Received: 15 March 2021 | Revised: 28 June 2021 | Accepted: 7 July 2021

DOI: 10.1111/aogs.14228

ORIGINAL RESEARCH ARTICLE

A value of soluble Toll-like receptor 2 and 4 in vaginally obtained amniotic fluid for the prediction of histological chorioamnionitis

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Funding information
This study was funded by the Research Council of Lithuania (RCL) under grant No. S-MIP-19-57.

Abstract

Introduction: This study was conducted to evaluate soluble Toll-like receptor 2 (sTLR-2) and soluble Toll-like receptor 4 (sTLR-4) levels in vaginally obtained amniotic fluid and investigate their value in the prediction of histological chorioamnionitis (HCA).

Material and methods: This prospective case–control study included patients who had been diagnosed with preterm premature rupture of membranes before 34 weeks of gestation and were admitted to Vilnius University Hospital Santaros Klinikos. Free leaking amniotic fluid was obtained vaginally using a sterile speculum up to 48 h before delivery. Amniotic fluid levels of sTLR-2 and sTLR-4 were determined using an enzyme-linked immunosorbent assay. The diagnosis of chorioamnionitis was confirmed by histological examination of the placenta and membranes after delivery.

Results: The study included 156 patients, 65 with (HCA Group) and 91 without (non-HCA Group) HCA. No statistically significant differences were noted in the concentrations of sTLR-2 and sTLR-4 in vaginally obtained amniotic fluid between patients with and without HCA: the median sTLR-2 level was 0.09 ng/mL in the HCA Group vs 0.1 ng/mL in non-HCA Group, and the median sTLR-4 level was 0.23 ng/mL in the HCA Group vs 0.28 ng/mL in non-HCA Group ($p > 0.05$). A positive correlation between sTLR-2 and sTLR-4 levels was identified ($\rho = 0.57$, $p < 0.001$), but no correlation was found between these markers and gestational age.

Conclusions: Concentrations of sTLR-2 and sTLR-4 in vaginally obtained amniotic fluid do not reflect the presence of HCA in pregnancies complicated by preterm premature rupture of membranes before 34 weeks of gestation.

KEYWORDS
chorioamnionitis, preterm premature rupture of membranes, sTLR-2, sTLR-4

Abbreviations: AUC, area under the curve; ELISA, enzyme linked immunosorbent assay; HCA, histological chorioamnionitis; IQR, interquartile range; MIAC, microbial invasion of the amniotic cavity; PPROM, preterm premature rupture of membranes; ROC, receiver operating characteristic; sTLR, soluble Toll-like receptor.

1 | INTRODUCTION

Preterm premature rupture of membranes (PPROM) is the spontaneous rupture of fetal membranes during pregnancy before 37 weeks of gestation. The longer latency of pregnancy following rupture of membranes is associated with a higher risk of chorioamnionitis.[1] The presence of chorioamnionitis in the setting of PPROM leads to an increased incidence of fetal inflammatory response syndrome and neurodevelopmental delay.[2] Therefore, a diagnosis of chorioamnionitis is a contraindication to the expectant management of pregnancy.

Some different methods exist to diagnose chorioamnionitis. The easiest, cheapest and fastest method is using clinical criteria, which are based on clinical signs of inflammation in the mother and fetus. There are various clinical criteria to diagnose chorioamnionitis. The most frequently used and oldest method is the Gibbs criteria: the presence of maternal fever (>37.8°C) and at least two of the following: maternal tachycardia (>100 beats/min), maternal leukocytosis (white blood cell count >15 000/mm^3), uterine tenderness, fetal tachycardia (>160 beats/min) and foul-smelling amniotic fluid.[3] Other criteria were proposed by Lencki et al.[4] in 1994. These consisted of maternal fever (>38.0°C) and more than one of the following clinical signs: maternal tachycardia (>100 beats/min), maternal leukocytosis (white blood cell count >15 000/mm^3), uterine tenderness and foul-smelling vaginal discharge.[4] Maki et al. found that both sets of criteria had a specificity of 98.8% but sensitivity was only 15.4%.[5]

Histological examination of the placenta is considered the gold standard for diagnosing chorioamnionitis,[6] but this option is only available after delivery. The gold standard method for prenatal diagnosis of chorioamnionitis could be direct testing of the amniotic fluid.[7] Infectious pathogens activate a cascade of events that lead to the release of numerous inflammatory mediators into the amniotic fluid. Therefore, amniotic fluid analysis has been recognized as an accurate method for the early prediction of chorioamnionitis.[8] Amniotic fluid for this analysis can be collected noninvasively (vaginally obtained) or invasively (by amniocentesis). Amniocentesis has been shown to be a safe procedure and is useful for improving neonatal outcomes.[9] However, it is more complicated in PPROM, as it requires higher surgical skill to insert the needle into the minimal amniotic fluid pocket.

Pattern recognition receptors are a first line of defence in the innate immune system. Among these receptors, the soluble Toll-like receptors (sTLRs) have been studied the most extensively.[10] Kim et al. demonstrated increased expression of TLR-2 and TLR-4 in the chorioamniotic membranes.[11] The aim of this study was to evaluate sTLR-2 and sTLR-4 levels in non-invasively obtained amniotic fluid and investigate their value in the prediction of histological chorioamnionitis (HCA).

Key message

sTLR-2 and sTLR-4 recognize the pathogens most frequently involved in microbial invasion of the amniotic cavity; however, the concentrations of these receptors in vaginally obtained amniotic fluid do not reflect the presence of histological chorioamnionitis.

2 | MATERIAL AND METHODS

2.1 | Patient population

This prospective cohort study was conducted in Vilnius University Hospital Santaros Klinikos between July 2017 and July 2019. Pregnant women were eligible if they provided informed consent and were admitted to the hospital for PPROM before 34 weeks of gestation. Other inclusion criteria were maternal age ≥18 years and singleton gestation. Patients with multiple gestations, fetal malformations, vaginal bleeding, placenta previa and non-reassuring fetal status were excluded. In total, 185 participants met the inclusion and exclusion criteria.

Amniotic fluid leakage was diagnosed via examination with a sterile speculum to verify the pooling of amniotic fluid in the vagina. Calculation of gestational age was based on the last menstrual period and confirmed or modified by ultrasound scan at 11+0 to 13+6 weeks of gestation. According to the institution's standard protocol, all patients were managed expectantly with antibiotics and a single course of antenatal corticosteroids. Clinical chorioamnionitis was diagnosed by the attending obstetrician-gynecologist and the diagnosis was based on the Gibbs criteria: maternal temperature >37.8°C, uterine tenderness, maternal leukocytosis, maternal and/or fetal tachycardia, and malodorous vaginal discharge.

2.2 | Amniotic fluid sampling and analyses

Free-leaking amniotic fluid was obtained vaginally with a sterile speculum. Samples were collected every second day, centrifuged at 3000 rpm for 5 min at 4°C, and stored at −80°C. Samples containing obvious mucus, blood or too little volume were excluded from the study. Thus, amniotic fluid samples from 156 participants, who delivered within 48 h following amniotic fluid collection, were included into the final analysis.

Immunological assays of amniotic fluid samples were performed using the enzyme-linked immunosorbent assay (ELISA) for human sTLR 2 and 4 (USCN Life Science & Technology Company), according to the manufacturer's instructions. The samples of amniotic fluid were not diluted for the determination of sTLR2 and sTLR4 concentrations. The concentrations of cytokines were calculated

according to standard curves using a special program for the evaluation of ELISA results (Gen5 Microplate Data Collection & Analysis Software; BioTek Instruments).

2.3 | Diagnosis of HCA

In the postpartum period, the placentas were examined histologically and HCA was diagnosed based on the presence of maternal neutrophil infiltration in the amnion, chorion and parietal decidua. According to histological analysis, patients were grouped into the HCA group or the non-HCA group. All maternal and neonatal medical records were reviewed and the perinatal outcomes were recorded.

2.4 | Statistical analysis

Statistical analysis was performed using R package (version 4.0.3) (R Core Team, 2020). The distribution of the data was determined by the Shapiro–Wilk test. The means were compared between the two groups using *t* tests when the data fit a normal distribution and using the Mann–Whitney *U* test when they did not. A Pearson Chi-square test was used to determine differences between the groups in categorical variables. Parametric continuous variables were expressed as means with standard deviations, nonparametric as median along interquartile range (IQR), and categorical variables as frequencies and percentages. The receiver operating characteristic (ROC) curves were constructed to estimate the ability of variables to differentiate between the groups. Spearman correlation analysis was used to identify the correlation between immunological markers and gestational age. A p value <0.05 was considered statistically significant for all tests.

2.5 | Ethical approval

This is a part of the study "Significance of biochemical and immunological markers in amniotic fluid in predicting intrauterine infection," which was approved by the Vilnius Regional Biomedical Research Ethics Committee on 4 July 2017 (No. 158200-17-931-434).[12]

3 | RESULTS

The study included 156 patients with PPROM before 34 weeks of gestation: 65 patients in the HCA Group and 91 patients in the non-HCA Group. The clinical characteristics of the patients and their distribution within the groups are listed in Table 1. Maternal age, gravidity, parity, comorbidities (gestational diabetes, hypertensive disorders), gestational age, latency between PPROM and delivery, and neonatal birthweight were similar between the groups and did not differ statistically. The colonization rate of Group B Streptococcus was higher and newborns had worse outcomes (lower Apgar score and umbilical pH) in the HCA Group ($p < 0.05$). Clinical chorioamnionitis was diagnosed in six participants (9.2%) in the HCA Group and in one participant (1.1%) participant in non-HCA Group.

TABLE 1 Characteristics of patients according to the groups

Characteristics	HCA group ($n = 65$)	Non-HCA group ($n = 91$)	p value
Maternal Characteristics			
Age of mother, years	30.6 ± 6.4	31.2 ± 5.8	0.53
Primiparous, *n* (%)	32 (49.2)	46 (50.6)	0.87
Multiparous, *n* (%)	33 (50.8)	45 (49.4)	0.87
Primigravida, *n* (%)	27 (41.5)	34 (37.4)	0.59
Multigravida, *n* (%)	38 (58.5)	57 (62.6)	0.59
Gestational age at birth, weeks	32+0 (27+2 to 33+0)	33+0 (28+1 to 33+5)	0.13
Latency between PPROM and delivery, h	43 (26–93)	41 (20–78)	0.29
Clinical chorioamnionitis, *n* (%)	6 (9.2)	1 (1.1)	0.002
Group B Streptococcus, *n* (%)	24 (36.9)	15 (16.5)	0.003
Gestational diabetes, *n* (%)	16 (17.6)	11 (16.9)	0.91
Hypertensive disorders, *n* (%)	12 (13.2)	8 (12.3)	0.87
Newborn characteristics			
Birthweight (g)	1768.2 ± 669.1	1872.7 ± 603.2	0.51
Apgar score at <7 at 5 min, *n* (%)	6 (9.2)	1 (1.1)	0.02
Umbilical cord arterial pH	7.31 ± 0.09	7.35 ± 0.09	0.003

The HCA Group, patients with diagnosed histological chorioamnionitis; the Non-HCA Group, patients without diagnosed histological chorioamnionitis; PPROM, preterm premature rupture of membranes.

No statistically significant differences were found in the sTLR-2 and sTLR-4 concentrations in vaginally obtained amniotic fluid between patients with and without HCA. The median of sTLR-2 was 0.09 ng/mL, IQR 0.01–0.35 in the HCA Group vs 0.1 ng/mL, IQR 0.03–0.34 in the non-HCA Group ($p = 0.403$). The median of sTLR4 was 0.23 ng/mL, IQR 0.07–0.52 in the HCA Group vs 0.28 ng/mL, IQR 0.13–0.78 in the non-HCA Group ($p = 0.123$) (Figures 1 and 2).

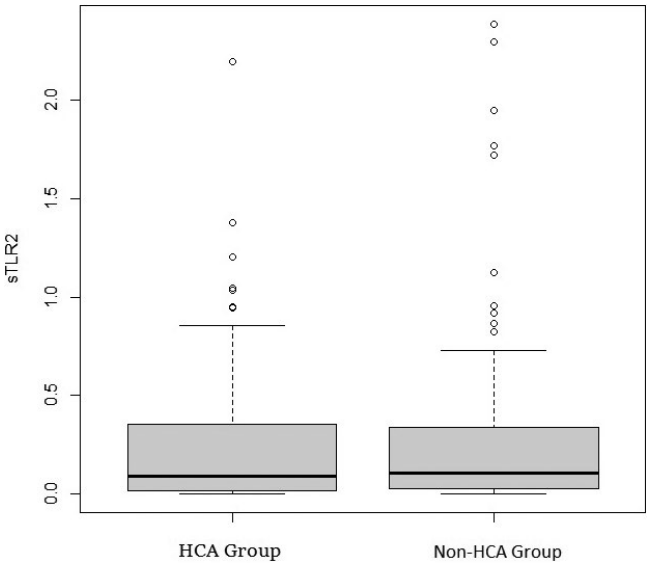

FIGURE 1 The soluble Toll-like receptor 2 (sTLR-2) levels in vaginally obtained amniotic fluid in the HCA Group and non-HCA Group. The HCA Group, patients with diagnosed histological chorioamnionitis; the non-HCA Group, patients without diagnosed histological chorioamnionitis; HCA, histological chorioamnionitis

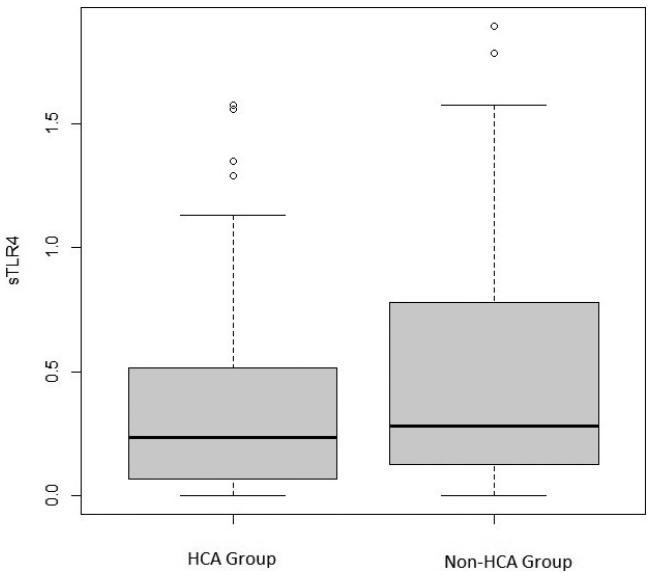

FIGURE 2 The soluble Toll-like receptor 4 (sTLR-4) levels in vaginally obtained amniotic fluid in the HCA Group and non-HCA Group. The HCA Group, patients with diagnosed histological chorioamnionitis; the non-HCA Group, patients without diagnosed histological chorioamnionitis; HCA, histological chorioamnionitis

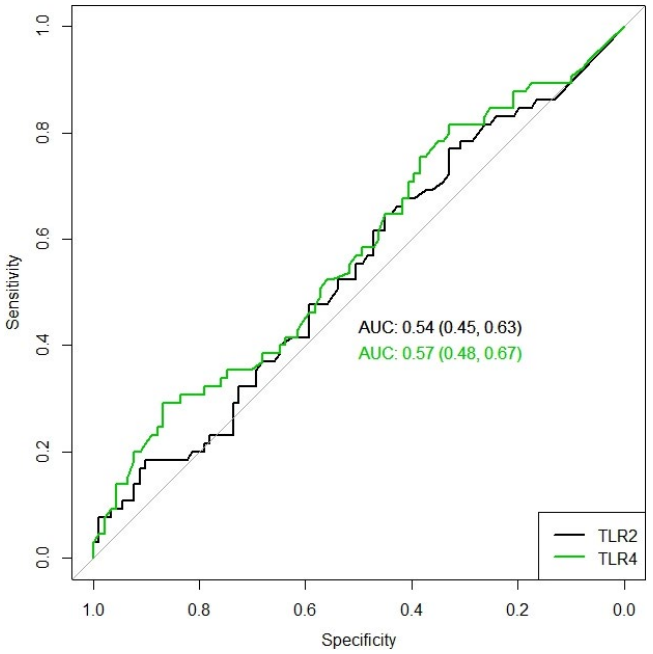

FIGURE 3 ROC curves of soluble Toll-like receptor 2 (sTLR-2) and soluble Toll-like receptor 4 (sTLR-4) levels following the diagnosis of chorioamnionitis. AUC, area under the curve

ROC curves were generated and the corresponding areas under the curves (AUCs) were calculated to determine the power of sTLR-2 and sTLR-4 to differentiate between the groups. The AUC of sTLR-2 was 0.54, and the AUC of sTLR-4 was 0.57 (Figure 3).

A positive correlation between sTLR-2 and sTLR-4 levels in vaginally obtained amniotic fluid was identified ($\rho = 0.57$; $p < 0.001$). Neither sTLR-2 nor sTLR-4 levels correlated with gestational age (sTLR-2 and gestational age: $\rho = 0.02$, $p = 0.86$; sTLR-4 and gestational age: $\rho = -0.02$; $p = 0.81$) (Figure 4).

4 | DISCUSSION

Soluble Toll-like receptors are transmembrane glycoproteins which recognize specific patterns of microbial components, such as unmethylated double-stranded deoxyribonucleic acid, single-stranded ribonucleic acid, lipoproteins, lipopolysaccharides and flagellin.[13] The activation of sTLRs by specific patterns of microbial components induces the secretion of inflammatory cytokines.[14] There are 10 functional sTLRs in humans (sTLR1–10).[15]

We chose to study sTLR-2 and sTLR-4 because these glycoproteins have been known to be expressed on chorioamniotic membranes and can recognize the pathogens frequently involved in microbial invasion of the amniotic cavity (MIAC): genital *Mycoplasma* and *Ureaplasma*, *Escherichia coli*, *Prevotella*, *Leptotrichia*, *Sneathia* and fungi.[11,16] Soluble TLR-2 is one of the most versatile sTLRs as it can recognize a large spectrum of specific patterns of microbial components by forming heterodimers with sTLR-1 and sTLR-6. Soluble TLR-2 recognizes lipoproteins and peptidoglycans of

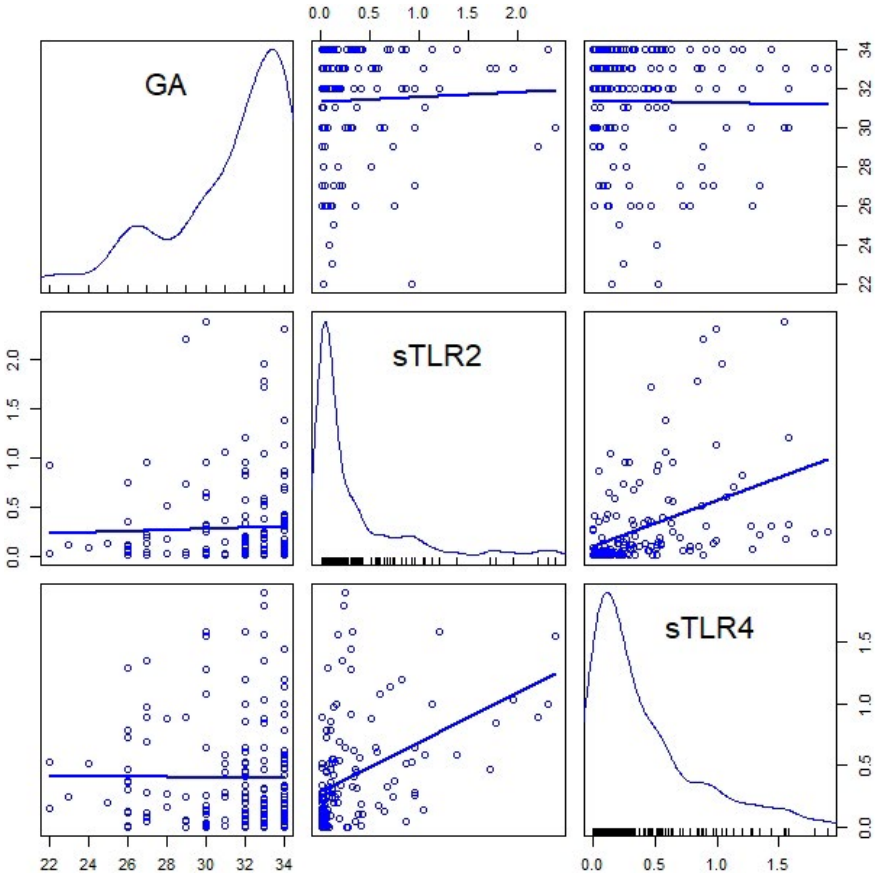

FIGURE 4 Correlations between soluble Toll-like receptor 2 (sTLR-2), soluble Toll-like receptor 4 (sTLR-4) and gestational age (GA)

gram-positive bacteria, lipoteichoic acid and fungal zymosan, and hence can recognize microbial products of genital *Mycoplasma* and *Ureaplasma* species, fungi and gram-positive bacteria such as Group B *Streptococcus*.[16,17] Soluble TLR-4 is crucial for responses to the lipopolysaccharides of gram-negative bacteria. Thus, sTLR-4 can recognize the other microorganisms frequently involved in MIAC, such as *E. coli*, *Prevotella*, *Leptotrichia*, *Sneathia* and other gram-negative species.[16–20]

To our knowledge, this is one of the largest reported studies that has sought sTLR-2, and the first study that has sought sTLR-4, in vaginally obtained amniotic fluid and investigated their predictive value in the diagnosis of chorioamnionitis. In this study, we found no statistically significant differences in sTLR-2 concentration in noninvasively collected amniotic fluid between the patients with and without a diagnosis of HCA. We found a positive correlation between sTLR-2 and sTLR-4 levels in vaginally obtained amniotic fluid, but no correlation between these markers and gestational age. Kacerovsky et al. also analyzed sTLR-2 concentrations in cervical and vaginal fluid and found that cervical and vaginal fluid sTLR-2 levels do not reflect the presence of MIAC and HCA. Moreover, they found a positive correlation between cervical and vaginal fluid sTLR-2 levels, but neither cervical fluid nor vaginal fluid sTLR-2 levels correlated with gestational age.[21] These differing results were also demonstrated in amniotic fluid collected by amniocentesis in a study performed by Andrys et al.[22] Those authors found that sTLR-2 was a promising predictor of both MIAC and HCA, with a high specificity in PPROM. They also found an optimal cut-off value of 222.7 ng/mL for sTLR-2, with a sensitivity of 63%, a specificity of 98%, and a likelihood ratio of 40.3 for the prediction of MIAC and HCA. Another study, performed by Dulay et al.,[23] indicated that sTLR2 levels are independent of intraamniotic infection or microbial category, but are gestational age-regulated, with sTLR-2 remaining elevated until 30 weeks of gestation and decreasing at term.

In our research, we found that the sTLR-4 concentration in vaginally obtained amniotic fluid did not differ significantly between patients with or without a diagnosis of HCA. To our knowledge, there are no other published studies investigating sTLR-4 levels in vaginally obtained amniotic fluid. Kacerovsky et al.[24] analyzed sTLR-4 concentrations in amniotic fluid obtained by amniocentesis. They found that patients with MIAC or HCA had statistically significant higher sTLR-4 levels than those without these complications.

We hypothesize that the different results between the studies analyzing sTLR-2 and sTLR-4 levels in amniotic fluid obtained by amniocentesis and those analyzing vaginally collected amniotic fluid could be influenced by sTLR presence in the epithelia of different regions of the female reproductive tract. Fazeli et al.[25] found that sTLR-2 was present in the epithelia of the uterine tubes and endometrium and was highly expressed in the vagina and ectocervix. Those authors also demonstrated that although the epithelia of the vagina and ectocervix seem to be lacking sTLR-4, intense staining was present in the epithelium and the cells lining

the endocervical glands. The vaginal microbiota is unique and dynamic, comprising a wide spectrum of aerobic and anaerobic bacteria, predominantly involving the *Lactobacillus* genus.[26] Free-leaking amniotic fluid passes through the cervical canal and vagina; thus, cervical and vaginal fluids contribute on the amniotic fluid composition. This could lead to a higher concentration of sTLR-2 and sTLR-4 in vaginally obtained amniotic fluid, independent of the presence of intrauterine infection.

The strength of this study is that it is one of the largest reported studies that has sought sTLR-2, and the first study that has sought sTLR-4, in vaginally obtained amniotic fluid and investigated their predictive value in the diagnosis of chorioamnionitis. All patients received standardized management with corticosteroids and antibiotics. Moreover, amniotic fluid samples were obtained up to 48 h before delivery; thus, the latency period between fluid collection and delivery was not long, which could otherwise predispose to incorrect results of immunological analyses.

The limitation of this study is that we used processed (centrifuged and frozen) amniotic fluid samples. However, Kacerovsky et al. demonstrated a strong correlation between the concentrations of immunological markers in fresh and processed amniotic fluid samples.[27] Another limitation of this study is that chorioamnionitis was defined by histological examination of the placenta. Although HCA is the gold standard for diagnosing intrauterine infection,[6] there is controversy regarding whether it correlates with a higher rate of neonatal morbidity and mortality.[6,28,29]

5 | CONCLUSION

Concentrations of sTLR-2 and sTLR-4 in vaginally obtained amniotic fluid do not reflect the presence of HCA in pregnancies complicated by PPROM before 34 weeks of gestation.

CONFLICT OF INTERESTS

None.

AUTHORS' CONTRIBUTIONS

Conceptualization, DR. Data curation, GSD. Formal analysis, GB, GK-B and VG. Funding acquisition, DR. Investigation, GB, VG and ID. Methodology, GB, ID and RV. Writing: original draft, GB; review & editing, IP, ID and DR. Project administration, IP. Supervision, GSD and DR.

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How to cite this article: Balciuniene G, Gulbiniene V, Kvederaite-Budre G, et al. A value of soluble Toll-like receptor 2 and 4 in vaginally obtained amniotic fluid for the prediction of histological chorioamnionitis. *Acta Obstet Gynecol Scand*. 2021;100:2209–2215. https://doi.org/10.1111/aogs.14228