Original Research

Novel inflammatory markers of labor following premature preterm rupture of membranes

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Summary

The aim of the study was comparison of levels of resistin (RE), soluble receptor for advanced glycation end products (sRAGE), soluble receptor activator for nuclear factor-κB ligand (sRANKL), osteoprotegerin (OPG), glucose (GL), total protein (TP), and albumin (AL) between women with preterm and term rupture of fetal membranes and evaluation of prognostic values of all above in diagnostic of premature labor. Ninety-eight women in singleton pregnancy presenting with premature rupture of fetal membranes were included and divided into two groups: A – 49 women after 37 weeks and B – 49 women before 36 weeks of gestation. Plasma levels of sRAGE, OPG, TP, and AL were significantly lower in group B, and RE and GL plasma levels were higher in this group. The high prognostic values for sRAGE and sRANKL in the prognosis of spontaneous delivery after preterm rupture of membranes (pPROM) were found. The authors concluded that sRAGE and sRANKL serum levels could be a useful prognostic factors after pPROM. Resistin can play role in pathogenesis of rupture of membranes in premature pregnancy.

Key words: Preterm labour; Resistin; sRAGE; esRAGE; sRANKL.

Introduction

According to the World Health Association's data, the prevalence of preterm delivery all over the world is as high as thirteen million per year [1]. Approximately 45–50% of preterm births are idiopathic, 30% are related to preterm rupture of membranes (pPROM), and another 15–20% are attributed to medically indicated or elective preterm deliveries [2-3]. Premature birth causes 28% of perinatal deaths of the neonates [4]. Despite many years of research, the actual cause of pPROM remains unclear. Most cases (60–80%) are associated with pathogenic vaginal microbial flora, including viruses, fungi, bacteria, and their components such as lipopolysaccharides, heat shock proteins, and peptidoglycans. Working as the activators of Toll-like receptors (TLRs), mainly TLR2 and TLR4, these agents increase cytokine production. This finally leads to the onset of arachidonic acid cascade, oxidative stress accumulation, and the rise in production of not only prostaglandins, but also different types of proteases, especially matrix metalloproteinase (MMP) [5-7]. The last group is believed to be the most important destructive factor in fetal membranes [8].

Usually labor does not occur until its natural term, even in case of vaginal and cervical microbial colonization. It is known that generally the immune response cascade is initiated only after maternal-placental-fetal unit maturation, close to the estimated day of delivery. An earlier activation of the immune response in pregnancy can be a consequence of preterm aging of the maternal-placental-fetal unit [9]. It is believed that factors known as classical to the increase in oxidative stress, such as smoking, drug addiction, psychical stress or malnutrition, are the same to accelerate maternal-placental-fetal unit aging. There are some studies in progress, evaluating an importance of the aforenamed aging factors for the pathogenesis and diagnostics of premature labor [9-10].

Receptors for advanced glycation end products (RAGE) are non-specific multi-ligand receptors that belong to the superfamily of immunoglobulin. Activation of RAGE induces and supports inflammatory response, mainly by nuclear factor kappa-β (NF-κB) and mitogen-activated protein-kinase (MAPK) activation [11-14]. There is a hypothesis regarding the protective function of negative RAGE isoforms, which would prevent PROM during gestation [15, 16].

Resistin (RE) is an adipokine from the family of cysteine-rich proteins, so-called resistine-like molecules (RELM) [17]. The importance of resistin in some inflammatory diseases has already been proven [18]. Some cytokines, including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and lipopolysaccharides (LPS) stimulate resistine gene expression. Some hypothesis postulate the potential role of RE in preterm labor [19-21].

Osteoprotegerin (OPG), receptor activator of NF-κB (RANK), and receptor activator of NF-κB ligand (RANKL) are glycoproteins that belong to the family of tumor necro-
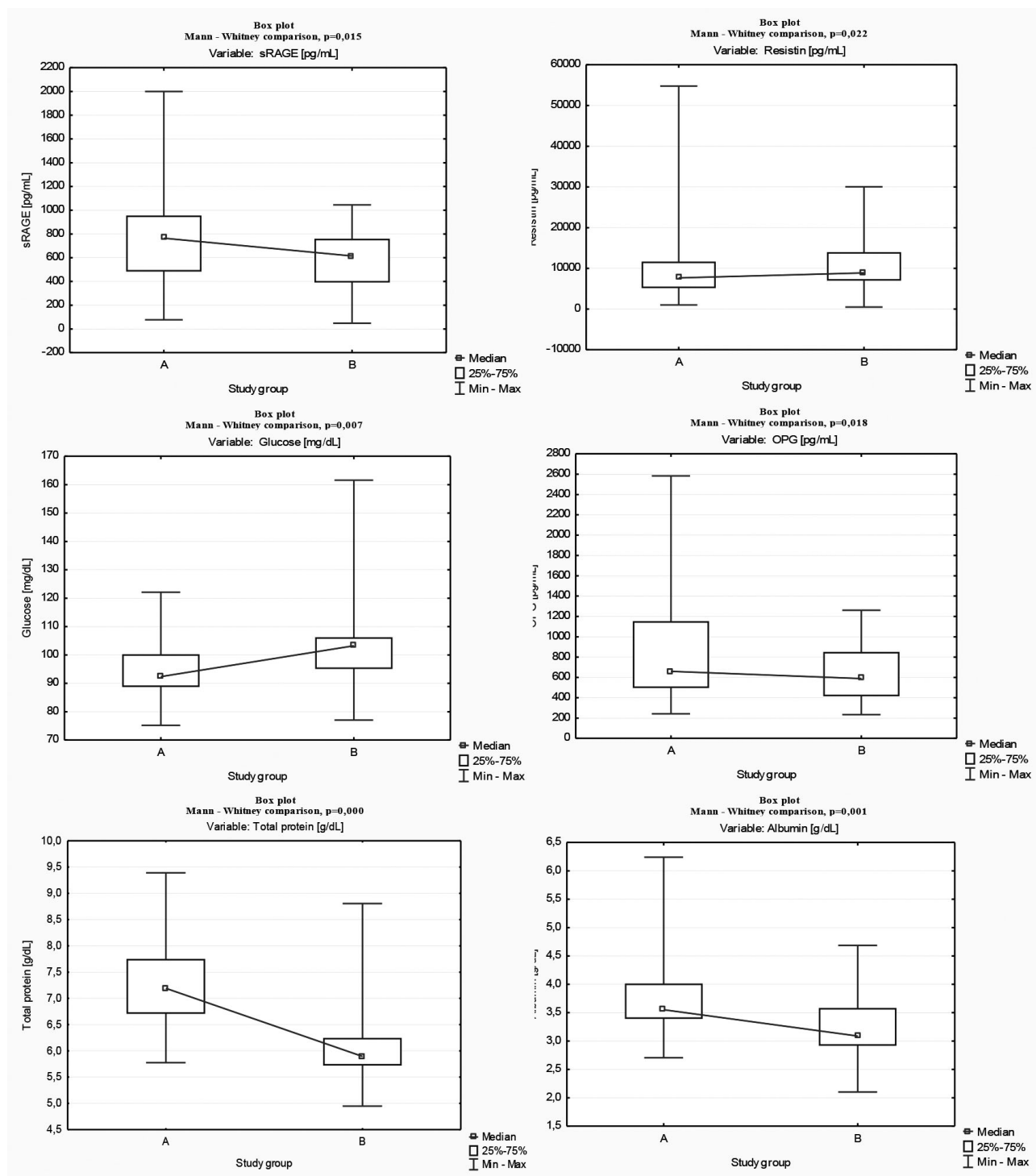
Figure 1. — Comparison of sRAGE, resistin, OPG, glucose, albumin, and total protein between the groups. The Mann–Whitney U-test was used for comparison.

sis factor receptors [22-24]. Some research suggests that the activation of the immune system can also noticeably affect the OPG/RANKL/RANK formation. It is known that even low levels of cytokines can influence components of the OPG/RANKL/RANK system [25-26]. However,

there is paucity of scientific data to support this hypothesis in obstetrics [15].

The aim of the study were 1) calculation and comparison of levels of resistin (RE), soluble receptor for advanced glycation end products (sRAGE), soluble sRANKL, OPG, glu-

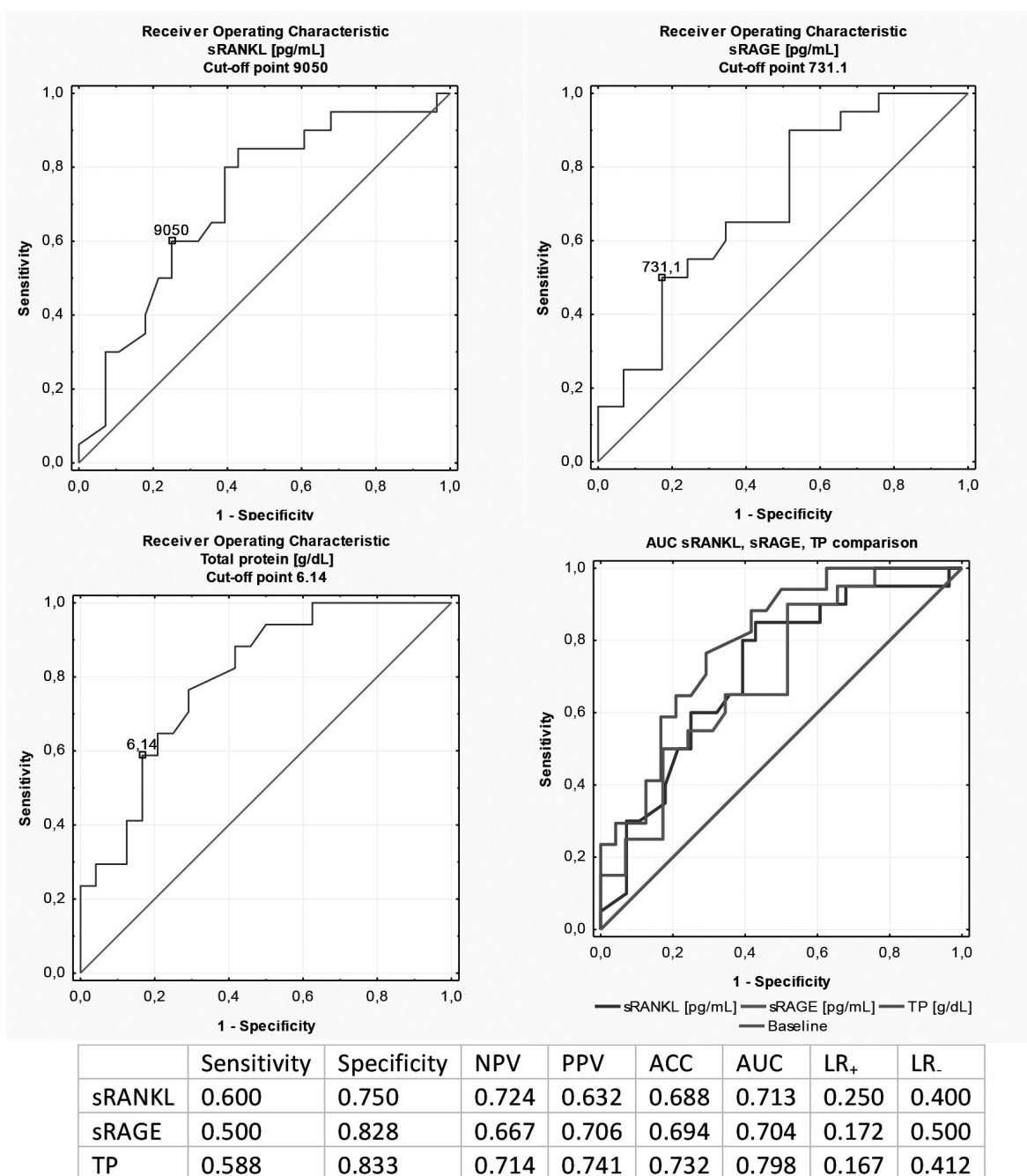|	Sensitivity	Specificity	NPV	PPV	ACC	AUC	LR+	LR-
sRANKL	0.600	0.750	0.724	0.632	0.688	0.713	0.250	0.400
sRAGE	0.500	0.828	0.667	0.706	0.694	0.704	0.172	0.500
TP	0.588	0.833	0.714	0.741	0.732	0.798	0.167	0.412

Figure 2. — ROC curves analysis of sRANKL, sRAGE, and total protein according to latent time from symptoms until delivery.

cose (GL), total protein (TP), and albumin (AL) among women with PROM that occurred about the estimated day of delivery and those who experienced it earlier in pregnancy and 2) evaluation of prognostic value of RE, sRAGE, esRAGE, sRANKL, OPG, GL, TP and AL in the diagnostics of premature labor following pPROM.

Materials and Methods

The study was conducted in the Department of Obstetrics and Gynecology and in the Department of Microbiology and Immunology Diagnostic of Pomeranian Medical University from January 01, 2014 to March 30, 2016. The study was approved by the Bioethical Committee of Pomeranian Medical University (KB-0012/121/12). All women gave their written informed consent prior to their inclusion in the study.

Ninety-eight women in singleton pregnancy experiencing pre-

mature rupture of fetal membranes were included and divided into two groups. Group A comprised of 49 women after 37 weeks of gestation, and group B consisted of 49 women between 22 and 36 weeks of gestation. Demographic and clinical characteristics of study groups are shown in Table 1.

The criteria of inclusion in the study were as follows: (1) clinical diagnosis of premature rupture of the membranes in singleton pregnancy, (2) confirmation of premature rupture of the membranes by a positive test result for the presence of insulin-like growth factor binding protein-1 in vaginal discharge, (3) absence of spontaneous uterine contractility with a negative tocodynamometric test result, and (4) the absence of fetal congenital malformations. Successive patients who reported to the departments and met the criteria for inclusion were entered into the study. Random selection was the method of assignment of participants to one of the two study groups.

No later than two hours after admission to the departments, peripheral maternal blood was sampled from the ulnar vein and placed into tubes containing EDTA-K2. After centrifugation (10 minutes, 5000 rps), plasma samples were stored at −80°C until measurement of RE, sRAGE, esRAGE, sRANKL, and OPG levels. Immunoassay methods were used to measure RE, sRAGE, esRAGE, sRANKL, and OPG levels. Human resistin ELISA was used for quantitative measurement of human RE levels, with a calibration range of 1,000–50,000 pg/mL with the limit of detection at 12 pg/mL. Human sRAGE ELISA was used for quantitative measurement of human sRAGE levels, with a calibration range of 50–3,200 pg/mL and a limit of detection at 19.2 pg/mL. Human esRAGE ELISA was used for quantitative measurement of human esRAGE. The calibration range for esRAGE was 0.625–40 ng/mL, with a limit of detection at 0.156 ng/mL. Human sRANKL (total) ELISA was used to establish sRANKL serum levels, with a calibration range of 31.25–2,000 pg/mL and a limit of detection at 25 pg/mL. Human OPG ELISA was used for quantitative measurement of human OPG. The calibration range for OPG was 180–7,200 pg/mL, with a limit of detection at 36 pg/mL. Coefficients of variation for the ELISA assays are shown in Table 2. The authors additionally measured the white blood cell count (WBC), the percentage of neutrophils in venous blood (BAND%), albumin (AL), glucose (GL), and total protein (TP).

In group B, the pregnant women were administered betamethasone in two 12-mg doses with a 24-hour interval to accelerate fetal lung maturation. Antibiotic agents were administered after diagnosis to extend the duration of pregnancy between rupture of the membranes and delivery. The authors administered 2 grams of ampicillin and 300 mg of erythromycin every 6 hours intravenously for 48 hours. They subsequently administered 500 mg of amoxicillin every eight hours and 250 mg of erythromycin every six hours for five days orally as a standard protocol.

To establish the prognostic values of analyzed substances, group B was categorized into subgroups by the duration of pregnancy from pPROM up to delivery, with a 48-hour and seven-day cut-off point.

Statistical evaluation was performed using Statistica 10.0 PL software. The distribution of variables was checked using the non-parametric Shapiro–Wilk test. The level of significance was set at $p < 0.05$. For the presentation of variables, the number of patients, range of values (minimum–maximum), median (Me), the first and third quartile values (Q1–Q3), arithmetical mean, and standard deviation (SD) were shown in accordance to its distributions. The Mann–Whitney U test for unpaired variables and Student t parametric test were used to assess the differences in the studied parameters between the groups. Receiver operating characteristic (ROC) curve analysis was used to determine the cut-off point, as well as the predictive value of tests, their sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively), accuracy, and positive and negative likelihood ratios (LR+, LR-, respectively). Comparison of the areas under the curves (AUC) was used to compare diagnostic tests.

Table 1. — *Demographic and clinical characteristics of study groups.*

Parameter	Group A	Group B	*p* value
Number of women	49	49	-
Age (years)	28.82±6.36	29,73±6,81	NS
Gestational age (weeks)	37.6±3.8	31,4±3,5	NS
Parity (median, range)	1 (1-6)	2 (1-7)	NS
Birth weight (grams)	3103.40±790,21	1939.37±801.77	0,000
Smoker (n)	11	14	NS
Non-smoker (n)	38	35	
Place of residence – city (n)	35	32	NS
Place of residence – village (n)	14	17	
Excellent socio-economic status (n)	21	20	NS
Mediocre socio-economic status (n)	28	29	

Table 2. — *Coefficients of variation for ELISA assays.*

Assay	Coefficient of variation	
	Intra-assay (%)	Inter-assay (%)
RE	5.2	7,0
OPG	3.53	5.78
sRANKL	9.38	12.00
sRAGE	4.00	7.15
esRAGE	5.20	8.50

RE - resistin; OPG – osteoprotegerin; sRANKL - soluble receptor activator for nuclear factor-κB ligand; sRAGE – secretory receptors for advanced glycation end products; esRAGE – endogenous secretory receptors for advanced glycation end products.

Results

Most of the studied variables were characterized by deviation from the normal distribution (Shapiro-Wilk test $p > 0.05$). Table 3 illustrates descriptive statistics of analyzed variables. Plasma levels of sRAGE, OPG, TP, and AL were significantly lower in group B than in group A (respectively: Me = 614.1 *vs.* 765.1 pg/mL; 589.0 *vs.* 659.3 pg/mL; 5,890 *vs.* 7,193 g/dL; 3,091 *vs* 3,553 g/dL). Resistin and glucose plasma levels were higher in those with rupture of membranes in immature pregnancy (respectively: 8,882 *vs.* 7,654 pg/mL; 103.2 *vs.* 100.25 mg/dL). Figure 1 shows non-parametric comparison of variables. As shown in Table 3, the value of the other variables did not differ between groups.

In group B, sRAGE plasma levels were lower, while sRANKL and TP levels were significantly higher in the subgroup of women who gave birth before 48 hours from pPROM, in comparison to those who delivered later (re-

Table 3. — *Comparison of study parameters between groups.*

Parameter	Group A					Group B					p
	N	Min–max	Q1	Q3	Median	N	Min–max	Q1	Q3	Median	
RE (pg/mL)	49	1020-54800	5166	11650	7654	49	479.4-30060	7000	14000	8882	0.022
OPG (pg/mL)	49	241.9-2583	496.0	1153	659.3	49	234.2-1261	415.2	850.5	589	0.018
sRANKL (pg.mL)	49	2577-77500	5228	19920	9581	49	1245-75875	5911	22643	11218	NS
sRAGE (pg/mL)	49	77.46-2000	484.2	955.5	765.1	49	48.99-4872	361.8	789.9	614.1	0.015
esRAGE (pg/mL)	49	410.4-2450	497.5	644.6	544.8	49	353.3-958.7	483.0	606.5	541.13	NS
Band (%)	49	60.20-91.50	66.90	79.80	73.00	49	55.70-91.00	66.45	80.41	71.35	NS
AL (g/dL)	49	2.707-6.240	3.391	4.010	3.553	49	2.103-4.422	2.917	3.580	3.091	0.001
TP (g/dL)	49	5.776-9.390	6.708	7.750	7.193	49	4.947-8.810	5.719	6.250	5.890	0.000
GL (mg/dL)	49	75.18-122.1	88.70	100.25	92.38	49	77.01-161.54	95.09	106.25	103.2	0.007
WBC (10^9/L)	49	8.060-21.08	9.970	14.04	11.43	49	6.800-21.91	9.980	14.16	11.52	NS

RE – resistin; OPG – osteoprotegerin; sRANKL - soluble receptor activator for nuclear factor-κB ligand; sRAGE – secretory receptors for advanced glycation end products; esrAGE - endogenous secretory receptors for advanced glycation end products; CRP – C-reactive protein; Band – banded neutrophils; AL – albumine; TP – total protein; GL – glucose; WBC – white blood cells.

spectively: 495.6 ± 272.1 *vs.* 687.4 ± 217.1; *p* = 0.014 for sRAGE, 22,697 ± 19,679 *vs.* 11,527 ± 14,356; *p* = 0.011 for sRANKL and 5.83 ± 0.41 *vs.* 5.68 ± 0.42 *p* = 0,001 for TP). The subgroups divided depending on a seven-day or longer interval from pPROM to delivery hardly differed in plasma levels of those substances. The only exception was sRAGE level, found to be significantly higher in those who gave birth later than seven days after pPROM (724.9 ± 262.5 *vs.* 535.2 ± 262.5; *p* = 0,02).

Prognostic levels of analyzed substances were calculated to estimate the occurrence of preterm birth in two intervals: less than 48 hours from pPROM and less than seven days from pPROM.

Figure 2 illustrates the analysis of the AUC ROC for the 48-hour cut-off point, including prognostic values for studied substances. Threshold values for the occurrence of labor within 48 hours of pPROM were established at a level of more than 9,050 pg/mL for sRANKL, less than 731.1 pg/mL for sRAGE, and more than 6.14 g/dL for TP. The comparison of the AUC ROC did not prove a superiority of any analyzed substance over others in the diagnostics of preterm birth.

The analysis of the area under the curve ROC for seven-day interval, with sRAGE plasma level 974.1 pg/dL as a threshold showed high sensitivity (0.975), but low specificity (0.200) of the test, with PPV 0.826, NPV 0.667, LR+ 0.800, ACC 0.816, and LR- 0.026.

Discussion

Most authors associate PROM in premature pregnancy with an infection of the lower pole of conceptus, and subsequent intrauterine infection [27-31]. Recent research has proved an important role of cytokines in the onset and development of this kind of pregnancy complication [32-34]. However, not always does vaginal and/or cervical microbial colonization result in pPROM

The primary objective of this study was to find some new markers, whose characteristic levels would be interpreted as predisposing to intrauterine infection and subsequent preterm delivery following pPROM.

While in animals resistin is mainly released from adipocytes, in humans it is produced mostly by monocytes and macrophages and its importance for inflammatory response is well proven [35-36]. Cytokines like IL-1, IL-6, and TNF increase transcription of the resistin gene (RENT). Resistin itself, operating in positive feedback, enhances the expression of not only the aforementioned cytokines, but also vascular and intracellular adhesion molecules and pentraxin 3 [37-39].

The animal experiments show an importance of resistin in inflammatory response modulation [40-41]. In their experimental study, Jang *et al.* proved the facilitated development of infection in transgenic mice with human RENT gene [40]. Otherwise, Razvi *et al.* showed that exogenous resistin does not increase pulmonary inflammatory response in mice exposed to ozone, but after such exposition the level of endogenous resistin in lungs increases [41].

There are some arguments for the importance of resistin as inflammation mediator in humans, such as the well-proven correlation between autoimmune diseases severity and resistin plasma level [42-45]. In normal pregnancy, resistin level in the pregnant women increases, therefore it can influence maternal-placental-fetal unit maturation [46]. In the present study, higher resistin levels were found in women with preterm rupture of membranes in premature pregnancy, comparing to those with PROM occurring just before the estimated day of delivery. It can be an indirect proof of resistin importance for inflammatory reaction associated with preterm premature rupture of membranes. There is a paucity of the literature on evaluation of the usefulness of resistin plasma level measurements in premature labor. Kusanovic *et al.* confirmed correlation of resistin level increase with intrauterine infection development in premature pregnancy [19]. On the other hand, Lee *et al.*, analyzing only twin pregnancies, did not find higher levels of resistin in women giving birth prematurely [47]. The results of the analysis of resistin level depending on latency

period from pPROM until delivery in the present study does not enable to qualify this parameter as a predictive marker for spontaneous uterine contractility occurrence following pPROM. There is a need for more research on the role of resistin in preterm labor diagnostics.

Negative soluble forms of receptors for RAGE, named esRAGE and sRAGE, are well-known inflammatory response modulators. High levels of sRAGE decrease systemic inflammatory response, improving natural history, and the prognosis in many disorders connected with endogenous inflammation, including diabetes mellitus, atherosclerosis, ischemic heart disease, inflammatory bowel diseases, some neoplasm, and many others [48-58]. There are very few available publications on RAGE receptors in the context of premature labor. In the present study sRAGE plasma levels in group B were significantly lower than in group A, which suggests its protective function in pregnancy. Romero *et al.*, although their own previous reports denied such hypothesis, recently have admitted that in women suffering from overt chorioamnionitis, the level of RAGE receptors in amniotic fluid was decreased [59, 60]. The current authors' former analyses showed that a high sRAGE level in women at risk of premature labor is associated with prolongation of an interval from the occurrence of symptoms of threatened preterm labor until the completion of delivery [15]. Bastek *et al.* confirmed the protective role of sRAGE in premature labor. Analyzing 529 women at risk of preterm delivery, they found that high sRAGE levels were rare among women who finally gave birth prematurely. Similarly to the present team, they concluded that the sRAGE evaluation can be a useful marker of near premature labor onset [61]. In the present work the authors established prognostic values of sRAGE levels for prediction of the interval from pPROM until spontaneous preterm delivery. The sensitivity of their method was much higher for seven-day latency period compared to this of 48-hour. This finding suggests that high sRAGE plasma concentration following pPROM has a protective effect, preventing overt intrauterine infection development, which would result in spontaneous uterine contractility. The present observations show that low sRAGE levels in pregnancy can participate in pPROM occurrence. Still, the evaluation of sRAGE as a prognostic factor of uterine activity following pPROM needs more prospective studies on large groups.

Analyzing the present study groups, the authors found a lower concentration of OPG in group B compared to group A. In the human system, OPG is present commonly in circulating blood, mainly as a free particle, not linked to cell membrane [62]. It is produced, inter alia, in endothelial cells and vascular smooth muscle cells (VSMC) of many organs, including the heart, kidneys, lungs, bowels, bones, as well as in stromal hematopoietic cells (megakaryocytes) and immune system cells [63-67]. There are many factors increasing the OPG gene expression, including some cytokines such as IL-1a, IL-18, TNF-α, and sex hormones

[68-69]. As well as in obstetrics, in other branches of medicine, the correlation between OPG concentration and inflammatory diseases is well known [70-81]. Nevertheless in the present study the lack of correlation between OPG level and latency period in this study group B seems to disqualify it as a pathogenetic factor of premature labor. Lower OPG concentration among women in premature pregnancy complicated with pPROM shown in this study is probably a consequence of low gestational age, which would be consistent with the literature [82].

In the human system, RANKL is present in three forms: as a cytoplasmic molecule, as an originally membrane-bound particle, and as a free plasmatic fraction, so-called sRANKL [24]. The last is formed from RANKL by cleavage from cell membrane with metalloproteinase, known as tumor necrosis converting enzyme (TACE) [24, 25]. RANKL can be found in osteoblasts, T-lymphocytes, in peripheral lymph nodes, bone tissue, as well as in fetal liver and many other organs and tissues [22]. The expression of the RANKL gene is enhanced not only by IL-1β, IL-11, TNF-α, E_2 prostaglandin (PGE2), LPS, but also by glucocorticosteroids, active form of D_3 vitamin and parathormone [83-85]. Such cytokine induced increase of RANKL gene expression can cause changes of plasmatic concentration of RANKL molecule in women threatened with premature labor. There is no research in literature that would evaluate sRANKL as a marker of premature labor. A few available reports postulate its role in other obstetric disorders of an inflammatory nature [86-92]. In the present material, higher levels of RANKL were found in premature pregnancies. However, the difference between study groups did not reach statistical significance. The authors also demonstrated that high RANKL concentration characterizes women developing spontaneous uterine contractility in less than 48 hours after pPROM. According to the result of the analysis of the AUC ROC for sRANKL, the authors admitted this parameter to show a sensitivity of 60% and specificity of 75% for uterine activity onset in less than 48 hours after pPROM. What should also be considered is decreased total protein and albumin level found in this study in women after pPROM. Although in this article the authors have not made more detailed analysis of this finding, they still consider it as very promising for further study. There is a need to continue the study on the proteome of pregnant women's plasma, amniotic fluid, and cervicovaginal secretions to identify new protein markers of premature labor [93].

The substances analyzed in this research may in the future become an alternative to traditional markers of preterm labor. The authors postulate the need for identification of molecules, the excess or deficiency of which induce preterm maternal-placental-fetal unit ageing, thus facilitating inflammation development, to finally cause preterm delivery.

Conclusions

The sRAGE and sRANKL levels evaluated in women suffering from PROM in premature pregnancy can be used as prognostic factors. Higher levels of resistin in study group B suggest its role in PROM in premature pregnancy. Research on this topic should be continued.

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