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Is it possible to use the Toll-like receptors as biomarkers for neonatal sepsis? Review of the recent literature

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ABSTRACT

Background. Sepsis continues to be one of the main death causes in the neonate population. The toll-like receptors are molecules that express in the plasma or endosomal membrane and recognize endosomal or microorganism components. While aiming at the identification of new neonatal sepsis biomarkers, the toll-like receptors (TLR) have been considered that some of them overexpress in contact with the bacterial components.

Methods. Research in the PubMed database has been made by the following criteria: Inclusion criteria (PubMed database, Period 2005-2022, English & Humans, generated 29, Meta-Analysis - 0, Review - 6, Systematic Review - 0), Exclusion criteria (Studies on animal models, Articles with merely didactical content, Articles regarding only one of the words researched either only neonatal sepsis or TLR in another context than together, Articles that are not directly connected with the topic). Based on the above-mentioned criteria 13 articles were consulted, of which 7 articles included in vivo studies, 6 with in vitro studies.

Conclusions. The data of the present review and the current diagnostic method point at the fact that TLRs increase in the conditions of the presence of the inflammatory syndrome. Their dosing during in the neonatal sepsis is possible, but the non-specific overexpression is not a diagnostic.

Keywords: neonatal sepsis, toll like receptors

Abbreviations:
TLR – toll-like receptor
NPV – negative predictive value
PPV – positive predictive value and specificity
LPS – lipopolysaccharides
PAMPs – Pathogen-associated molecular patterns
RNA – Ribonucleic acid
DNA – Deoxyribonucleic acid
PRR – pattern recognition receptors
GPI – Glicosilfosfatidilinizotol
GN – Gram negative
GP – Gram positive
RSV – Respiratory Syncytial Virus
NEC – necrotizing enterocolitis
MAS – Meconium aspiration syndrome
GBS – group B streptococci
BMI – Body mass index
SE – Staphylococcus epidermidis

INTRODUCTION

Objectives

At the end of this review article, readers will have an updated and documented overview of the importance of TLR dosing during neonatal sepsis. Based on this article, it will be possible to start research directions in the identification of new biomarkers for the accurate diagnosis of neonatal sepsis.

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Sepsis

Sepsis continues to be one of the main causes of death in the newborn population. In 2018 it was estimated that approximately 22/1000 newborns develop sepsis with a death rate of 11% and 19%, respectively [1,2].

The definition of neonatal sepsis appears under several forms depending on the quoted sources, but most of them claim that the neonatal sepsis represents a complete physiopathological answer to the systemic infection which is associated with neonatal morbidity and mortality, especially within the population of preterm newborns [3].

The gold standard in the diagnostic of neonatal sepsis is represented by blood culture. But it has its limitations, since we cannot collect an enough amount of blood from a neonate, and due to the low bacterial load, the intrauterine exposure to antibiotics therapy, it leads towards a bigger rate of false negative results. According to some studies, this would lead to a failure to diagnose the clinically manifested sepsis to a 75% extent [4].

Depending on the onset moment, the neonatal sepsis is divided in early sepsis, originating in the perinatal period; it is defined as the apparition of the clinical manifestations in the first 72 hours of life [5]. The basic aetiology is with pathogens from the maternal genital-urinary tract with group B streptococcus (GBS) and Escherichia coli [6].

Late sepsis is diagnosed after more than 72 hours of life and it is usually the prerequisite of invasive procedures and of the need to grant complex medical assistance, being assigned to the nosocomial infections. The main causing agents are the coagulation negative Staphylococci, Staphylococcus aureus or Gram-negative (GN) bacteria [7].

The clinical diagnostic of sepsis and the use of empirical antibiotics therapy lead to the increase of adverse reactions and the increase of antimicrobial resistance. Therefore it is important to find new biomarkers of the neonatal sepsis. Up until now, other than blood culture, different parameters were tried for a quick and accurate diagnosis, such as blood count, the absolute number of neutrophils, the immature/total neutrophil ratio, the platelet absolute number. The acute phase reactants, the C reactive protein, the procalcitonin, the serum A amyloid, proteins that recognize lipopolysaccharides (LPS). Cytokines and chemokines such as IL6, IL 8, IL-1β, TNF-α but up until the current moment a general consensus has not been found for the accurate, feasible and rapid diagnostic of the neonatal sepsis [4].

Toll-like receptors

The toll-like receptors are molecules that express in the plasma or endosome membrane and recognize endosome or microorganisms components. While aiming at the identification of new biomarkers in neonatal sepsis, the toll-like receptors have been taken into account considering that some of them overexpress in contact with the bacterial components. It is already quite known the fact the Gram-positive (GP) microorganisms and their cellular wall are made of lipoteichoic acid signals through TLR-2 receptors, while the GN microorganisms and their secreted LPS signal through the TLR-4 receptors.

The more studied TLRs are TLR2 and TLR4.

TLR 2 is known mostly because it recognizes the lipoteichoic acid and the peptidoglycan of the GP bacteria [8].

TLR 4 recognizes the LPS on the surface of the GN bacteria. LPSs which are components of the external membrane of the GN bacteria show a strong immunostimulating activity, leading to the excessive activation of the monocytes and macrophages which can lead to a septic shock leading to the death of the host. It intervenes in the recognition of the "heat shock proteins" within the inflammation and the cell lesions and even in the absence of the infection [9,11].

MATERIAL AND METHOD

The current review aims at answering the question whether TLR can be considered biomarkers of the neonatal sepsis?

Therefore a synthesis of the specialized literature has been carried out.

Research in the PubMed database has been made by the following criteria:

Search keywords (toll-like receptors, neonatal sepsis);

Inclusion criteria (PubMed database, Period 2005-2022, English & Humans – generated 29, Meta-Analysis – 0, Review – 6, Systematic Review – 0);

Exclusion criteria (Studies on animal models, Articles with merely didactical content, Articles regarding only one of the words researched either only neonatal sepsis or TLR in another context than together, Articles that are not directly connected with the topic)

Based on the above-mentioned criteria 12 articles were consulted, of which 6 articles included in vivo studies, 6 with in vitro studies.

RESULTS

In vivo studies

Vieman et al. [12] studied 20 young adults and 85 at term newborns without congenital malformations. Of the 85 newborns, 53 were considered control lot and 32 were study lot with signs, symptoms

and paraclinical samples of sepsis. Except for the TLR2 expression on adult monocytes, the TLR2 and TLR4 expression level and was low in the phagocytic cells of the healthy individuals. TLR2 was expressed in somewhat higher levels in adults than the newborns phagocytes. The TLR4 expression level showed a greater variety between granulocytes and monocytes in newborns and there are no significant differences between adults and newborns. A correlation was made between the level of the leukocyte lines and TLR, therefore it was noted in order to differentiate whether the TLR modifications are directly correlated with the absolute number of granulocytes. Flow cytometry analyses were carried out in the days 0,1,4-6,7-10 and it was noted that the absolute number of monocytes did not change during sepsis. This suggests that the over expression of TLR is due mostly to the inflammatory activation process than a quantitative increase of the monocytes. During this study a modification of the TLR 4 expression during early sepsis was not proven [12].

In a study carried out in 22 newborns with sepsis, Banupriya et al, divided in two groups, some who received an antibiotic treatment and a group that received antibiotic treatment and Zinc supplements. They noted that the TLR 4 expression was not influenced by the administration of zinc during sepsis, but it modulates some genes involved in the inflammatory cascade [13].

The overexpression of TLR1 and TLR4 was noted in a group of 39 newborns with meconium aspiration syndrome, without chorioamnionitis, asphyxia, sepsis or malformations, in comparison with 17 healthy newborns. They proved that is that TLRs act as endogenous ligands for various components of meconium which initiate the inflammatory cascade of the meconium aspiration syndrome and contribute to its pathogenesis [14].

Another study on 20 patients with late sepsis showed that TLR is overexpressed during late sepsis, interacting with the NFKBIA, MYD88, CEBPB, STAT1, IRF7, IRAK2, IRAK4 and TBK1 genes which code the innate immune answer and lead to the production of pro-inflammation cytokines and type I IFN [15,16].

Also it seems that during the necrotizing enterocolitis (NEC), TLR 4 received the biggest attention, being the one that is activated in the presence of LPS of the GN bacteria, a trigger factor in the NEC development. The results of the study of Cho et al, [17] shown that the TLR4 signalling increased abnormally has a pathogenic role in NEC, while TLR9 and then TLR5 acts as counter-regulators of TLR4. The functional relevance of other TLRs in the disease remains poorly defined [17].

Silviera et al, tested if there was a difference between the TLR expression and the phagocytosis capacity of the neutrophils and monocytes. The study objective was to investigate if the phagocytes in healthy and with sepsis newborns show a development deficiency in their capacity to recognize, phagocyte and generate hydrogen peroxide (H2O2) as an answer to Escherichia coli and Staphylococcus aureus. Were taken into study 44 healthy newborns, 13 newborns with late onset sepsis and 24 healthy adults. TLR-2 and TLR-4 were similar between groups of healthy and sepsis newborns. The phagocytosis capacity of the monocytes and neutrophils exposed to E. coli and S. aureus in healthy and septic newborns was significantly reduced in comparison with the adults [18].

In a prospective study on 27 term newborns with clinical and paraclinical neonatal sepsis and in the control lot included healthy adults Redondo et al, noted that the levels of TLR 4 were higher in the case of patients with positive blood cultures compared to the subjects in the control group, while there were no modifications in the levels of TLR2. This study did not show differences in the TLR2 expression, which contradicts some of the studies so far [19].

See the results of studies in vivo on patients with confirmed sepsis in Table 1.

In vitro studies showed the following:

An in vitro study of 20 healthy term neonates sought to find the utility of identifying new biomarkers by collecting blood from the umbilical cord, which was then stimulated with subtype III group B streptococci colonies (GBS), from a newborn with confirmed early neonatal sepsis. The expression of TLR2 and TLR6 was higher in samples stimulated with subtype III GBS compared with the PBS controls (bacteria in saline solution buffered with phosphate) for 2 hours ($P<0.001$ and 0.038, respectively). TLR4 was not different in the samples stimulated with GBS subtype III in comparison with the PBS controls of 2 hours ($P = 0.73$) [20].

Another in vitro study on whole blood taken from the umbilical cord at birth from children and adult blood collected and stimulated with TLR agonists. Post-stimulation, the TLR4 level increased, but at the administration of pentoxifylline its levels decreased. The study featured the pentoxifylline capacity to modulate the production of cytokines mediated by TLR in newborn and adult whole blood tested in vitro. The neonatal inflammation is determined partly by the TLR signalling and it can contribute to the defence of the host against the infection. However, the TLR-mediated cytokine induction can also contribute to pathology and disease, including inflammatory disease of the early life, such as sepsis BPD and perinatal cerebral damage [21].

Raymond SL et al [22], aimed to determine the impact of TLR4 stimulation impact on the whole blood in adults, at term and preterm babies by

TABLE 1. TLR expression in patients from in vivo studies with confirmed sepsis

No.	Study	No of patients included	Presence or absence of sepsis	Result
1.	Vieman	85	Confirmed sepsis	TLR4 was not modified.
2.	Banupriya	22	Confirmed sepsis and zinc administration	TLR 4 expression was not influenced by the zinc administration.
3.	Anand	39	MAS without sepsis	TLR1 and TLR 4 overexpressed in absence of the sepsis. They are the inflammation cascade initiators.
4.	Ng	20	Confirmed late sepsis	Overexpressed TLR determine the cytokine production.
5.	Silviera	14	Confirmed sepsis	TLR2 and TLR2 expression similar between healthy and septic groups.
6.	Redondo	27	Confirmed sepsis	TLR4 - higher levels No differences of the TLR2 levels between the subjects and control.

measuring the spontaneous migration of the neutrophils, transcriptomics of neutrophils and cytokine production. Surprisingly, ex vivo LPS stimulation only had minimum effects on the spontaneous migration speed of the neutrophils and no effect on the number of migrating neutrophils.

With the stimulation of TLR4 the amount of spontaneous migration of the neutrophils remained low among the preterm newborns, but the speed was not significantly different between the stimulated groups. Finally, age specific, unique transcriptomic profiles and cytokines were proven as a response to the TLR stimulation, suggesting that newborns have a mitigated Th2 response depending on interferon [22].

In an in vitro study Schuller et al, collected whole blood samples from the umbilical cord and blood samples from control adults which were incubated with LPS and pentoxifylline. The expression of the surface markers, phagocytosis, cytokine secretion and TLR4 signalling, TLR4 of the monocytes was evaluated through flow-cytometry. The modifications of the mRNA and TLR4 were confirmed through PCR with reverse transcriptase. Pentoxifylline reduced the expression of TLR4 off the surface of the monocytes and at cellular and mRNA level it decreased the signalling and suppressed phagocytosis [23].

Knowing the essential role of the monocytes in the innate defence immune system, a study of Sureshchandra, wished to see the way in which a pre-pregnancy obesity status and implicitly the proinflammatory status has in the development of the immune response. The underlying mechanisms of suppression of the immune answer of the monocytes in the blood collected from the umbilical cord of the newborns from obese mothers after the LPS stimulation [24]. Genomic and functional determinations were carried out in order to discover the impact of pre-pregnancy obesity on the purified response of the monocytes in the LPS umbilical cord. 18 of mononuclear samples were collected from the umbilical cord, monofetal pregnancy, without complications, from non-smoking mothers, eight women with an average age of 31.25 ± 4.9 years and a pre-pregnancy BMI of 21.8 ± 1.9 kg/m^2 (thin); and ten women with an average age of 30.5 ± 5.6 and a BMI before the pregnancy of 36.6 ± 4.5 kg/m^2 (obese).

Ex vivo samples were stimulated with LPS, while mitigated answers were noted in several mediators secreted after the stimulation of LPS, including proinflammatory mediators. Despite the genic expression comparable of TLR4, after the LPS stimulation, gene expressions were noted only in the thin group, not in the obese one as well [24].

Knowing that SE rarely causes infection in at term infants, but it is a main cause of the late onset sepsis in preterm babies. Strunk et al issued the hypothesis that the innate immune responses at SE in preterm newborns are affected in a matter depending on the pregnancy age.

Therefore, mononuclear cells from the umbilical cord and the peripheral blood were stimulated in vitro with SE and a series of innate immune responses were assessed.

In order to investigate the differential expressions of the innate immune receptions potentially involved in the recognition of SE, mRNA and the expression of the TLR protein were analyzed. There were no significant differences in the genic expressions of the TLR2, TLR4 or TLR6 expression, between the preterm and at term newborns. Then the expression levels of the TLR2 and TLR4 proteins on monocytes were determined, using the flow cytometry where a growth tendency was noted of the TLR4-positive monocyte count with the increase of gestational age; however, this association was not statistically significant. Moreover, there were no differences between the groups of various pregnancy age in the medial intensities of the TLR2-positive or TLR4-positive monocytes [25].

See the results of TLR expression in patients from in vitro studies, in the absence of sepsis in Table 2.

TABLE 2. The TLR expression in patients from in vitro studies, in the absence of sepsis

No.	Study	No. of patients included	Stimulating agent	No. of patients included
1.	Naksad	20	GBS III	TLR2 – higher sensitivity and specificity in the case of stimulation with GBS III TLR4 and TLR6 – statistically insignificant reaction
2.	Speer	20	Pentoxifylline	TLR4 mediator of the inflammation possibly influenced by the administration of pentoxifylline.
3.	Schüller	13NNT/14NNP	Pentoxifylline	It reduces the expression of TLR4.
4.	Sureshchandra	18	LPS	TLR4 expression comparable between the study and control lots.
5.	Strunk	15	Staphylococcus Epidermidis	There were no significant differences in the genic expressions of the TLR2, TLR4 or TLR6 expression, between the preterm and at term newborns.
6	Raymond	43	LPS	After the stimulation of TLR4 the amount of spontaneous migration of the neutrophils remained low among the preterm newborns, but the speed was not significantly different between the stimulated groups.

DISCUSSIONS

The reaction modes of the TLRs are insufficiently known, it requires deeper studies on bigger groups of in vivo patients. Since the in vivo reaction way is not always overlapping on what happens in the body.

The entire microbiota of the preterm and at term newborns and the changes it undergoes are still to be studied, as well as whether these changes bring modifications in the reaction of TLRs.

The immune answer and the behavior way of the neonatal immune system is still insufficiently clarified.

Although the identification of new biomarkers of neonatal sepsis is a challenge for researchers and clinicians, multiple studies are still necessary to identify the reaction way of the organism in front of the bacterial aggressions. The possibility of a precise diagnosis of neonatal sepsis included the corroboration of several biomarkers.

The limitation of the current review are represented by:

- the small number of in vivo studies on preterm and at term newborns
- the small groups of patients in the study
- the multiple acute phase reaction in which that the toll-like receptors are overexpressed, being found in the inflammatory cascade, in the cellular lysis processes such as heart attacks, tumor processes, infective processes which make them rather unspecific.

CONCLUSIONS

Of the 6 studies which included newborns with confirmed sepsis, only 2 studies concluded that the expression of TLR2 and, respectively TLR4, modifies during the neonatal sepsis. TLR 2 is overexpressed in the phagocitary lines during sepsis, but the series research during the septic episodes could not confirm the direct causal relationship between the bacterial infection and TLR2.

The overexpression during the inflammatory episodes in the context of the meconium aspiration syndrome or ulcerous-necrotic enterocolitis comes to confirm the proinflammatory behavior of these receptors. The activation of TLR seems to be present in the context of the inflammatory cascade.

A single study sustains the differentiated behavior of TLR2 which grows during the infection with Gram positive bacteria and TLR4 during the infection with Gram negative bacteria. But the same thing was also seen in the in vitro studies and on the adult blood which does not make it specific enough for the neonatal period.

The current data sustain the hypothesis according to which TLR behave differently in the case of preterm newborns compared to the ones at term. In the case of at term newborns, TLRs behave just as in the case of adults, while in the case of preterms their expression seems increased. Differences in the cytokine production between adult and neonatal innate mononuclear cells have been reported after the activation of bacterial ligands of the TLRs.

However, there is not enough evidence to prove the specific behavior in the neonatal period or different depending on the pregnancy age.

Of the 6 in vitro studies on the blood collected from health newborns, subsequently stimulated with bacterial agents only one sustains the modification of the expression of TLR2, TLR4, TLR6 in the case of stimulation with GBS colonies. The TLR-mediated inflammatory answer at the neonatal pathogenic agents seem to be adjusted differently by the various pathogens and the TLR expression seem to be modified by the anti-inflammatory agents such as pentoxifylline which sustains rather the behavior as proinflammatory agent than as infective reactant. The authors of this study issued the hypothesis that pentoxifylline can lower the production of TLR-mediated cytokine production with a higher effi-

ciency in human newborns in comparison with the adult.

A similar conclusion, about TLR mediated inflammatory responses it was that pentoxifylline reduces the expression and the signalling of TLR4, determining the monocytes to exert their strongly anti-inflammatory properties. The effect was less obvious among the preterm newborns.

The recognition of the bacterial products, for example LPS by TLR4 in monocytes/macrophages leads to the production of cytokines and nitric oxide thus contributing to the defence of the organism against the pathogenic microorganisms. Which leads to the conclusion that TLR play a crucial role in the detection of bacteria and, in case of sepsis, in the stimulation of a pathogenic answer of the innate immunity system.

Preterm newborns susceptibility to sepsis is caused by a low number of spontaneous migratory neutrophils in comparison with adults and at term newborns.

Preterm newborns proved to have a significant reduction of the number and the speed of migration of neutrophils in comparison with adults which, together with a low capacity to follow a chemoattractant gradient can help explain their increased susceptibility to infections

But this study contradicts the previous study which claims that TLR2 which recognizes the lipoteichoic acid from the constitution of the GP bacterial wall and TLR4 which recognizes the lipopolysaccharides from the GN bacteria, leads to the overexpression during septic episodes. Both have a similar behavior in adults and at term or preterm newborns.

In summary, the accurate and quick diagnosis of neonatal sepsis is still a challenge considering that one must take into account the immunological particularities of newborns, the undeveloped immune system of the premature newborns. The definition of TLR expression patterns might open a new field of therapeutic targets for neonatal sepsis. The distinct expression patterns of TLR2 and TLR4 in the course of neonatal sepsis, rapid and high increase of their expression at initial symptoms suggests this parameter as a candidate for early sepsis marker.

It is also important to study the way of transmission of the immunological baggage of the preterm and the at term newborns, since in the studies carried out through in vitro stimulation with pro TLR agents no statistically significant differences were noted, which raises the issue of interposed in vivo mechanisms which lead to the overexpression of TLR during the sepsis episodes.

The data of the present review and the current diagnostic method point at the fact that TLRs increase in the conditions of the presence of the inflammatory syndrome. Their assessment during in the neonatal sepsis is possible, but the non-specific overexpression is not a diagnostic.

Future research should focus on the identification of biomarkers with increased specificity and sensitivity so that the diagnostic of neonatal sepsis makes possible the targeted treatment and to reduce the administration of empirical antiobiotherapy as well as the morbidity and mortality related to sepsis.

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