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The role of TLR-2 and TLR-4 gene polymorphisms in the development of sepsis in children with severe burns

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Abstract

Recently, many studies are based on the study of innate immunity genes, namely the role of TLRs in the development of various diseases. Severe burn injury is characterized by the development of hyperimmune reactions, which further leads to the development of multiple organ complications and sepsis. **The aim** of our study was to identify the frequency and prognostic value of polymorphism of TLR-2 Arg753Gln and TLR-4 Thr399 Ile genes and their role in the development of sepsis and MOD in children with severe burns. **Materials and methods:** genetic analysis of TLR-2 Arg 753 Gln and TLR-4 Thr399 Ile gene polymorphisms was performed in children with severe and extremely severe burns (n = 22) who were treated in the anesthesiology department with intensive care beds. **Results:** as a result of the obtained data, the heterozygous genotype TLR-2 Arg 753 Gln was detected in 69.5% (n = 16), in 31.8% (n = 7) patients with burn injury polymorphism was not detected. In 13.6% (n = 3) cases, polymorphism was associated with sepsis and MOD. Sepsis and normal homozygous genotype were diagnosed in 4.5% (n = 1). Heterozygous genotype TLR-4 Thr399 Ile was observed in 18.2% (n = 4) patients, of which only 4.5% (n = 1) patients had

polymorphism associated with sepsis and MOD. Analysis of the association of TLR 2 genotypes and markers of acute inflammation revealed statistically significant differences between the heterozygous TLR 2 Arg 753 Gln genotype and C-reactive protein (CRP) levels in the blood of patients on day 3 of burn disease. **Conclusions:** The study of the role of innate immune system genes is promising in predicting the development of sepsis and complications in severe burns and requires further careful study.

Key worlds: burns; Toll- like receptors; TLR-2 Arg 753 Gln and TLR-4 Thr399 Ile genes; sepsis; MOD; children; SIRS.

Topicality: The innate immune system is represented by a family of Toll-like receptors (TLRs), transmembrane proteins that are located on monocytes, macrophages, neutrophils, dendritic and other cells. Most TLRs transmit a signal via the Myd88 pathway (myeloid differentiation factor-88), which promotes the production of pro-inflammatory cytokines (leukotrienes, prostaglandins, chemokines). Activated TLRs initiate the activation of protection against pathogens, phagocytosis, leukocyte activation, antigen presentation by T-cells, which activates adaptive immunity [1]. When pathogens enter the body, they create a pattern - associated molecular patterns (PAMPs) are recognized by the immune system using image recognition receptors (PRRs), which include TLRs that are part of the interleukin-1 (IL-1R) superfamily, releasing proinflammatory cytokine factors. tumor necrosis, interleukin 1, 6, 8), inflammatory mediators (coagulation factor, nitric oxide, complement) and chemokines. In particular, the recognition of PAMP, TLR are involved in the recognition of endogenous released molecular patterns associated with danger (DAMP), which mediates "sterile" inflammation in the absence of infection [2]. Some authors have found that the patterns of expression of cytokines and chemokines of protein and gene transcripts demonstrate a state of inflammatory dysregulation of wound healing [3].

Severe injury is a test for the immune system. Post-traumatic hyperinflammation in combination with ischemic/reperfusion lesions mediated by complement system that activate the innate immune system and early post-traumatic inflammatory response, leading to indirect remote organ damage and multiorgan failure syndrome (MOD). In this process, an important role is played by TLR-4, the expression of which was detected in the liver, lungs and myocardium in hemorrhagic shock and resuscitation [4]. Increased TLR activation leads to systemic inflammation and sepsis. TLR-2 and TLR-4 play an important role in this [5].

An important role in the development of septic shock belongs to TLR, as noted in the publications of some authors who studied the effect of TLR-4 polymorphism on the development of gram-negative bacteremia and sepsis [6].

Recently, many studies have linked the polymorphism of TLR genes and their relationship to susceptibility to disease. The molecular weight of TLR-2 is 89.8 kDa, it can form heterodimers from TLR-1 or TLR-6 and interacts with lipoproteins, peptidoglycans and membrane fatty acids of gram-positive bacteria. The Arg753Gln polymorphism of the TLR-2 gene was described in 2000, is characterized by the replacement of arginine by glutamine at residue 753 and is associated with increased susceptibility to staphylococcal infections and candidiasis [7].

TLR-4 protein weighing 95.6 kDa creates dimers and recognizes proteins of heat shock, eyelashes of gram-positive bacteria, is closely associated with the development of sepsis caused by gram-negative bacteria, as well as systemic candidiasis [8].

Recent studies have shown that TLR-4 recognizes molecules released by stress and necrotic cells, as well as degradation products of endogenous molecules [9].

The most studied polymorphism TLR-4 - Asp299Gly is identified by the replacement of A → G in nucleotide 896 from the start codon, which leads to the substitution of aspartic acid for glycine at position 299 of amino acid sequences. However, the prevalence of homozygous mutations in the population is low, only 9.4%. Some studies have shown that the association of polymorphisms in TLR-4, A1287G / Asp299Gly, and C13174T / Thr399Ile alters the extracellular structure, making the response to lipopolysaccharides slow and increasing the incidence of septic shock [10].

TLRs have been shown to be produced by renal cells and involved in protection against pathogens. In addition, TLR-4 has been shown to trigger endothelial activation, which is required for inflammation in ischemic kidney damage [11].

Some sources indicate that TLRs affect myelopoiesis, a process that is due to the fact that TLRs are expressed on hematopoietic stem cells (HSCs) that respond to the release of proinflammatory cytokines. The influence of pathogens on HSCs reduces their function and ability to hematopoiesis [12].

Dysfunction of neutrophils in sepsis, which leads to their apoptosis is associated with the activation of TLR-2 by LPS [13].

Impaired intestinal epithelial permeability to endotoxins produced by severe burns, Kupffer cells are activated in the liver via TLR-4 receptors, which activate proinflammatory

cytokines that affect liver endothelial cells and lead to inflammation hepatitis and fibrosis (Mookerjee, 2011).

The development of septic lung disease and respiratory distress syndrome is the activation of alveolar macrophages, the release of inflammatory mediators and the involvement of blood leukocytes in the lungs. This process causes the destruction of epithelial and endothelial barriers and pulmonary edema [14].

Impaired hemodynamics in sepsis and, as a consequence, septic cardiomyopathy depends on many pathophysiological processes associated with dysfunction of endothelial cells, cardiomyocytes due to the release of pro-inflammatory mediators and impaired hemostasis [15].

The nervous system is tightly connected and regulates the immune system through the autonomic nervous system, as all cells in the body have cytosolic and membrane receptors for neurotransmitters. Defeat of the nervous system in response to the release of a large number of pro-inflammatory mediators, leads to impaired autonomic and neurohumoral regulation, which leads to changes in the immune response, metabolic disorders and organ dysfunction [16].

The development of sepsis and mortality in patients with severe trauma, according to some authors, is realized in several ways. In one case, massive trauma leads to a hyperimmune response, namely systemic inflammatory response syndrome (SIRS) and dysfunction of many remote organs of MOD and mortality in the first 24 hours after injury. Another way to develop complications in patients with severe trauma is hyperimmune reaction / SIRS with subsequent development of compensatory anti-inflammatory syndrome (CARS) and immunosuppression, which leads to the inability to re-adequate adequate immune response to secondary infection. These immune responses lead to the development of late septic complications, MOD and a high risk of mortality. Also, the development of cachexia in patients with sepsis indicates an effect on the violation of cellular immunity, the so-called catabolic immune suppression syndrome (PICS) and is an important factor in increasing mortality [17].

The role of CRP in the development of the primary immune response is determined by activating TLR to stimulate the production of TNF, IL 1β, IL-23 and activation of inflammatory macrophages [18]. Thus, the role of CRP in the primary immune response explains the enhancement and maintenance of systemic inflammation through constant stimulation of Toll-like receptors and the synthesis of proinflammatory cytokines.

Given that the development and outcome of sepsis in burn patients depends not only on the severity of burn injury, burn depth, intensity and duration of systemic inflammatory response, but also on the patient's immune system and genetic background, the detection of TLR-2 and TLR-4 polymorphism is relevant.

The aim of our study was to identify the frequency and prognostic value of polymorphism of TLR-2 Arg753Gln and TLR-4 Thr399 Ile genes and their role in the development of sepsis and MOD in children with severe burns.

Materials and methods

Genetic analysis of TLR-2 Arg 753 Gln and TLR-4 Thr399 Ile gene polymorphisms was performed in children with severe and extremely severe burns (n = 22) who were treated in the anesthesiology department with intensive care beds of the regional burn department of the 1Communal Nonprofit Enterprise "Odessa Regional Clinical Medical Center" of the Odessa Regional Council, Odessa, Ukraine. Collection of genetic material was performed on the first day after burns in children with severe and extremely severe burns by removing the buccal epithelium on an empty stomach with an applicator. Isolation of the polymorphic region of TLR-2 Arg 753 Gln and TLR-4 Thr399 Ile from epithelial cells was performed in several stages:

1. Collection of material: performed on an empty stomach, by buccal scraping of the mucous membrane of the right and left cheeks with a cotton swab.

2. DNA isolation: from buccal epithelium was performed with 5% solution of Chelex. 200 μl of 5% Chelex solution was collected in a separate tube, where the applicator with biomaterial was placed and incubated on a thermal shaker at 60 ° C for 30 minutes, and then at 95 ° C for 30 minutes. The cooled tube was centrifuged for 2 minutes at 12 thousand revolutions per minute. The selected supernatant was transferred to a new tube and the DNA concentration was determined on an Implen spectrophotometer. After that, the liquid was diluted with sterile distilled water to a concentration of 60-70 mg / μl. The resulting solution is stirred with a Vortex centrifuge.

3. Isolation of DNA from epithelial cells: performed by PCR reaction on the amplifier Tertsyk TP-4 PCR01, produced by "DNA technology", Russia. To prepare the reaction mixture, the PCR tubes were placed on an ice bath and distilled deionized water, DNA template, 0.5mM dNTP mixture, forward and reverse primers, 10X Taq buffer, 25mM MgCl2, Taq DNA polymerase (manufactured by Rosia, Syntol) were added. Stir the sample gently and centrifuge briefly to collect all droplets from the walls of the tube. The sample was

covered with mineral oil (three quarters of the sample volume) and placed in an amplifier for PCR.

PCR reaction program:

- for TLR-2: 95 ° C - 5 minutes, 95 ° C - 15 minutes, 65 ° C - 30 sec, 72 ° C - 40 sec.
- for TLR-4: 95 ° C - 5 minutes, 95 ° C - 15 minutes, 60 ° C - 30 sec, 72 ° C - 40 sec.

4. Restriction:

After completion of the amplification program, the samples were restricted by enzymes: for TLR-2 enzyme MSP1, for TLR-4 enzyme HINF1.

5. Electrophoresis:

Separation of the amplification products was performed in a horizontal 2% agarose gel prepared on disposable tris-borate buffer (1xTBE). Molecular weight marker - pUC19 DNA: Msp1. Agarose gel was stained with ethidium bromide and visualized in transmitted ultraviolet light. to obtain the final products. At the norm of TLR-2 the length of the fragment is 129 bp, at mutation the fragment as a result of restriction is cut into 2 fragments of length 104 and 25 bp.

At the norm of TLR-4 the fragment as a result of restriction is cut into 2 fragments of length 98 and 26 bp, at mutation the fragment as a result of restriction is not cut, and its length makes 124 bp.

The research was performed on the basis of the testing laboratory of G-LAB LLC, Odessa, Ukraine.

The results were evaluated using the program STATISTICA 13.3 (developer - StatSoft.Inc) and the spreadsheet "Exel". Categorical data were described with absolute and relative frequencies. Quantitative variables were assessed for normality using the Shapiro-Wilk test. Quantitative variables following non normal distribution were described using median (Me) and lower and upper quartiles (Q1 – Q3).

Mann-Whitney U-test was used to compare two groups on a quantitative variable whose distribution differed from the normal distribution.

Estimation of the frequency of gene polymorphism distribution was performed using the exact Fisher's test, reliable took into account $p < 0,05$. The relative risk of sepsis and mortality was assessed using the odds ratio.

ROC analysis was used to assess the diagnostic performance of quantitative variables in predicting a categorical outcome. The optimal cut-off value of the quantitative variable at was estimated using the Youden's J statistic.

Results:

As a result of the obtained data, the heterozygous genotype TLR-2 Arg 753 Gln was detected in 69.5% (n = 16), in 31.8% (n = 7) patients with burn injury polymorphism was not detected. In 13.6% (n = 3) cases, polymorphism was associated with sepsis and MOD. Sepsis and normal homozygous genotype were diagnosed in 4.5% (n = 1).

When comparing Sepsis depending on TLR-2, we could not detect significant differences (p = 0.318, Fisher's exact test).

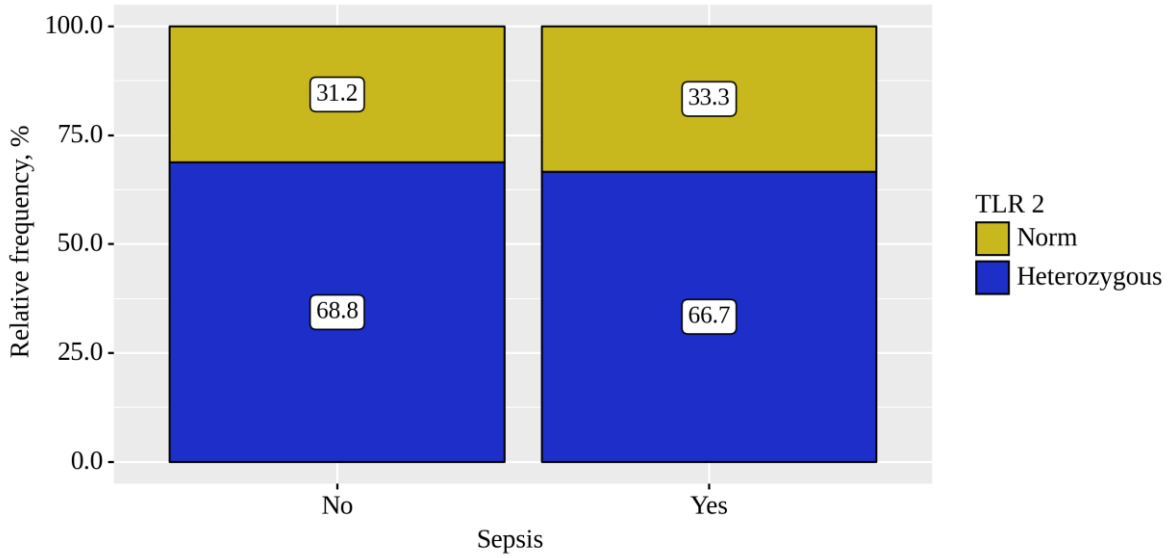

Figure 1 – Analysis of TLR 2 conditioning on Sepsis

Chances Yes in the Heterozygotes group were lower 3,250 times, compared with the Norms group, the difference in chances was not statistically significant (HS = 0.308; 95% CI: 0.044 - 2.171).

Heterozygous genotype TLR-4 Thr399 Ile was observed in 18.2% (n = 4) patients, of which only 4.5% (n = 1) patients had polymorphism associated with sepsis and MOD.

In the analysis of Sepsis depending on the TLR 4 genotype, we were unable to establish statistically significant differences (p = 1,000, Fisher's exact test).

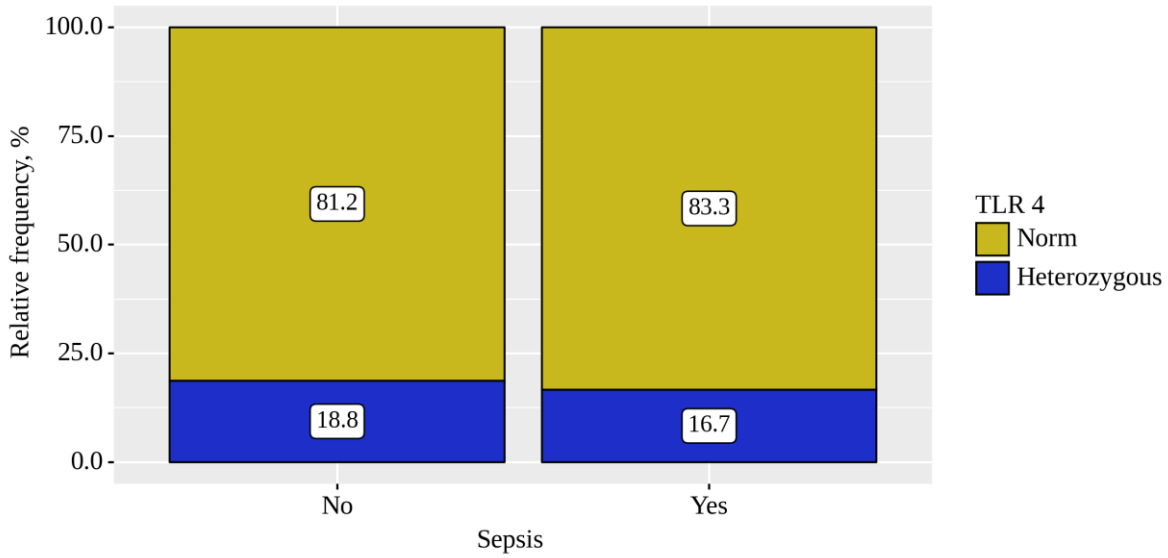

Figure 2 – Analysis of TLR 4 conditioning on Sepsis

Chances Thus, in the Heterozygotes group they were 1,071 times lower than in the Norms group, the difference in chances was not statistically significant (HS = 0.933; 95% CI: 0.078 - 11.177). Thus, in children with a heterozygous immune genotype, the response to severe burn injury does not lead to the development of long-term CVD.

Among children with severe burns who were diagnosed with sepsis, the heterozygous TLR-2 Arg 753 Gln genotype was diagnosed in 75% and the TLR-4 Thr399 Ile genotype in 25%, respectively.

Correlation analysis comparing developed complications and TLR 2 and TLR 4 genotypes did not reveal statistically significant differences.

Table 1

Analysis of CRP conditioning on TLR 2

Variables	Categories	TLR 2			p
		Me	$Q_1 – Q_3$	n	
CRP1	Norm	2	1 – 4	7	0.113
	Heterozygous	5	4 – 7	15	
CRP3	Norm	2	2 – 5	7	0.045*
	Heterozygous	8	7 – 9	15	
CRP7	Norm	2	2 – 7	7	0.084
	Heterozygous	10	5 – 13	15	
CRP14	Norm	1	0 – 2	7	0.535
	Heterozygous	3	0 – 9	15	
CRP21	Norm	0	0 – 2	7	0.838
	Heterozygous	0	0 – 1	15	

* – differences are statistically significant ($p < 0.05$)

Analysis of the association of TLR 2 genotypes and markers of acute inflammation revealed statistically significant differences between the heterozygous TLR 2 Arg 753 Gln genotype and C-reactive protein (CRP) levels in the blood of patients on day 3 of burn disease.

Based on the results obtained, it can be concluded that CRP and TLR2 are involved in the primary immune response in OT, and the strength and duration of this response as well as SIRS depends on the TLR2 genotype. Thus, the heterozygous TLR2 genotype is activated not immediately after receiving OT, but based on the results obtained at a later date, which may affect the duration and outcome of burn disease.

The following curve was obtained by estimating the dependence of the probability of the heterozygous TLR2 genotype on the CRP3 index using ROC analysis.

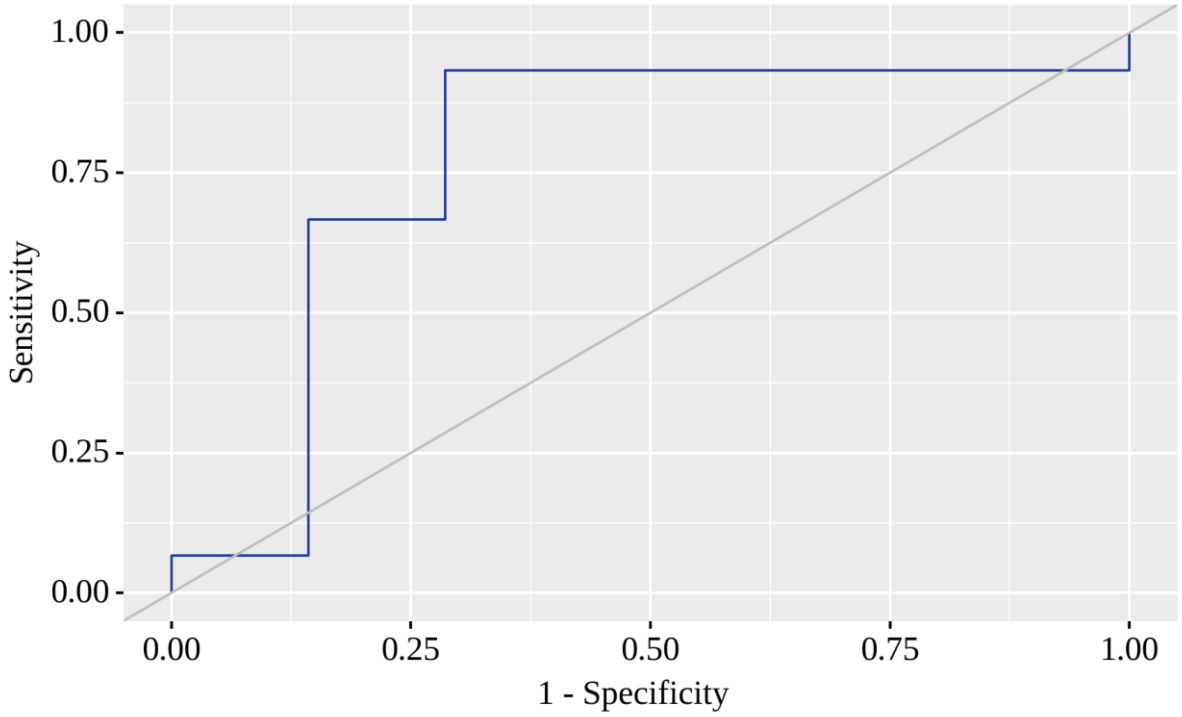

Figure 3 – ROC-curve characterizing the dependence of the probability TLR 2 on CRP3

The area under the ROC curve was 0.771 ± 0.102 with 95% CI: 0.571 - 0.972. The obtained model was statistically significant (p = 0.045).

The cut-off threshold at the cut-off point, which corresponded to the highest value of the Juden index, was 3,870. Heterozygote was predicted when the value of "CRP3" is higher

than or equal to this value. The sensitivity and specificity of the model were 93.3% and 71.4%, respectively.

No significant differences were found between other indicators of inflammation and TLR2 and TLR4 genotypes.

Conclusions:

1. We found that different genotypes of TLR 2 and TLR 4 have different effects on the chances of developing sepsis in patients with severe burn injury due to the intensity of the immune response, but are not specific markers of sepsis.

2. Heterozygous type TLR 2 is involved in the development of SIRS in severe burns and depending on the term of activation regulate the timing and outcome of OX.

3. The study of the role of innate immune system genes is promising in predicting the development of sepsis and complications in severe burns and requires further careful study.

References:

1. Hari A, Flach TL, Shi Y, Mydlarski PR. Toll-like receptors: role in dermatological disease. Mediators of inflammation. 2010 Oct; 2010.

2. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling**.** Nat Rev Immunol **7**: 353–364, 2007.

3. G. Hahm, J. J. Glaser, and E. A. Elster, "Biomarkers to predict wound healing: the future of complex war wound management," Plastic and Reconstructive Surgery, vol. 127, pp. 21S–26S, 2011.

4. L. J. McGhan and D. E. Jaroszewski, "The role of toll-like receptor-4 in the development of multi-organ failure following traumatic haemorrhagic shock and resuscitation," Injury, vol. 43, no. 2, pp. 129–136, 2012.

5. El-Zayat SR, Sibaii H, Mannaa FA. Toll-like receptors activation, signaling, and targeting: an overview. Bulletin of the National Research Centre. 2019 Dec; 43(1):1-2.

6. Assenzio B, Martin-Conte EL. Cellular Mechanisms of MOF during Severe Sepsis and Septic Shock. INTECH Open Access Publisher; 2012 Feb 10.

7. Hernandez-Sancen P, Maldonado-Bernal C. Relevance of single-nucleotide polymorphisms in human TLR genes to infectious and inflammatory diseases and cancer. Genes & Immunity. 2014 Jun;15(4):199-209.

8. Lei HE, Maohu LI, Wensheng FA, Yunxi LI, Jijiang SU, Yubin XI, Ning JI. Association of Single Nucleotide Polymorphisms in Toll-like Receptors with Acinetobacter baumanii Infectionin a Chinese Population. Iranian journal of public health. 2016 Jan;45(1):20

9. Zhao H, Perez JS, Lu K, George AJ, Ma D. Role of Toll-like receptor-4 in renal graft ischemia-reperfusion injury. American Journal of Physiology-Renal Physiology. 2014 Apr 15;306(8):F801-11.

10. Yuan FF, Marks K, Wong M, Watson S, de Leon E, McIntyre PB, Sullivan JS Clinical relevance of TLR2, TLR4, CD14 and FcgammaRIIA gene polymorphisms in Streptococcus pneumoniae infection. Immunol Cell Biol. 2008 Mar-Apr; 86(3):268-70.

11. Gluba A, Banach M, Hannam S, Mikhailidis DP, Sakowicz A, Rysz J. The role of Toll-like receptors in renal diseases. Nat Rev Nephrol 6: 224–235, 2010.

12. Boiko JR, Borghesi L. Hematopoiesis sculpted by pathogens: Toll-like receptors and inflammatory mediators directly activate stem cells. Cytokine. 2012 Jan 1;57(1):1-8.

13. Navarini AA, Lang KS, Verschoor A, et al. Innate immune-induced depletion of bone marrow neutrophils aggravates systemic bacterial infections. Proc Natl Acad Sci U S A 2009;106:7107-12. 10.1073/pnas.0901162106 .

14. Assenzio B, Martin-Conte EL. Cellular Mechanisms of MOF during Severe Sepsis and Septic Shock. INTECH Open Access Publisher; 2012 Feb 10.

15. Bouhemad B, Nicolas-Robin A, Arbelot C, Arthaud M, Feger F, Rouby JJ. Acute left ventricular dilatation and shock-induced myocardial dysfunction. Crit Care Med 2009;37:441-447.

16. Streck EL, Comim CM, Barichello T, Quevedo J. The septic brain. Neurochem Res 2008;33:2171-2177.

17. Wischmeyer PE. Malnutrition in the acutely ill patient: is it more than just protein and energy? S Afr J Clin Nutr. 2011;24:1–7.

18. Newling M, Sritharan L, van der Ham AJ, Hoepel W, Fiechter RH, de Boer L, Zaat SA, Bisoendial RJ, Baeten DL, Everts B, den Dunnen J. C-reactive protein promotes inflammation through FcγR-induced glycolytic reprogramming of human macrophages. The Journal of Immunology. 2019 Jul 1;203(1):225-35.

19. Tsimogiannis KE, Tellis CC, Tselepis AD, Pappas-Gogos GK, Tsimoyiannis EC, Basdanis G. Toll-like receptors in the inflammatory response during open and laparoscopic colectomy for colorectal cancer. Surgical endoscopy. 2012 Feb;26(2):330-6.

20. Di S, Wang Y, Sun L, Zhao H, Guo L. The value of TLR-4, CRP, PCT and WBC levels in assessing the diagnosis and prognosis of sepsis patients. International journal of clinical and experimental medicine. 2020 Jan 1;13(12):9419-28.

21. Haider DG, Leuchten N, Schaller G, Gouya G, Kolodjaschna J, Schmetterer L, Kapiotis S, Wolzt M. C-reactive protein is expressed and secreted by peripheral blood

mononuclear cells. Clinical & Experimental Immunology. 2006 Dec;146(3):533-9.

22. Newling M, Sritharan L, Baeten D, Den Dunnen J. P080 C-reactive protein: not only a marker, but also a cause of inflammation through metabolic reprogramming of human macrophages. Annals of the Rheumatic Diseases. 2018 Mar 1;77: A47.

23. Yurko K, Kozko V, Solomennik A, Bondar O, Sokhan A, Gavrylov A. The role of polymorphism asp299gly of the gene tlr 4 in patients co-infected with hiv/hcv. Georgian medical news. 2018 jul 1(280-281):138-41.

24. AbdAllah NB, Toraih EA, Al Ageeli E, Elhagrasy H, Gouda NS, Fawzy MS, Helal GM. MYD88, NFKB1, and IL6 transcripts overexpression are associated with poor outcomes and short survival in neonatal sepsis. Scientific Reports. 2021 Jun 28;11(1):1-4.