PREDICTING PRETERM BIRTH BASED ON VAGINAL MICROBIOTA ASSESSMENT BY REAL-TIME PCR IN THE FIRST TRIMESTER

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Detecting high risk of preterm birth (PB) early makes its prevention possible. The aim of the work was to develop a mathematical predictive model for assessing the risk of preterm birth based on a quantitative analysis of the vaginal microbiota in the first trimester of pregnancy. The study included 199 pregnant women, i.e. 41 pregnancies that ended in preterm birth, and 158 — in term birth. Vaginal microbiota was analyzed in all patients in the 1st trimester of pregnancy by quantitative real-time PCR (qPCR). The method of dicriminant analysis was used to develop a predictive model. A method for predicting PB was developed with the calculation of the PRIMA prognostic index (Premature Birth. Index Of Microbiological Analysis). If the value of PRIMA > 0 – the risk of premature birth is low, if PRIMA < 0 – the risk is high. The sensitivity and specificity of the method are respectively 70.7% and 79.75%, the effectiveness is 77.89%. Evaluation of vaginal microbiota in the 1st trimester makes it possible to identify a high-risk group of PB and perform timely preventive measures.

Keywords: vaginal microbiota, premature birth, Femoflor-16, prediction, real-time PCR, discriminant analysis

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Compliance with ethical standards: the study was approved by the Ethics Committee of Ural State Medical University, Federal State Budget Educational Institution of Higher Education under the Ministry of Health of the Russian Federation (Protocol № 7 dated March 28, 2011). All patients signed the informed written consent to participation in the study.

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ПРОГНОЗИРОВАНИЕ ПРЕЖДЕВРЕМЕННЫХ РОДОВ НА ОСНОВАНИИ ИССЛЕДОВАНИЯ МИКРОБИОТЫ ВЛАГАЛИЩА МЕТОДОМ ПЦР В РЕАЛЬНОМ ВРЕМЕНИ В ПЕРВОМ ТРИМЕСТРЕ БЕРЕМЕННОСТИ

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Раннее выявление высокого риска развития преждевременных родов (ПР) дает возможность их предупреждения. Целью работы было разработать математическую прогностическую модель для оценки риска преждевременных родов на основании количественного анализа микробиоты влагалища в первом триместре беременности. В исследование было включено 199 беременных, из которых у 41-й беременность закончилась преждевременными родами, у 158 — срочными родами. Всем участницам проводили исследование микробиоты влагалища методом ПЦР в реальном времени (ПЦР-РВ). Для разработки прогностической модели использовали метод дискриминантного анализа. Был разработан способ прогнозирования ПР с расчетом прогностического индекса ПРИМА (сокр. от «Преждевременные роды. Индекс микробиологического анализа»). Если значение ПРИМА > 0, риск ПР низкий, если ПРИМА < 0, риск высокий. Чувствительность и специфичность метода составляют соответственно 70,7 и 79,75%, эффективность — 77,89%. Оценка микробиоты влагалища в первом триместре дает возможность определения группы высокого риска ПР и проведения своевременных профилактических мероприятий.

Ключевые слова: микробиота влагалища, преждевременные роды, прогнозирование, ПЦР в реальном времени, дискриминантный анализ

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Prevention and early diagnosis of gestational complications are crucial for reducing maternal and neonatal mortality rates, as well as for reducing morbidity throughout the rest of their lives [1]. Preterm birth (PB) accounts for estimated 70% of neonatal mortality and 36% of child mortality. Long-term consequences of PB take the form of neurological disorders in children in 25–50% of cases [2], and therefore, it represents not only a medical, but also a social problem worldwide.

Preterm birth occurs during 22nd to 36th weeks of pregnancy [2]. PB is one of the great obstetrical syndromes, which are characterized by a long preclinical stage [1, 2, 3, 4]. The prevalence of PB in Russia is about 6% of the total number of births [2]. Moreover, in 70–80% of PB cases, the labor occurs spontaneously, and only 20–30% of the cases manifest with early signs from mother and/or fetus [2, 3].

In recent years, a number of the most significant complications of pregnancy (premature birth, placental insufficiency, fetal growth retardation syndrome, and preeclampsia) are increasingly considered as a single group called "the great obstetrical syndromes" [4, 5].

These pathological conditions are based on placental disorders in the early stages of pregnancy, associated with defects in the gestational transformation of spiral arteries, which in turn can be caused by a number of etiological factors (luteal insufficiency, disorders in the formation of immunological tolerance, acquired or congenital thrombophilia, direct or indirect effects of infectious agents) [6, 7]. In clinical practice, as a rule, there is a complex combination of several etiological factors and pathogenetic mechanisms.

The 2020 clinical recommendations on preterm birth management emphasize the scientifically proven relationship between PB and genital tract infections (GTIs). The course of GTIs in most cases is asymptomatic [2, 8]. A change in the microbiota of the genital tract leads to an increase in the contractile activity of the myometrium and degradation of the extracellular matrix with the remodeling of the cervix and amniotic membrane [2, 8, 9].

The structure of genital infections in pregnant women is dominated by vaginal microbiota disorders. More than half of women have at least one episode of genital pathology associated with dysbiotic disorders during pregnancy [10]. At the same time, pregnancy itself is a risk factor for the development of a pathology caused by opportunistic microbiota and microorganisms with weak virulence and aggression factors [11].

Vaginal dysbiosis is caused by an imbalance between the opportunistic and physiological microflora, the extreme degree of which is pronounced dysbiosis, which definitely requires treatment [12, 13, 14]. The state of vaginal microbiocenosis, where the proportion of lactobacilli is in the range of 20–80% of the total bacterial load (TBL), is regarded as moderate dysbiosis [15]. Whether this option is normal or requires treatment to date remains unclear.

The emergence of quantitative molecular based methods has significantly expanded our understanding of the quantitative and qualitative vaginal microbiota composition in normal and pathological conditions, including pregnancy [16].

Given the long preclinical stage of PB, development of new prognostic models for this state is crucial. Vaginal microbiota analysis and timely treatment in high-risk women may lead to a decrease in the preterm birth rates and thus improve the perinatal outcomes.

The aim of the work was to develop a mathematical predictive model for assessing the risk of preterm birth based on a quantitative analysis of the vaginal microbiota in the first trimester of pregnancy.

METHODS

Research design

A single-center cohort retrospective comparative study included 199 patients who received prenatal care at the Garmonia Medical Center (Yekaterinburg) in the period from 2012 to 2021. The study was conducted on the basis of the laboratory department of the "Garmonia" Medical Center.

Inclusion and exclusion criteria

Inclusion criteria for the main group: age from 20 to 42 years, spontaneous preterm birth (28–36 weeks gestation), consent to participate in the study.

Inclusion criteria for the control group: age from 20 to 42 years, term birth, consent to participate in the study.

Exclusion criteria for the main group: severe endocrine diseases; arterial hypertension of 2nd–3rd degrees; thyroid diseases in the decompensation stage; coagulopathy; antiphospholipid syndrome; intrauterine infectious processes during pregnancy; severe chronic diseases (kidneys, liver, respiratory organs, cardiovascular system, central nervous system); mental ilnesses; present or history of oncological diseases; chronic alcohol or nicotine intoxication, substances abuse; uterine malformations.

Exclusion criteria for the control group: refusal to participate in the study, indications for induced preterm birth or preterm surgical delivery, infections of the genital tract caused by obligate pathogens.

Groups of patients

The main group (group 1) included patients with PB (n = 41), the control group (group 2) consisted of women with successful full-term delivery (n = 158).

Research methods

All participants of the study underwent a clinical and laboratory examination, in accordance with the current standards.

All patients underwent vaginal microbiota analysis by qPCR at 6–12 weeks of gestation (I trimester). The material for the study was collected in an Eppendorf tube containing 1 ml of sterile saline solution from the posterolateral vaginal wall. DNA was extracted using the PREP GS PLUS reagent kit (DNA Technology LLC; Russia), was performed using Femoflor ® 16 REAL-TIME PCR Detection Kit (DNA Technology LLC; Russia) according to the manufacturer's instructions. 16 groups of bacteria were typed and quantified. According to the test results, each vaginal sample was automatically characterized using the software provided by the manufacturer.

Ethical aspects

The design of the study was reviewed and approved by the local ethics committee of the Federal State Budgetary Educational Institution of the Ministry of Health of the Russian Federation, Protocol No. 7 of 28.03.2011. All participants of the study were informed about the methods and nature of the study, the inclusion of the survey results in the scientific study and signed a voluntary informed consent.

Methods of statistical analysis

Statistical processing of the study results was carried out using computer programs Statistica 7.0 (StatSoft Inc.; USA), Microsoft Excel 2016 and StatPlus:mac 8.0.3 (AnalystSoft; USA). The normality of the distribution was checked using the Kolmogorov–Smirnov criterion. The distribution was not normal. For quantitative indicators, the median value (Me) and the interquartile range (Q_1–Q_3) were indicated, and the nonparametric Mann–Whitney criterion was used to assess the statistical significance of the differences. Absolute and relative values (%) were presented for qualitative indicators, the statistical significance of the differences was determined using the criterion χ^2. The strength of the association of the obtained values was estimated in the values of the odds ratio indicator (OR) with a 95% confidence interval (95% CI). The differences were considered statistically significant at $p < 0.05$. To form the regression equation, which formed the basis of the predictive model, the method of discriminant analysis with the calculation of canonical coefficients of the discriminant function (CCDF) was used. ROC analysis was applied to evaluate the effectiveness of the proposed predictive model.

RESULTS

Clinical and anamnesis characteristics of the examined patients

Initially, the groups were compared according to their medical history parameters. The data of somatic and obstetric-gynecological history of the patients were taken into account. The average age of the examined patients was 29.04 ± 4.62 years for the main group and 28.53 ± 4.03 years for the control group ($p > 0.05$). In terms of weight and height parameters, the differences were not significant ($p > 0.05$). The frequency of detection of somatic pathologies (diseases of the cardiovascular, urinary, respiratory, nervous systems, endocrinopathies, pathology of the gastrointestinal tract and autoimmune diseases) also did not differ between the groups ($p > 0.05$). Thus, the groups were clinically comparable.

Vaginal microbiota analysis

Vaginal microbiota composition was assessed by qPCR. In accordance with the previously proposed criteria [16], 4 variants of vaginal microbiocenosis were distinguished, the frequency of detection of which differed in pregnant women of the analyzed groups (Fig. 1).

In the main group, dysbiosis of varying severity was determined in 16 (39.0%) patients, in the control group it was determined in 18 (11.39%) patients (χ^2 =17.457; $p < 0.001$ [2.9E-5]). The detection rates of *Ureaplasma spp* in an amount of more than 10^4 genomequivalents in ml (GE/ml) differed significantly: 18 (43.9%) in the main group, 39 (24.7%) in the control group ($\chi^2 = 5.853$; $p = 0.016$). Moreover, in group 1 dysbiosis in combination with *Ureaplasma spp*. in an amount of more than 10^4 GE/ml was detected in 11 (26.8%) women, and in the control group — in 6 (3.8%) pregnant women ($\chi^2 = 21.991$; $p < 0.001$ [2.7E-6]). At the same time, detection rates of conditional normocenosis associated with *Ureaplasma spp*. in the amount of more than 10^4 GE/ml, did not differ between the groups. Also, there were no statistically significant differences in the detection rates of absolute normocenosis and conditional normocenosis associated with *Candida spp*.

Discriminant analysis

For the convenience of assessing the risk of PB and the distribution of pregnant women into low- or high-risk groups, based on the results of vaginal microbiota analysis by using qPCR, we have developed a prognostic method with the calculation of the prognostic index PRIMA (Preterm birth. Index of Microbiological Analysis). To develop a prognostic index a discriminant analysis was carried out in the obtained matrices of laboratory parameters for patients of the analyzed groups.

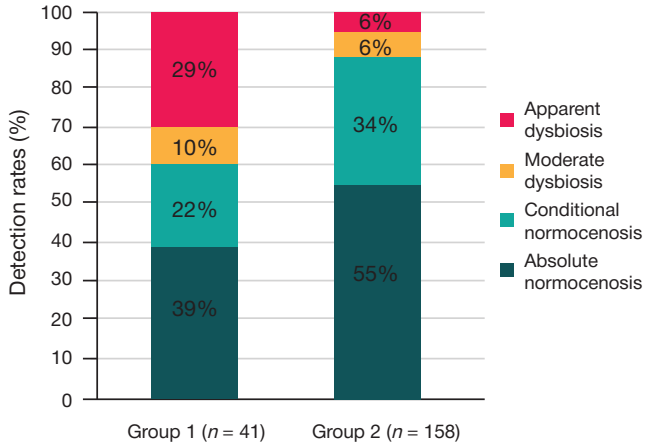

Fig. 1. The structure of vaginal microbiocenosis in the examined groups of pregnant women ($\chi^2 = 19.066$; $p < 0.001$ [1.2E-5])

Lactobacillus spp., *Staphylococcus spp*., *Sneathia spp*. / *Leptotrichia spp*. / *Fusobacterium spp*., *Gardnerella vaginalis* / *Prevotella bivia* / *Porphyromonas spp*., *Eubacterium spp*., *Lachnobacterium spp*. / *Clostridium spp*., *Mobiluncus spp*. / *Corynebacterium spp*., *Ureaplasma spp* were the most significant in predicting the risk of PB. The total bacterial load (TBL) was also taken into account. The data were determined in the Lg GE/ml format.

CCDF are presented in Table 1.

The PRIMA index is calculated by the following formula:

$PRIMA = 0.25 - 0.49 \times X_1 + 0.74 \times X_2 - 0.16 \times X_3 - 0.22 \times X_4 - 0.04 \times X_5 - 0.22 \times X_6 - 0.03 \times X_7 - 0.03 \times X_8 + 0.3 \times X_9$, where

X_1 is TBL, Lg GE/ml;

X_2 is *Lactobacillus spp*., Lg GE/ml;

X_3 is *Gardnerella vaginalis* / *Prevotella bivia* / *Porphyromonas spp*., Lg GE/ml;

X_4 is *Lactobacillus spp*., Lg GE/ml;

X_5 is *Sneathia spp*. / *Leptotrichia spp*. / *Fusobacterium spp*., Lg GE/ml;

X_6 is *Lachnobacterium spp*. / *Clostridium spp*., Lg GE/ml;

X_7 is *Mobiluncus spp*. / *Corynebacterium spp*., Lg GE/ml;

X_8 is *Ureaplasma spp*., Lg GE/ml;

X_9 is *Staphylococcus spp*., Lg GE/ml;

If the value of PRIMA > 0 — the risk of preterm birth is low, if PRIMA < 0 — the risk is high.

The average PRIMA value in groups 1 and 2 was –0.39 (–1.43 –0) and 0.33 (–0.15 –0.84) respectively. The differences were statistically significant ($p < 0.001$). Graphically, the value of the PRIMA index is represented by Fig. 2.

To assess the effectiveness of the presented predictive model, ROC analysis was performed. The ROC curve for the PRIMA index is represented by Fig. 3. The area under the curve (area under curve, AUC) was 0.76 (95% CI 0.68–0.84), which corresponds to the good quality of the predictive model.

Table 1. CCDF when calculating the PRIMA index

Parameter, Lg GE/ml	Non-standardized CCDF	Standardized CCDF
TBL	0.46	0.33
Lactobacillus spp.	–0.69	–0.76
Staphylococcus spp.	–0.28	–0.44
Sneathia spp. + *Leptotrichia spp.* + *Fusobacterium spp.*,	0.04	0.07
Gardnerella vaginalis + *Prevotella bivia* + *Porphyromonas spp.*	0.15	0.29
Eubacterium spp.	0.2	0.36
Lachnobacterium spp. + *Clostridium spp.*	0.21	0.36
Mobiluncus spp. + *Corynebacterium spp.*	0.03	0.04
Ureaplasma spp.	0.03	0.06

To determine the sensitivity and specificity of the presented prognostic method, a holdout sample was used. The sensitivity and specificity parameters were respectively 70.7% and 79.75%, the effectiveness of the method was 77.89% (Table 2).

Sensitivity and specificity at different values of the PRIMA index are shown in Fig. 4.

DISCUSSION

According to previous studies, the lactobacilli-dominated type of the vaginal microbiota, which is established by the beginning of the second trimester, is the most favorable for the successful course and timely completion of pregnancy [17]. Normal microbiota in such pregnant women, as a rule, persists in the second and third trimesters [18]. In our study, we did not find statistically significant differences in the incidence of absolute and relative normocenosis in the 1st trimester of pregnancy, however, it was shown that severe dysbiosis is a risk factor for preterm birth: in group 1, it was significantly more common.

The presence of various opportunistic microorganisms, especially those associated with bacterial vaginosis, in the vaginal microbiota is prognostically unfavorable [17]. At the same time, treatment of bacterial vaginosis during pregnancy is often unsuccessful, and the proven elimination of *G. vaginalis* does not reduce the risk of PB due to the presence of other obligate anaerobes with a reduced proportion of lactobacilli [12]. Earlier studies have shown that the presence of *Ureaplasma spp* in a high titer is a risk factor for PB [19]. In our work, we confirmed these data — in the main group, *Ureaplasma spp*. in the amount of more than 10^4 GE/ml was detected significantly more often. Moreover, in group 1, dysbiosis in combination with *Ureaplasma spp*. in an amount of more than 10^4 GE/ml was detected in 11 (26.8%) women, and in the control group — in 6 (3.8%) women ($\chi^2 = 21.991$; $p < 0.001$). At the same time, detection rates of conditional normocenosis associated with *Ureaplasma spp*. in the amount of more than 10^4 GE/ml, did not differ between the groups. Probably *Ureaplasma spp*. has the greatest significance in patients with dysbiosis.

Other opportunistic microorganisms have no independent significance, however, together they can form the type of vaginal microbiota, which increases or, conversely, reduces the risk of PB. To effectively predict this condition, a comprehensive assessment is necessary.

Previously, an attempt was made to develop a prognostic model for assessing the risk of PB based on the results of vaginal microbiota evaluation [17]. However, the authors of the proposed method used the NGS sequencing method (the target site of the bacterial genome is the 16S rRNA gene). Despite high informativeness, this approach has a number of disadvantages: complex sample preparation, difficulty in controlling the collection of material, long duration of analysis, difficulty in interpreting the results, high cost of equipment and reagents for research. These disadvantages make it almost impossible to use NGS sequencing in routine medical practice. For this purpose, real-time PCR method is much more convenient.

On this basis, we have developed a method for predicting preterm birth, taking into account data on the qualitative and quantitative composition of the vaginal microbiota of pregnant women obtained using qPCR. The novelty of the proposed method is that for the first time, a highly effective prediction of preterm birth is carried out solely on the basis of the qualitative and quantitative composition of vaginal microbiota. The proposed prognostic PRIMA index takes into account the role of 8 out of 16 determined parameters in the complex vaginal microbial

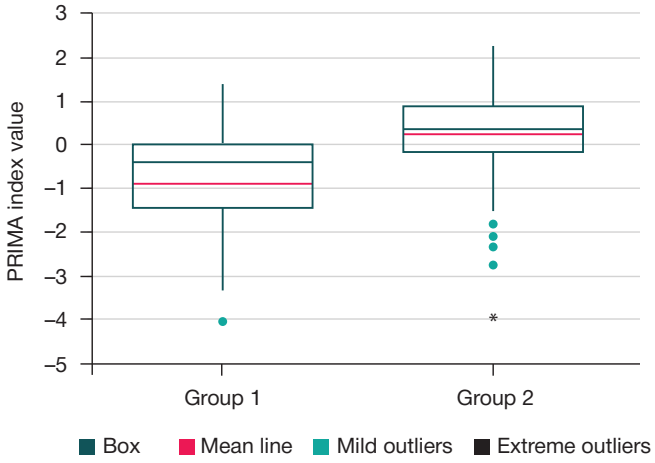

Fig. 2. The value of the PRIMA index in the analyzed groups

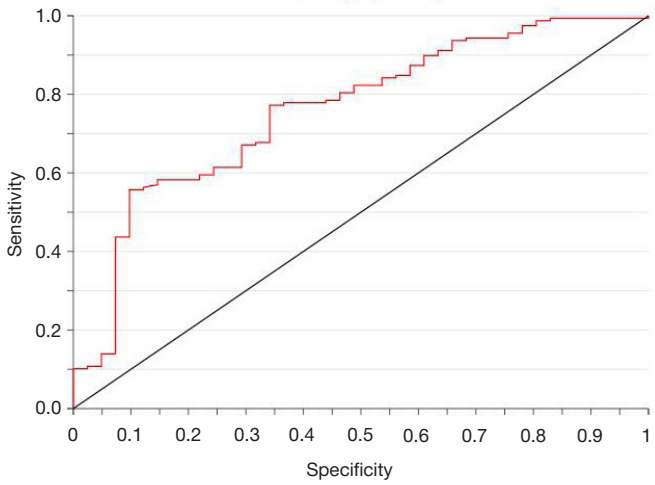

Fig. 3. ROC curve of the PRIMA index

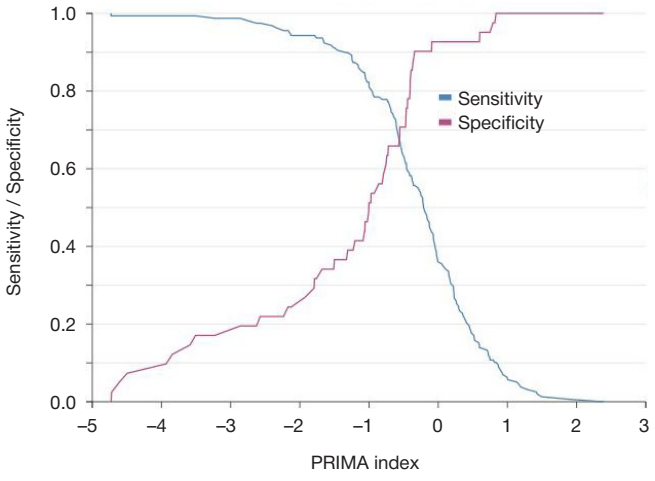

Fig. 4. Sensitivity and specificity at different values of the PRIMA index

community. It is noteworthy that in addition to *G. vaginalis*, which is traditionally associated with an increased risk of PB [17], 4 other groups of obligate anaerobes were significant in the formation of the prognostic index. This fact emphasizes the expediency of a comprehensive analysis of vaginal microbiota with an assessment of the significance of all potentially present microorganisms.

The limitation of this study is the exclusion of clinical and medical history data, as well as the results of other analyses from the number of parameters used to compile a prognostic index. We understand that preterm births cannot be entirely attributed to vaginal microbiota disorders. Therefore, it is possible to increase

Table 2. Sensitivity and specificity of the PRIMA index

Group / Prognosis	1	2	Total	% correct
1	29	12	41	70.7
2	32	126	158	79.75
Total	61	138	199	77.89

the sensitivity and specificity of the predictive model by including additional markers.

CONCLUSIONS

The predictive model developed by us makes it possible to detect pregnant women at risk of PB early on the basis of a comprehensive assessment of the vaginal microbiota by real-time PCR in the first trimester of pregnancy. The use of the proposed prognostic PRIMA index justifies the need to treat vaginal dysbiosis already in the first trimester of pregnancy to reduce the risks of preterm birth and, consequently, reduce perinatal morbidity and mortality. Distinguishing patients at risk for preterm birth based on the PRIMA index creates prerequisites for the use of already known preventive methods, for example, vaginal micronized progesterone from 22 to 34 weeks of pregnancy. The proposed prognostic method can be widely used in clinical practice, does not require significant additional costs.

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