Eotaxin-2 as a potential marker of preterm premature rupture of membranes: A prospective, cohort, multicenter study

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Abstract

Background. Despite advances in medicine, there is currently no effective procedure for predicting and the early diagnosis of preterm premature rupture of membranes (pPROM).

Objectives. To apply measurements of selected biochemical markers of inflammation for diagnosing cases of pPROM without clinical signs of infection.

Materials and methods. This is a prospective cohort study. Three groups were compared, a study group: 82 women between 22 and 37 weeks of pregnancy hospitalized due to pPROM, a control group: 64 women between 22 and 37 weeks of pregnancy in the 1st stage of preterm labor with intact fetal membranes, and a reference group: 99 women between 37 and 42 weeks of pregnancy in the 1st stage of physiological term labor and intact fetal membranes. To assess the concentration of cytokines, a multiplex method was used for measurement of: IGFBP-1, IGFBP-2, BDNF, L-selectin, E-selectin, PECAM-1, ICAM-1, and VCAM-1, MIP-1d, MIP-3b, BLC, eotaxin-1, and eotaxin-2.

Results. Out of the studied molecules, we found that eotaxin-2 concentrations in the study group were significantly lower than in the control group and the reference group: 9.22 pg/mL compared to 13.76 pg/mL and 14.14 pg/mL ($p = 0.014$), respectively. We also showed that serum concentration of eotaxin-2 below 8.24 pg/mL could be used as a cut-off level of pPROM (sensitivity: 0.58; specificity: 0.57).

Conclusions. Findings of significant differences in eotaxin-2 can be the basis for further studies on the use of this molecule as a biochemical marker of pPROM.

Key words: preterm rupture of membranes, cytokine, preterm labor, molecular biology

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Background

Premature rupture of membranes (PROM) is one of the major complications of physiological pregnancy resulting in the leakage of amniotic fluid. It occurs in around 3% of singleton pregnancies and around 7–20% of multiple ones.[1–3] Despite application of advanced immunohistochemical tests, including detecting vaginal fetal fibronectin, insulin-like growth factor binding protein-1 and placental alpha-microglobulin-1, in some clinical cases diagnosis of PROM still remains difficult. An extremely important event in the context of obstetric complications is preterm PROM (pPROM), defined as the rupture of fetal membranes before the 37th week of pregnancy, or more than 24 h before the onset of labor.[4] Preterm PROM is the cause of about 1/3 of premature births.[5] The diagnosis of pPROM mainly depends on a medical interview carried out with the patient. Up to 90% of cases can be diagnosed on this basis.[6,7] The diagnosis of pPROM is usually confirmed based on accessory examinations, which include: nitrazine test and/or immunochromatographic tests, based on the detection of placental alpha-microglobulin-1 (PAMG-1) or insulin-like growth factor binding protein-1 (IGFBP-1), present in vaginal secretion.[13,14] In contrast to the rupture of membranes during term labor, in the etiology of pPROM inflammatory, mechanisms linked to infection are far more common. Cytokines, including chemokines produced by activated immune cells, are the largest group of biochemical factors involved in the pathogenesis of pPROM and can potentially be used as valuable biomarkers of preterm labor.[15]

In the fetal membranes, cytokines may exhibit direct autocrine or paracrine activity. Endocrine cytokines might also be released by cells located within other sites of the pregnant woman's body.[8,9] Despite advances in medicine, there is as yet no effective test to predict pPROM. Endothelial cells express endothelial-specific adhesion molecules (integrins): L-selectin, E-selectin, ICAM-1, VCAM-1, and PECAM.[8,9] They are involved in the regulation of migration as well as permeability of blood cells through the endothelium. This process may play an important role in the pathogenesis of pPROM by participating in the formation of inflammation in the placenta and fetal membranes.

Chemokines: MIP-1d, MIP-3b, eotaxin-1, eotaxin-2, and B lymphocyte chemoattractant (BLC), as factors associated with inflammatory processes, may also play a role in the pathogenesis of pPROM through chemotactic effects on inflammatory cells. In the reproductive system, MIP-1d, MIP-3b, eotaxin-1, eotaxin-2, and BLC interact with numerous peptides that induce smooth muscle contractions. They are biologically active by interacting with G-protein binding membrane receptors (chemokine receptors) that are selectively located on the surfaces of target cells.

The 3rd group of potential biochemical markers that were the subject of research in this work are circulating proteins with variable expression during the inflammatory phase: IGFBP-1, IGFBP-2 and BDNF. They can be associated with the formation of inflammatory infiltrates within the fetal membranes, placenta and/or umbilical cord, and thus are involved in the pPROM pathogenesis.

The development of research methods, including multiplexed technologies,[10] may be the basis for implementation of new diagnostic methods for early diagnosis of this complication or even identification of high-risk groups, which could contribute to improvement in antenatal monitoring.

Objectives

This paper attempts to apply paralleled measurements of selected biochemical markers of inflammation for diagnosing pPROM in pregnant women without clinical signs of infection.

Materials and methods

This is a prospective cohort study reviewed and approved by an institutional review board of a Local Bioethics Committee before it began. The study group consisted of 82 women between 22 and 37 weeks of pregnancy hospitalized due to pPROM. The control group consisted of 64 women between 22 and 37 weeks of pregnancy in the 1st stage of preterm labor with cervical dilatation of up to 3 cm, and intact fetal membranes. We used the reference group consisting of 99 women between 37 and 42 weeks of pregnancy in the 1st stage of physiological term labor, with cervical dilatation of up to 3 cm, and intact fetal membranes. As exclusion criteria we considered antibiotic treatment up to 3 weeks prior to the study, placenta previa, multiple pregnancy, and clinical symptoms of infection: temperature above 37°C, cough, chills, and fetal tachycardia. The diagnosis of pPROM was based on ACOG recommendations,[11] that is: 1) gynecological examination – visualization of amniotic fluid passing from the external cervical os; 2) nitrazine test (change in the color of paper impregnated with nitrazine from yellow to blue); 3) immunochromatographic tests.

The diagnosis of pPROM was confirmed by the presence of at least 2 of the 3 criteria. The average time between fetal membrane rupture and the patient's inclusion was 3 h 46 min (min. 30 min, max. 26 h). A total of 79 women were in early pPROM (up to 18 h) and 3 women were in late pPROM (more than 18 h passed between the rupture and the onset of labor).

Samples collection

Whole blood samples of 15 mL were collected from the ulnar vein of each pregnant woman using 2 tubes: 5 mL for serum into a test tube with a serum separating

tube (SST) and 10 mL for plasma into an EDTA tube. In the study group, blood was collected immediately after pPROM diagnosis. In the control and reference groups, blood was collected at the onset of labor with cervix dilatation below 3 cm. In all 3 groups, samples were taken before pregnant women were given any medication. From the EDTA tube, 2 × 2.5 mL were pipetted and the rest was centrifuged together with the serum (taken to the separator tube), at 3000 rpm for 5 min at 4°C. Serum and EDTA treated plasma were titrated 10 × 250 µL, each separately. Samples were preserved immediately at –80°C. The concentrations of the following biochemical markers were determined in the patients' blood serum: 1) Circulating proteins expressed during inflammation: IGFBP-1, IGFBP-2, and BDNF; 2) Adhesion molecules involved in leukocyte-endothelial transduction: L-selectin, E-selectin, PECAM-1, ICAM-1, and VCAM-1; 3) Chemokines: MIP-1d, MIP-3b, BLC, eotaxin-1, and eotaxin-2.

Determination of the level of biochemical markers was made using protein macroarrays in accordance with the detailed specifications of the manufacturer (RayBiotech, Norcross, USA).

Statistical analyses

We used the Shapiro–Wilk test for analysis of normality. We compared study group with the control group, and study group with the reference group. When comparing 2 groups for quantitative data that did not come from a normal distribution, the U Mann–Whitney test was used. For data with a normal distribution, Student's t-test was used to evaluate eotaxin-2 threshold value; receiver operating characteristic (ROC) curve was performed. Statistical significance level was assumed at $p < 0.05$.

Results

Characteristics of the groups

The mean age of women with pPROM was 26.4 years (ranging from 18 to 41 years), and was not statistically significantly different ($p > 0.05$) from the age of women in the control and reference groups. There were no statistically significant differences ($p > 0.05$) between the groups in the number of pregnancies, urine pH, hemoglobin, and white blood cells levels. There were important differences in smokers in the study group compared to reference group and vaginal pH between study group compared to control and reference groups due to pPROM (Table 1).

Analysis of the concentration levels of selected biochemical markers

Serum concentrations of IGFBP-1, IGFBP-2; L-selectin, MIP-1d, BDNF, BLC, eotaxin-1, E-selectin, ICAM-1, MIP-3b, PECAM-1, and VCAM-1 were not significantly different ($p > 0.05$) between the study group, control group and reference group (Table 2,3). Women with pPROM showed lower mean eotaxin-2 concentrations compared

Table 1. Characteristic of the groups

Factor	Study group	Control group	Reference group	p_1	p_2
Age [years]	26.4 (18–41)	25.1 (18–39)	24.4 (19–40)	0.241	0.182
Smokers	11	7	5	0.072	0.034
Alcohol abuse	0	0	0	NA	NA
Drug abuse	0	0	0	NA	NA
Number of pregnancies	2 (1–6)	1.8 (1–4)	1.6 (1–3)	0.112	0.075
Pregnancy age [weeks]	31.1 (22–36)	28.3 (23–36)	39.6 (38–41)	0.068	0.003
Fetal weight USG [g]	1761 (507–2482)	1642 (612–2412)	3620 (3238–4216)	0.136	<0.001
BMI	27.3 (24.4–28.3)	25.8 (23.6–29.12)	32.1 (29.8–35.2)	0.061	0.042
Vaginal pH	6.4 (5.9–7.2)	4.3 (4.0–5.2)	4.8 (4.4–5.5)	0.026	0.044
Hemoglobin [g%]	11.6 (10.3–14.3)	11.2 (10.6–13.9)	11.5 (11.2–12.3)	0.072	0.103
WBC [/1 mm^3]	1071 (7358–13235)	9836 (8532–14533)	12300 (9275–13288)	0.211	0.178
Urine pH	6.3 (5.7–6.7)	5.9 (5.3–7.2)	6.0 (5.5–7.6)	0.06	0.078

p_1 – study group compared to control group; p_2 – study group compared to reference group; NA – not applicable.

Table 2. Analysis of concentration levels of selected biochemical markers

Concentration of markers [pg/mL]	Mean			Student's t-test p_1	Student's t-test p_2
	study group	control group	reference group		
iGFBP-1	177.97	144.57	158.93	0.056	0.096
IGFBP-2	429.72	424.00	422.36	0.695	0.753
L-selectin	2369.2	2379.9	2456.4	0.888	0.687
MIP-1d	25.01	27.79	24.78	0.092	0.132

p_1 – study group compared to control group; p_2 – study group compared to reference group.

Table 3. Analysis of concentration levels of selected biochemical markers – cont.

Concentration of markers [pg/mL]	Median			Mann–Whitney U test (p_1)	Mann–Whitney U test (p_2)
	study group	control group	reference group		
BDNF	42.22	38.82	40.93	0.062	0.112
BLC	32.25	34.66	31.78	0.491	0.621
Eotaxin-1	1.42	1.78	1.66	0.178	0.225
E-selectin	380.24	398.18	376.15	0.953	0.871
ICAM-1	435.66	420.22	341.61	0.699	0.575
MIP-3b	22.50	17.02	19.50	0.093	0.182
PECAM-1	26.34	29.15	31.91	0.248	0.154
VCAM-1	37.42	36.85	33.89	0.158	0.344

p_1 – study group compared to control group; p_2 – study group compared to reference group.

Table 4. Comparison of eotaxin-2 concentrations in the study group vs the control and reference groups (pg/mL)

Concentration of markers [pg/mL]	Mean	Median	Lower quartile	Upper quartile	Standard deviation	Standard error	p_1	p_2	p_3
Study group	9.22	7.45	5.03	11.0	5.64	0.66	0.037	0.014	0.075
Control group	13.76	10.22	7.34	17.46	8.31	2.29			
Reference group	14.14	11.90	9.78	16.88	7.26	1.94			

p_1 – study group compared to control group = 0.037 (Mann–Whitney U test); p_2 – study group compared to reference group = 0.014 (Mann–Whitney U test); p_3 – control group compared to reference group = 0.075 (Mann–Whitney U test).

Table 5. Analysis of sensitivity and specificity of pPROM dignosis based on Eotaxin-2 concentrations

Eotaxin-2 threshold value [pg/mL]	Sensitivity	95% CI	Specificity	95% CI
8.24	0.58	0.496 to 0.821	0.57	0.433 to 0872

with women with preterm labor and intact membranes and to women with physiological term labor (Table 4). Serum concentrations of eotaxin-2 below 8.24 pg/mL show significance as a potential pPROM biomarker with sensitivity of 0.58 and specificity of 0.57 (Fig. 1, Table 5).

Discussion

This study has shown that eotaxin-2 exhibits statistically lower concentrations in women with pPROM than in women in preterm labor and intact membranes or women in physiological term labor. This may be an important factor in the pathogenesis of pPROM, because eotaxin- 2, like eotaxin-1, is responsible for the activation and chemotaxis of eosinophils,[12] as well as neutrophils, macrophages and T lymphocytes.[13] Its activity was observed in decidual fibroblasts. By modifying the apoptosis of decidual cells, eotaxin-2 may contribute to premature membrane weakening and rupture. It is worth noting that the concentration of eotaxin-2 receptors is dependent on estrogen, progesterone and placental gonadotropin.[14]

The obtained results indicate that eotaxin-2 may be a potential marker of pPROM, and may also play a role in the pathogenesis of premature rupture of membranes, which could increase the predictive value of this molecule. The biomarker potential of eotaxin-2 is to confirm rupture but not to predict latency between rupture and

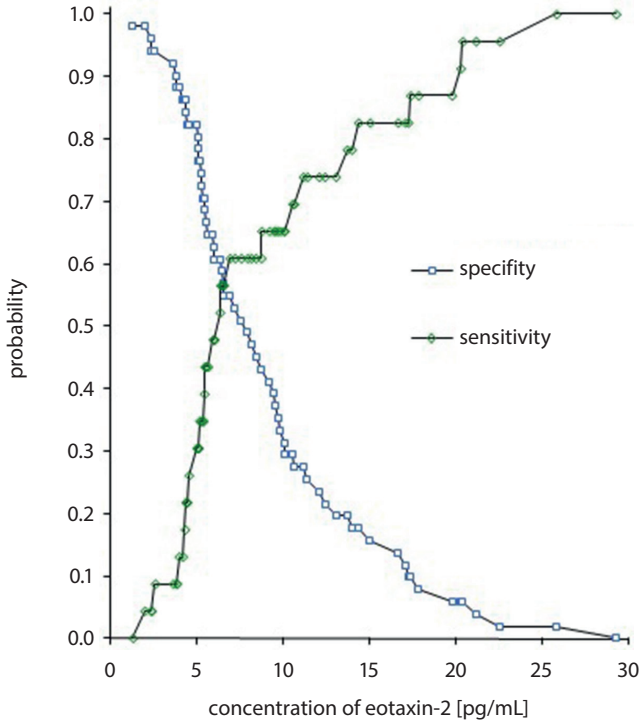

Fig. 1. ROC analysis of sensitivity and specificity of pPROM diagnosis based on eotaxin-2 concentrations

delivery. Despite excluding patients with clinical and laboratory symptoms of infection from the study, the existence of asymptomatic inflammation of the fetal membranes in women with pPROM cannot be ruled out, which might have affected the concentrations of biochemical markers. It remains unknown whether the reduction in eotaxin-2 concentrations in women with pPROM resulted from latent infection or the onset of labor.

When interpreting the results of concentrations of eotaxin-2 in women with pPROM, one should take into account physiological changes in the concentration of some proteins during pregnancy. However, regardless of gestational age, there are other mechanisms that affect the concentration of individual biochemical markers in the blood serum. Liu Y et al.[15] showed a change in calgranulin B concentrations (a protein whose expression appears on the surface of macrophages and epithelial cells of tissues in the acute phase of inflammation) synthesized in the fetal membranes and decidua in the course of intrauterine infections. These changes occur before the onset of clinical symptoms of infection, which gives grounds for searching for biochemical markers of pPROM among cytokines. In contrast, not all cytokine concentrations undergo modulation in the course of intrauterine inflammatory processes.[16]

Likewise, in this study no significant changes have been found in the concentrations of IGFBP-1, IGFBP-2, BDNF, L-selectin, E-selectin, ICAM-1, PECAM-1, VCAM-1, as well as MIP-1d, MIP-3b, eotaxin-1, and BLC. However, the role of expression of some cytokines in the diagnosis of early stages of intrauterine inflammations is indisputable and some have already been studied in preterm labor.[17] Despite no women exhibiting symptoms of chorioamnionitis in the examined group (patients with clinical signs of infection were excluded from the study), the presence of such infections cannot be ruled out. Asymptomatic inflammatory changes in afterbirth can affect the immune system. So far, no pathomechanisms are known to occur with the reduction of eotaxin-2 concentration, directly corresponding to pPROM. There are only suggestions that alterations of immune reactivity, such as decreased cervical cytokines, may predispose to urinary tract infections, pPROM and preterm labor.[18] Physiological body mass index (BMI) alteration is evidenced during pregnancy. Although BMI, as a chronic stress state, is a strong pro-inflammatory nature disorder, little is known about how body composition interferes with inflammatory markers during pregnancy. Therefore, in this study, we evaluated the biomarkers in the reference group, which exclusively include obese subjects. This work demonstrates how the amount of fat mass interferes with the balance of cytokines. Changes in the concentration of pro-inflammatory cytokines may result in a reduced immune response, thus contributing to a symptomatic infection.[19–21] Chronic immune response may lead to a decrease in reactivity, manifested by a decrease in the concentration of some cytokines.[22,23] This study has shown eotaxin-2 to be such a phenomenon. In women with pPROM, eotaxin-2 concentrations were significantly lower compared with the values in the group of women with physiological labor. This interpretation of the reduced level of eotaxin-2 in women with pPROM is not the only one. Simhan et al. found that a reduced level of some cytokines may be a primary phenomenon resulting from genetic conditions, which causes decreased intrinsic resistance to intrauterine inflammations and promotes the occurrence of pPROM.[24] This is consistent with the studies of Dizon-Townson et al., according to which premature labor, at least in part, is genetically determined,[25] which allows a hypothesis that genetically determined decreased levels of eotaxin-2 may lead to pPROM. In view of encouraging results, the role of cytokines in the course of pPROM should be the aim of further research, as well as the use of eotaxin-2 as a pPROM marker.

Limitations

The weakness of the study is a poor sensitivity and specificity of this test and its small sample size, which is why the presented results should be considered as pilot ones. Further studies are necessary on larger groups, which would help define the exact role particular cytokines play in premature rupture of membranes.

Conclusions

We concluded that eotaxin-2 could be the basis for further studies on the use of this molecule as a biochemical

marker of pPROM. Defining the potential for eotaxin-2 as a biochemical marker of pPROM requires further investigation on a larger group of patients.

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