. Rev. Eukaryot. Gene Expr 8: 19–42.

174. Verlinden L, Verstuyf A, Quack M, Van Camp M, Van Etten E, De Clercq P, et al. (2001) Interaction of two novel 14-epivitamin D3 analogs with vitamin D3 receptor-retinoid X receptor heterodimers on vitamin D3 responsive elements. J Bone Miner Res 16: 625–638.

175. Hewison M, Freeman L, Hughes SV, Evans KN, Bland R, Eliopoulos, AG, et al. (2003) Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. J. Immunol 170: 5382–5390.

176. Veldman CM, Cantorna MT, and DeLuca HF (2000) Expression of 1,25- dihydroxyvitamin D(3) receptor in the immune system. Arch Biochem Biophys 374: 334–338.

177. Mathieu C and Adorini L (2002) The coming of age of 1,25-dihydroxyvitamin D(3) analogs as immunomodulatory agents. Trends Mol Med 8: 174–179.

178. Van Etten E and Mathieu C (2005) Immunoregulation by 1,25-dihydroxyvitamin D(3): Basic concepts. J Steroid Biochem Mol Biol 97: 93–101.

179. Fang Y, van Meurs JB, d'Alesio A, Jhamai M, Zhao H, Rivadeneira F, et al. (2005) Promoter and 30-untranslated-region haplotypes in the vitamin D

receptor gene predispose to osteoporotic fracture: The Rotterdam Study. Am J Hum Genet 77: 807–823.

180. Nejentsev S, Godfrey L, Snook H, Rance H, Nutland S, Walker NM, et al. (2004) Comparative high-resolution analysis of linkage disequilibrium and tag single nucleotide polymorphisms between populations in the vitamin D receptor gene. Hum Mol Genet 13: 1633–1639.

181. Arai H, Miyamoto K, Taketani Y, Yamamoto H, Iemori Y, Morita K, et al. (1997) A vitamin D receptor gene polymorphism in the translation initiation codon: Effect on protein activity and relation to bone mineral density in Japanese women. J Bone Miner Res 12: 915–921.

182. Zmuda JM, Cauley JA, and Ferrell RE (2000) Molecular epidemiology of vitamin D receptor gene variants. Epidemiol Rev 22: 203–217.

183. Jurutka PW, Remus LS, Whitfield GK, Thompson PD, Hsieh JC, Zitzer H, et al. (2000) The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB. Mol Endocrinol 14: 401–420.

184. Whitfield GK, Remus LS, Jurutka PW, Zitzer H, Oza AK, Dang HT, et al. (2001) Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene. Mol Cell Endocrinol 177: 145–159.

185. Colin EM, Wee AE, Uitterlinden AG, Buurman CJ, Birkenhager JC, Pols H, et al. (2000) Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D3. Clin Endocrinol 52: 211–216.

186. Gross C, Krishnan AV, Malloy PJ, Eccleshall TR, Zhao XY, Feldman D (1998) The vitamin D receptor gene start codon polymorphism: A functional analysis of FokI variants. J Bone Miner Res 13: 1691–1699.

187. Alroy I, Towers TL, and Freedman LP (1995) Transcriptional repression of the interleukin-2 gene by vitamin D3: Direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. Mol Cell Biol 15: 5789–5799.

188. Takeuchi A, Reddy GS, Kobayashi T, Okano T, Park J, Sharma, S (1998) Nuclear factor of activated T cells (NFAT) as a molecular target for 1alpha,25-dihydroxyvitamin D3-mediated effects. J Immunol 160: 209–218.

189. Adorini L, Penna G, Giarratana N, Roncari A, Amuchastegui S, Daniel KC, et al. (2004) Dendritic cells as key targets for immunomodulation by vitamin D receptor ligands. J Steroid Biochem Mol Biol 89/90: 437–441.

190. Griffin MD, Xing N, and Kumar R (2004) Gene expression profiles in dendritic cells conditioned by 1alpha,25-dihydroxyvitamin D3 analog. J Steroid Biochem Mol Biol 89/90: 443–448.

191. van Halteren AG, Tysma OM, Van Etten E, Mathieu C, and Roep BO (2004) 1alpha,25-Dihydroxyvitamin D3 or analogue treated dendritic cells modulate human autoreactive Tcells via the selective induction of apoptosis. J Autoimmun 23: 233–239.

192. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. (1998) Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. J Clin Invest 101: 252–262.

193. Overbergh L, Decallonne B, Waer M, Rutgeerts O, Valckx D, Casteels KM, et al. (2000) 1alpha,25-Dihydroxyvitamin D3 induces an autoantigen-specific T-helper 1/T-helper 2 immune shift in NOD mice immunized with GAD65 (p524–543). Diabetes 49: 1301–1307.

194. Komine M, Watabe Y, Shimaoka S, Sato F, Kake K, Nishina H, et al. (1999) The action of a novel vitamin D3 analogue, OCT, on immunomodulatory function of keratinocytes and lymphocytes. Arch Dermatol Res 291: 500–506.

195. Towers TL, Staeva TP, and Freedman LP (1999) A two-hit mechanism for vitamin D3-mediated transcriptional repression of the granulocyte-macrophage colony- timulating factor gene: Vitamin D receptor competes for DNA binding with NFAT1 and stabilizes c-Jun. Mol Cell Biol 19: 4191–4199.

196. Yu XP, Bellido T, and Manolagas SC (1995) Down-regulation of NF-kappa B protein levels in activated human lymphocytes by 1,25- dihydroxyvitamin D3. Proc Natl Acad Sci USA 92: 10990–10994.

197. Harant H, Wolff B, and Lindley IJ (1998) 1alpha,25-Dihydroxyvitamin D3decreases DNA binding of nuclear factor-kappaB in human fibroblasts. FEBS Lett 436: 329–334.

198. Alagarasu K, Selvaraj P, Swaminathan S, Narendran G, and Narayanan PR (2009) 5' regulatory and 3' untranslated region polymorphisms of vitamin D receptor gene in south Indian HIV and HIV-TB patients. J Clin Immunol 29: 196–204.

199. Cantorna MT (2011) Why do T cells express the vitamin D receptor? Ann NY Acad Sci 1217: 77–82.

Curriculum Vitae

Personal data:

Name: Atia Ramadan M. Elkilany

Born: 11 – 12 – 1969 S.KHAMIS – KHOMS

Nationality: LIBYAN

Marital status: married

Position: UNIV staff member

Phone: + 381 61 6906996

Passport No: 559601

email: atiaelkilany@yahoo.com

Education Background

BSc: 1990, Omar AL-Mukhtar University, Libya

MSc: 2004, „Evaluation of some tumor indicators for detection of tumor bladder", AL-Mergab University, Faculty of Arts and Science

MSc nostrification: 2009, University of Belgrade, Faculty of biology

PhD program: Immunobiology, University of Belgrade, Faculty of biology

Academic Employment

1994 – 1999, researcher in the lab at AL-Mergab University, Libya

2004 – 2008, Head of Biology at AL-Mergab University, Libya

The published papers in extenso in international journals

1. Elkilany A, Zeljic K, Surbatovic M, Djordjevic D, Magic Z, Bozic B. Toll-like receptors (TLR) 2, 3, and 4 genes polymorphisms in critically ill patients. Archives of biological archives. 2014; 66(3): in press

The published papers in extenso in international journals

1. Bozic B, Elkilany A, Zeljic K, Surbatovic M, Djordjevic D, Magic Z. Toll-like receptors genes polymorphisms in sepsis. Third Pan European Congress of Military Medicine, Belgrade, 2014: 115.

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