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TLR2 AND TLR4 AS A BIOMARKER IN ADULT AND CHILDREN IRAQI BACTERIAL SEPSIS SYNDROME PATIENTS

Farah Q. Younis*, Ali Hussein. Alwan*, Neihaya H. Zaki*

*Biology Department, College of Science, University of Mustansiriya, Iraq
E.mail: farahyounis89@gmail.com

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ABSTRACT

This study aimed to investigate the possibility using of TLR2 and TLR4 gene expression as an early biomarkers for diagnosis bacterial septic syndrome in children and elderly. The causative agents of infection was determined by blood culture. TNF-α, IL-10, and sHLA-DR were measured by ELISA and TLR2, TLR4 expression was determined by quantitative RT-PCR. about 75 patients was diagnosed with sepsis syndrome included in this study . The age range of patients (13days-92years) with mean 56.3±13.9 and matched to 55 healthy volunteers. Depending on age, patients were divided into 4 age groups (group A <1 year, group B 1-13 year, group C 14-40 year and group D >40 year). The results indicated that the levels of TNF-α, IL-10 and sHLA-DR in all age groups were significantly increased except the level of TNF-α in group B compared with control groups. A positive correlation has observed between levels of TNF-α and sHLA-DR with patients age respectively. TLR2 and TLR4 mRNA expression was significantly increased in all age groups with significant difference between group A and groups D. TLR2 expression highly increases in G+ve infection while TLR4 expressed highly in G-ve bacteria. can be conclude that TLR2 and TLR4 expression in bacterial sepsis patients indicates the strong possibility of using them as biomarkers in the early diagnosis of bacterial sepsis syndrome, in children and elderly patients

Keyword: TLR2, TLR4, Soluble HLA-DR, Sepsis biomarker, bacterial sepsis

INTRODUCTION

The human innate immune system is the first defense line against invasive pathogens. Infant's immune system is differing from that of adult but, both very young children, and older adults both have a relatively poor immune system ((Simon *et al*., 2014). Both immune responses (innate and adaptive) are irregulated by aging, which contributes to increase the occurrence of infection in the elderly ((Starr and Saito, 2014). Bacterial sepsis syndrome is a systemic inflammatory response to an infectious stimulus orchestrated by the bulk of pro-inflammatory mediators production as a response to pathogen associated molecular patterns (PAMP) such as lipopolysaccharide (LPS) and peptidoglycan which the main component of bacterial cell wall of G-ve and G+ve bac-teria (Rittirsch *et al*., 2008). The innate immune response very important in the defense against bacterial pathogens in early phases of infection. Pattern recognition receptors (PRRs) are part of these defense, which expressed on leukocytes, and one of the most important PRRs is Toll-like receptors (TLRs), which is important as sentinel receptors have increased appreciation that which recognized PAMPs (Zhang *et al*., 2010). LPS is primarily sensed by TLR4 while, peptidoglycan, has been sensed by TLR2 (Kumar *et al*., 2011). TLR cascade of cellular signals leads to active-tion of pro-inflammatory cytokines such TNFα, and anti-inflammatory cytokines like IL- cytoki-nes like IL-10 and this TLRs activation not only leads to inducing of inflammatory responses but also to development of antigen-specific adaptive immunity (Lu, 2009).

Soluble HLA-DR is one of the numerous mark-ers that have been tested for their capacity to predict mortality in septic patient's molecules acts a central role in the specific immune resp-onse to infection. The important of soluble HLA-DR molecules can function as ligands for super-antigens and, thus, may play a role in the "detoxi-fication" of super-antigens (Perry *et al*., 2004).

Studies that combine children and elderly with bacterial sepsis are generally low. This study came to illustrate differences between them in their immune response to sepsis infection and determine a better biomarker for early diagnosis of bacterial septic syndromes in Iraqi's patients.

MATERIALS AND METHODS

Study Design and Population: Seventy five blood samples were collected from different pat-ients who diagnosed clinically by a specialized physician with sepsis from intensive care units of Baghdad Hospitals, People's Republic of Iraq, during the period March 2016 to October 2016. Patients are characterized by having more than two criteria of sepsis with mean 56 ± 13 year. Control group consists of 55 healthy volunteers, with mean of age 35.7 ±17.2 years.

The patients have been divided into 2 main groups' adults and children and each group was subdivided into two groups according to their ages.

For adults, sepsis has been defined by pre-sents of two from the four following criteria: fever (>38˚C or <36˚C), respiratory rate of >20 breaths/minute or arterial carbon dioxide tension ($PaCO_2$) <32mm Hg, heart rate of >90 beats/ minute, and abnormal white blood cell count (>12,000/µL or < 4,000/ µL or >10% immature forms). For children, sepsis was defined as two of the four following criteria: temperature >38.5˚C <36˚C, respiratory rate >2 (SD) above normal for age in the absence of external stimulus, heart rate >2 standard deviations (SD) above normal for age, and white blood cell count elevated or depre-ssed for age, or > 10% bands (Rudd *et al*., 2017**).**

Sample Collection: Five ml of blood from adults and 3 ml form children has been collected by vein puncture after prepared it by using a bacterial disinfectant 2 % solution peripheral venous blood samples were collected from each participant by using sterile single use needles. This blood was used for blood cultures, cytokines measurement and RNA extraction.

Cytokines and soluble HLA-DR measurement: Serum IL-10, TNF-α and soluble HLA-DR concentrations were determined by ELISA using a commercial human ELISA kit (R&D system Inc, USA) for IL-10 and TNF-α and (MyBio-Sou rce Company, USA) for sHLA-DR, in accordance with the manufacturer's instructions. Concentrations were calculated by using of the mean optical density of two wells and comparison with a standard curve.

RNA extraction: RNA was extracted manually by TRIzole provides by (Invitrogen Life Technologies, USA) an efficient method for purifying total RNA from whole blood, and also the procedure of extraction according to the manufacturer's instructions. RNA concentration was measured by nano-drop spectrophotometer (Quawell Q5000, USA) and the purity detected by noticing the ratio of optical density (O.D.) at wave length 260/280.

qReal-Time PCR: TLR2 and TLR4 mRNA expression were determined using a (KAPA SY- BR FAST one-step qRT-PCR kit, Canada) is a sensitive and convenient solution for real-time PCR using RNA as template. The kit comprises KAPA SYBR FAST master mix (2 X) and KA-PA RT mix (50 X). The KAPA RT mix comprises.

RT-PCR was performed using designated primers for TLR2 (forward: **5'**TGTGGATGGTGTGG GTC TTG**3'**, Reverse: 3'ATATGCAG CCTCCGGATTGT5') and TLR4 (forward: 5'ATATTGACAGG-AAACCCCATCCA3', Reverse: 3' A GAGAGATTGAGTAGGGGCATTT5'), with (GAPDH) (forward: 5'ATCACTGCCACCCAGAAGACTG3', reverse: 3'AGGTTTTTCTAGACGG-CAGGTCAG5') (Alpha DNA technologies). Relative gene expression to an internal calibrator was determined using the $2^{-\Delta\Delta CT}$ method. The Ct value of the target genes was normalized (ΔCt) to the Ct value of the TLR 2, 4 gene of the samples.

Statistical analysis: Student *t*-test was used when comparing two groups and ANOVA /Bonferroni test when comparing more than two groups. Data were expressed as mean±S.E. Correlations were determined using a Spearman correlation test. *P*-values lower than 0.05 were considered statistically significant. The data were analyzed by the statistical software (SPSS 22.0, SPSS Inc., Chicago, IL, USA).

RESULTS

The study included 75 patients with suspicion of septicemia who admitted to ICU and hospital lounges, and 55 healthy controls were enrolled in the study. Table 1 shows the demographic characteristics of the patients.

Table 1: Demographic data of patients with suspected sepsis

Characteristic	Adult	Children
No.	41	34
Age (yr)	56.5 ± 22.26	2.6 ± 3.18
Male / female	28/13	17/17
Infection lead to sepsis		
Ural Sepsis	18 (43.9%)	-
Immune suppression patient	4 (9.7%)	10 (29.4%)
Wound infection	6 (14.6%)	-
Post operation sepsis	5 (12.1%)	-
Renal failure, Hepatic failure, Brain failure	8 (19.5%)	-
Neonatal sepsis	-	10 (29.4%)
Meningitis	-	5 (14.4%)
Respiratory tract infection	-	5 (14.7%)
Gastrointestinal infection	-	4 (11.7%)

Blood culture was positive in 49/75 (65.3%) of septic patient's. All infections were due to only one organism. About the types of bacteria that isolated from positive culture, G-ve constituted 59.1%, while G+ve constituted 40.8%. A total of 49 organisms were isolated, the commonest organism was *E.coli* 17 (34.6%) followed by *S.aureus* 14 (28.5%). These bacteria and other bacteria which result from blood culture shown in table 2.

Table 2: Types of isolated bacteria in patients enrolled in the present study

Type of Bacteria	No. (%)
G-ve	**29 (59.1)**
E.coli	17 (34.6)
A.baumannii	4 (8.1)

K.pneumonia	4 (8.1)
P.aeroginosa	3 (6.1)
C.freundii	1 (2)
G+ve	**20 (40.8)**
S.aureus	14 (28.5)
E.fecalis	3 (6.2)
S.pneumoniae	3 (6.1)
Total	49 (100)

Level of IL-10, TNF-α and sHLA-DR in serum of patients with different age group: According to their ages, the patients have been divided into 4 groups (<1 year (group A), 1-13 year (groups B), 14-40 year (groups C), >40 year (groups D).

TNF-α and IL-10 concentration was significantly increased *P*-value< 0.05, *P*-value< 0.001 in all age groups compared with their controls. Except level of TNF- α in group C (P=0.123) as shown in table 3.

sHLA-DR concentration was significantly increased *P*-value < 0.05, *P*-value< 0.001 in age groups (group A, C and D) compared with their controls, but no significant has been shown in age group B when it's compared with their control group as shown in table 3.

Table 3: Level of IL-10, TNF-α and sHLA-DR in patient's serum with different age group.

Age group	No.	IL-10 Mean ± S.E	P.value	TNF-α Mean ± S.E	P.value	sHLA-DR Mean ± S.E	F
group A	24	49.50 ± 9.36	**0.000**	56.1 ± 5.74	**0.001****	9.06 ± 1.75	**0.031***
	18	3.84 ± 0.49	**	28.63 ± 4.10		4.49 ± 0.16	
group B	10	41.16 ± 13.04	**0.033**	62.48 ± 11.71	**0.123**	9.19 ± 2.63	**0.128**
	7	3.32 ± 0.89	*	39.80 ± 2.04		4.59 ± 0.16	
group C	12	77.97 ± 21.43	**0.011**	77.97 ± 9.08	**0.009***	15.97 ± 2.36	**0.021***
	8	2.54 ± 0.49	*	43.90 ± 4.00		8.52 ± 0.45	
group D	29	60.33 ± 12.24	**0.000**	81.19 ± 9.62	**0.000****	20.00 ± 1.88	**0.000****
	22	2.91 ± 0.56	**	34.00 ± 3.28		9.30 ± 0.47	

*** *P*-value<0.05 ** *P*-value<0.001**

increased with increasing of age (R= 0.2332, *P*-value =0.044) and also increasing of sHLA-DR in serum of patients with increasing of their ages (R =0.42832, *P*-value = 0.00013). Figure 1B. No correlation has been observed between the level of IL-10 of patient's serum and their age.

Correlation between cytokines and sHLA-DR and age: The results of study has been shown, there were a significant positive correlation *P*-value<0.05 between level of both TNF-α and sHLA-DR in patients serum and their age. As shown in figure 1A, the concentration TNF-α was

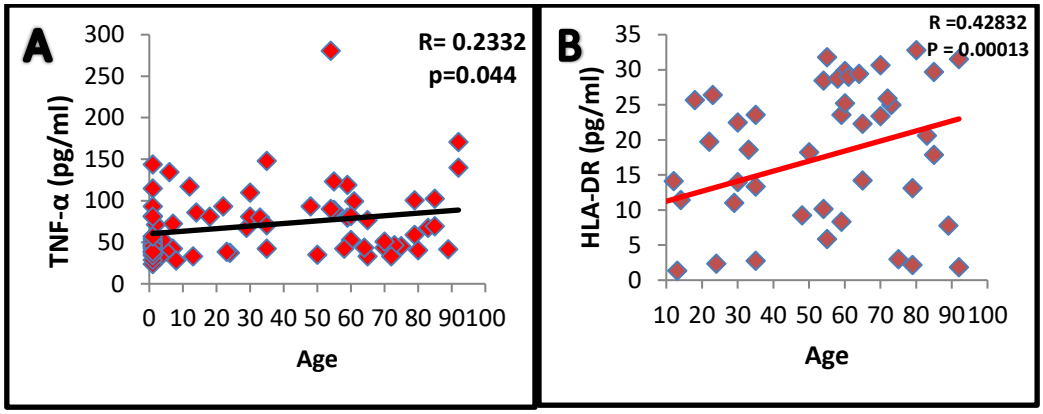

Figure 1A: correlation between TNF-α concentration and patients age. B: correlation between sHLA-DR concentration and patients age

difference among all groups (*P*-value < 0.049). As shown in table 4.

TLR4 expression has been shown with high level in group D (7.669 ± 0.803) and the lowest level of expression shown in group B (1.575 ± 0.296), and also a significant difference among all groups 0.001 (*P*-value<0.000). As shown in table 4.

Expression of TLR2 and TLR4 in different age groups: TLR2 and TLR4 mRNA have been expressed in all age groups. TLR2 expression has been shown with high level in group D (4.458 ±0.650) and the lowest level of expression shown in group A (2.436 ±0.298), and also a significant

Table 4: fold genges in expression of of TLR2 and 4 in patients according to their age

Age group	No.	TLR 2 (fold change ± S.E)	P.value	TLR 4 (fold change± S.E)	P.value
Group A	21	2.436 ±0.298	**0.049***	2.497 ± 0.281	**0.000****
Group B	5	2.763 ±0.697		1.575 ± 0.296	
Group C	8	3.021 ±0.664		3.255 ± 0.616	
Group D	26	4.458 ±0.650		7.669 ± 0.803	

*** *P*-value < 0.0 5 ***P*-value< 0.000**

Expression of TLR 2 and 4 according to type of bacteria: present results indicated that the expression of TLR2 significantly increases in septic patients (*P*-value<0.000) who infected by G+ve compared with G-ve 5.570 ± 0.712 , 2.333 ± 0.225) respectively.

On the other hand, the results showed that the expression of TLR4 significantly increases in septic patients (*P*-value<0.000) who infected by the G-ve compared with G-ve (8.120 ± 0.684, 2.231 ± 0.257) respectively.

DISCUSSION

In present trial, the relationship between sepsis and age, grow in blood culture, type of growing pathogen, cytokines (TNF-α, IL-10, sHLA-DR) and TLR expression in patients were investigated. (Artero *et al*.,2012) indicated a direct relationship between progress age and the occurrence of sepsis, with a sharp increase in incidence in elderly people, and also the incidence of sepsis in infants is also elevated. Burrell *et al*., (2016) reported that the patients are reached at the age of 65 and above, the relative risk for sepsis was 13 times higher than younger patients.

In the infant sepsis the group A who >1year was 32% of all patients and 70 % of children's groups and this result similarity with Wacker *et al*., who finding that 76% of children less than 1 year of age from their cases of sepsis are most likely to be infected **(**Wacker *et al*., 2013). (Angus *et al*., 2001) founded the incidence of sepsis was highest in infants (5.3/1,000 aged, 1y), and also, rising sharply in the elderly (26.2/1,000 aged 85y) and also the number of cases also increased with increasing age so that, this result in line with our study**.** Children from 1-4 years old are clearly different than adults in terms of underlying disease, mortality and sites of infection, and also those younger than 35 years of age, in fact, these two groups more similar to each other than they are to either infants or adults over the age of 60 years (Watson *et al*., 2003)**.**

As a result of our study G-ve bacteria has been the most predominant causative pathogens of septicemia in the present study with (59.1%), whereas G+ve (40.8%) this result correspondingly with (Sahoo *et al*., 2016) who find G+ve organisms were found to be 30.8% among all isolates causing sepsis, whereas the G-ve bacteria were 69.2% of all isolates.*E.coli* was most com-mon causative of sepsis in patients with (34.6%) followed by *S. aureus* with (28.5%) this result was identical to Kotgire *et al*., (2017) who found that *E.coli* constituted (34.6%) and *S.aureus* (23.1%). Zhang *et al*., (2015) shown in their study that *S.aureus* and *E.coli* were the prevalent isolates from the total isolate that constitute for G+ve and G-ve, respectively.

The results of study have been indicated that pro-inflammatory and anti inflammatory cytokines levels were significantly higher in septic patients compared with healthy controls. As well; the level of IL-10 significantly higher in the elderly compared to children patients. These results are in accordance with previous studies of Bruunsgaard *et al*., (1999) and Krabbe *et al*., (2004) reported no age-related difference in TNF-α , or IL-10 on admission in hospitalized patients with *S.pneumonia* infection; and also the elderly patients had significantly higher levels of TNF-α comparing with younger patients, suggesting once again a prolonged period of inflammatory response in elderly investigators and other investigators (House and Descotes, 2007) who indicated that all cytokine levels were measured significantly higher in septic patients compared with healthy individuals and the monitoring of serum cytokines levels were a good marker for the immune status**.**

Soluble HLA-DR level had significantly higher in all age groups except group B.Our results accordance with (Perry *et al*., 2004) who has been reported that sHLA-DR increased in the infectious and inflammatory diseases or diseases that activeted immune profile. Hietbrink, (2008) reported the increases of soluble HLA-DR in adult patients after surgery prior to clinical signs of bacterial sepsis. High levels of sHLA-DR has been shown in septic patients compared with healthy controls may therefore be indicative of infection and their response to inflammation**.** Rebmann *et al*., (2002) has been indicated the soluble forms of HLA in serum and other body fluids can perform functions as immune response modulators. There is an

increasing support that the serum-soluble HLA forms play a role in the pathophysiology of many diseases.

The data of this study has been shown a positive correlation between TNF-α, sHLA-DR in the patient's serum and their age. The expression of HLA-DR in both term and preterm newborns with or without signs of infection showed a lower level comparison with adults during the first day of life ($p<0.0001$). Prematurity associates with lowering expression of neonates with gestational age less than 32 weeks ($p=0.0008$) (Zhuang *et al*., 2017). Thevaranjan *et al*., (2017) was reported that ageing is related to increasing of inflammatory activity in the blood, including increased circulating levels of TNF-α compared with the young group. While (Kleiner *et al*., 2013) did not show any differences between young children and adults, but were all upregulated in children between TNF-α levels and showed more complex profiles.

The results of our study have been shown increasing expression of TLR2 and TLR 4 in age groups with significant differences between group A and group D. This result consistent with the results of (Akira and Takeda, 2004 and Zhang *et al*., 2010), who founded that TLR2 and TLR4 mRNA expression in peripheral blood significantly increasing in the infections of neonatal. This result indicates that the status of infection is a main parameter affecting in the expression of TLR2 and TLR4, which can use it as a septic syndrome biomarkers. De Gaudio *et al*., (2009) has been reported that expression of TLRs and their function due to aging affects not only the magnitude but also the quality of the host immune response to pathogens by the altered inflammatory and priming environment.

Furthermore, TLR 2 has increased in cases of infection with G+ve bacteria, while TLR4 generally increased in the cases of infection with G-ve bacteria. This result agreed with (Das., 2000) who reported that TLR-4 is one of the receptor which specifically for LPS and signal transduction cascades activation may be lipoteichoic acid. Moreover, the specificity of TLR-2 is much less, and responds to a different number of antigens including peptidoglycan and G+ve. This evidence suggests that it is important to look at each TLR separately and in their combination of the possibility responses to specific microorganisms to discover specific protein pathways for the differrent types of pathogens that will enable us to understand the pathways of the innate immune system responds to different stimuli**.**

Conclusion

present investigation showed a significant increase in the gene expression of TLR2 and TLR4 in different age groups of patients and also, between the children who under 1 year of age and those above 40 years. The TLR2 gene expression was also increased in G+ve compared to G-ve and the increase in TLR4 gene expression in the G-ve compared with G+ve. This indicates the strong possibility of using TLR2 and TLR4 vital indicators in the early diagnosis of bacterial sepsis syndrome, especially in children and elderly patients, and to determine the type of bacterial infection

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REFERENCES

Akira, S. and K.Takeda, Toll-like receptor signalling. Nat. Rev. Immunol. 4(7):499-511 (2004)

Angus, D.C., W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo and M.R. Pinsky, Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med. 29(7): 1303-10 (2001)

Artero, A., R. Zaragoza and J.M. Nogueira, Epidemiology of severe sepsis and septic shock. In Severe Sepsis and Septic Shock-Understanding a Serious Killer. InTech. (2012).

Bruunsgaard, H., P. Skinhøj, J. Qvist and B.K. Pedersen, Elderly humans show prolonged in vivo inflammatory activity during pneumococcal infections. J. Infect. Dis. 180(2): 551-4 (1999)

Burrell, A.R., M.L. McLaws, M. Fullick, R.B. Sullivan and D. Sindhusake, Sepsis Kills: early intervention saves lives. Med. J. Aust. 204(2): 73 (2016).

Das U.N., Critical advances in septicemia and septic shock. Critical care 4(5): 290 (2000).

De Gaudio, A.R., S. Rinaldi, C. Chelazzi and T. Borracci, Pathophysiology of sepsis in the elderly: clinical impact and therapeutic considerations. Curr Drug Targets 10(1): 60-70 (2009)

Hietbrink, F., Kinetics of the excessive cellular innate immune response after injury (Doctoral dissertation, Utrecht University (2008)

House, R.V. and J. Descotes, Cytokines in human health: immunotoxicology, pathology and clinical applications. Humana 366 (2007)

Kleiner, G., A. Marcuzzi, V. Zanin, L. Monasta and G. Zauli, Cytokine levels in the serum of healthy subjects. Mediators Inflamm. 2013 (2013).

Kotgire, S. and S. Hatkar, Aerobic bacteriological profile and its antimicrobial sensitivity pattern from blood culture specimens in a tertiary care hospital. APALM 4(1): A23-27 (2017).

Krabbe, K.S., M. Pedersen and H. Bruunsgaard, Inflammatory mediators in the elderly. Exp. Gerontol. 39(5): 687-99 (2004)

Kumar, H., T. Kawai and S. Akira, Pathogen recognition by the innate immune system. Int Rev Immunol. 30(1): 16-34 (2011).

Lu, Y.C., Regulation of toll-like receptor signal transduction pathways, Doctoral dissertation, University of Toronto (2009).

Perry, S.E., S.M. Mostafa, R. Wenstone, A. Shenkin and P.J. McLaughlin, HLA-DR regulation and the influence of GM-CSF on transcription, surface expression and shedding. Int. J. Med. Sci. 1(3): 126 (2004).

Rebmann, V., S. Ugurel, W. Tilgen, U. Reinhold and H. Grosse-W andilde, Soluble HLA-DR is a potent predictive indicator of disease progression in serum from early-stage melan-oma patients. IJC 100(5): 580-5 (2002).

Rittirsch, D., M.A. Flierl and P.A. Ward, Harmful molecular mechanisms in sepsis. Nat Rev Immunol. 8(10): 776 (2008).

Rudd, K.E., L.K. Tutaryebwa, and T.E. West, Presentation, management, and outcomes of sepsis in adults and children admitted to a rural Ugandan hospital: A prospective observational cohort study. PloS one 12(2): e0171422 (2017).

Sahoo, D., L. Mohanty, S.S. Panda and S.N. Mishra, Bacteriological analysis of blood culture isolates in patients with sepsis in a tertiary care hospital of eastern India. IJCMR 3: 3448-50 (2016).

Simon, A.K., G.A. Hollander and A. McMichael, Evolution of the immune system in humans from infancy to old age. In Proc. R. Soc. B. 282(1821): 20143085 (2015).

Starr, M.E, and H. Saito, Sepsis in old age: review of human and animal studies. Aging Dis. 2: 126 (2014).

Thevaranjan, N., A. Puchta, C. Schulz, Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. Cell Host & Microbe. 21(4): 455-66 (2017)

Wacker, C., A, Prkno, F.M. Brunkhorst and P. Schlattmann, Procalcitonin as a diagnostic marker for sepsis: a systematic review and meta-analysis. Lancet Infect. Dis. 13(5): 426-35 (2013).

Watson, R.S., J.A. Carcillo, W.T. Linde-Zwirble, G. Clermont, J. Lidicker and D.C. Angus, The epidemiology of severe sepsis in children in the United States. AJRCCM 167(5): 695-701 (2003).

Zhang, J.P., Y. Yang, O. Levy and C. Chen, Human Neonatal Peripheral Blood Leukocytes Demonstrate Pathogen-Specific Coordinate Expression of TLR2, TLR4/MD2 and MyD88 During Bacterial Infection In Vivo. Pediatr. Res. 68(6): 479 (2010).

Zhang, X. D.Liu, Y.N. Liu, R.Wang and L.X. Xie, The accuracy of presepsin (sCD14-ST) for the diagnosis of sepsis in adults: a meta-analysis. Crit. Care 19(1): 323 (2015).

Zhuang, Y., H. Peng, Y. Chen, S. Zhou and Y. Chen, Dynamic monitoring of monocyte HLA-DR expression for the diagnosis, prognosis, and prediction of sepsis. Front Biosci. 22: 1344 (2017).