Section 4. Medical science

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BACTERIAL DYSBIOSIS RISK PREDICTION ACCORDING TO VAGINAL NORMOBIOTA INDICATOR

Abstract. Vaginal bacterial dysbiosis is evident by the progressive growth of obligate and optional anaerobic bacteria, myco- and ureaplasmas and fungi against the background of a sharp decrease in the number of lactobacilli.

The aim of those work was to establish prognostic factors and the risk of bacterial dysbiosis development according to the vaginal normobiota indicator based on the comprehensive analysis of the state of systemic and local immunity and hormonal regulation. As conclusions, with bacterial dysbiosis the pathological regulatory hormonal-immune system is formed, which contributes to the vaginal dysbiosis progression. In accordance with this, dysbiosis can be considered as a dysregulatory pathology, and the identified indicators of the "interleukin cascade" as its markers.

Keywords: bacterial dysbiosis, IL2, IL4, TNFα.

Introduction

Despite certain success achieved in the treatment of female genital organs inflammatory diseases, the prevalence of these diseases is increasing steadily and, according to various authors, ranges from 26% to 40–45% [1, 472; 2]. Internal female genital organs non-specific inflammatory diseases occupy one of the main places in the gynecological pathology structure, which is one of the main medical problems and has a significant impact on the health of millions of childbearing age women [3]. These processes occur against the background of a very noticeable deterioration in reproductive health and determine the development of obstetric-gynecological pathology that determines the health of a mother and fetus [4, 143–148; 5, 859–864].

Women internal genital organs chronic inflammatory processes should be considered as a common multisystem disease. It is accompanied by the involvement in the pathological process of all parts of the systemic regulation of organs and body systems [6, 1399–1405].

Bacterial vaginosis (BV) is an infectious non-specific non-inflammatory syndrome, which is the final variant of the bacterial dysbiosis development, and is evident by the growth of obligate and optional anaerobic bacteria, myco- and ureaplasmas, fungi against the background of a sharp decrease in the number of lactobacilli [1, 472; 3; 7, 555–563]. The main place in the BV pathogenesis takes the disorder of the coordinated functioning of the hormonal and immune regulation of the vaginal secretions coloni-

zation resistance, leading to the shift in the microbiota towards the pathogenic factors [4, 143–148; 5, 859–864]. The local and systemic immunodeficiency is accompanied by the disorder of the vagina normal microbiocenosis and the antimicrobial substances secretion [8, 283–291].

According to the data [9, 1–5], BV is characterized by a significantly reduced systemic and local inflammatory response against the activation of cytokine cascades, corresponding to the increase in BV-associated microflora [10, 481–487].

Based on this concept, it seems relevant within the framework of one comprehensive study to establish the main links in the pathogenesis of bacterial dysbiosis and select marker factors for its development.

Purpose of the study is to establish prognostic factors and the risk of developing bacterial dysbiosis according to the vaginal normobiota indicator based on the comprehensive analysis of the state of systemic and local immunity and hormonal regulation.

Material and methods

This study uses examination data of 298 women aged from 16 to 64 who saw gynecologist for a preventive examination or with complaints of genital discomfort. Criterion for exclusion was the presence in the vaginal epithelium scrapings of definitely pathogenic microorganisms (*Trichomonas vaginalis, Neisseria gonorrhoeae, Chlamydia trachomatis and Herpes Simplex Virus 1,2*). Presence in the smear of more than 15–20 leukocytes, which indicated of an inflammatory reaction, was also the reason for exclusion from the number of patients.

During the examination, scraping of epithelium from the posterolateral vaginal paries was made using a urogenital probe. Molecular and genetic studies were performed by the real-time Polymerase chain reaction (PCR). DNA was extracted using the Proba-GS reagent kit (LLC DNA Technology, RF). Amplification of the tubes with the reaction mixture was carried out in a DTLite thermocycler (DNK-Technologiia LLC, RF). Investigation of vaginal biocenosis status was performed using Femoflora 16

test system, designed to perform real-time PCR. Microbiota was quantified by the following indicators [11, 30]: Total bacterial mass (TBM), normobiota (*Lactobacillus spp.*), Obligate anaerobes (ObA; *Atopobium vaginalis, Eubacterium spp., Gardnerella vaginalis, Prevotella bivia, Porhhyromonas spp., Lachnobacterium spp., Clostridium spp., Megasphaera spp., Veilonella spp., Dialister spp., Mobiluncus spp., Corynebacterium spp., Peptostreptococ spp., Sneathia spp., Leptotrihia spp., Fusobacterium spp.*), optional anaerobes (*OpA; Enterobacteriaceae spp., Staphylococcus spp., Streptococcus spp.*), myco- and ureaplasmas (MU; *Ureaplasma urealiticum + parvum, Mycoplasma hominis + genitalium*) and yeast-like fungi (*YF; Candida spp.*).

According to the PCR results, the Opportunistic pathogenic microflora index (OPMI) and normobiota index (PNB) were calculated: $OPMI = \log((\Sigma 10OA + \Sigma 10\Phi A + \Sigma 10MP + 10DG) - 10LB)$ NBI = $= \lg 3БМ - \lg ЛБ$, where ObA means obligate aerobes; OpA – optional anaerobes; MU – myco and ureaplasmas; YF – yeast-like fungi; LB – lactobacilli; TBM – total bacterial mass. According to OPMI, patients were divided into two groups: with normocenosis (OPMI was lower than –1 lg GE / sample; n = 53) and dysbiosis (higher than –1 lg GE / sample; n = 245) [11, 30]. With dysbiosis, the NBI index reflected its degree and ranged from 0 lg GE / sample to 7.2 lg GE / sample. The NBI value above 1 log GE / sample (the number of such cases was amounted to 83) indicated the maximum degree of dysbiosis and corresponded to the state of BV [12, 54–57; 13, 103–7].

According to the standard immunological methods [14, 960; 15, 576], there was determined the content of immunoglobulins A(IgA), M(IgM) and G(IgG) in the blood serum and vaginal secretions (Granum NVL test systems; Ukraine) and the content of immunoglobulin G2(IgG2) and secretory IgA(sIgA) (Hema, LLC; RF); the transforming growth factor content 1β(TGF-1β) (DRG; USA); immune complexes (IC, in vaginal secretion) and circulating IR(CIR, in blood) by the method of selective precipitation in polyethylene glycol solution;

the interleukins content 1β (IL1β), 2 (IL2), 4 (IL4), 6(IL6), 8(IL8), 10(IL10), tumor necrosis factor α (TNFα) and γ-interferon (γ-INF) (Vector-Best, LLC; RF); the complement components content C3 and C4("PLIVA-Lachema Diagnostica s.r.o"; Czech Republic); lysozyme (DRG; USA).

The leukocyte phagocytic activity (LPA) was determined using the yeast cells suspension (Granum, NPL, Ukraine); LPA was calculated as the average number of particles absorbed by one active neutrophil per 100 cells, the LPA index (ILPA) as the percentage of phagocytes from the number of counted neutrophils. The number of lymphocytes in the blood (L) was calculated [14, 960]; quantitative determination of CD3 +, CD4 +, CD8 +, CD16 + and CD22 + cells was carried out using erythrocytic diagnosticum of Granum, NPL (Ukraine), the immunoregulatory index (IRI) was calculated as the ratio of CD4 + / CD8 +. The pH of the vaginal secretion was determined using the Kolpo-Test Ph test strips manufactured by Biosensor AN, LLC (RF). By applying the enzyme-linked immunosorbent assay there was determined the content of hormones in the blood serum:: luteotropic (LT), follicle-stimulating (FS), prolactin (PL), cortisol (C), progesterone (PG), estradiol (E2), testosterone (TS), free triiodothyronine) and free thyroxine (T4) using reagent kits manufactured by the Granum NPL (Ukraine).

The influence of factor variables on dependent indicators was studied using one- and multi-factor linear and non-linear regression analysis [16]. There were calculated the regression coefficients (β), the reliability of their differences from the null hypothesis, the correlation coefficients (R) and determination (R2) for linear models, as well as the value of Wald statis-

tics and the maximum likelihood coefficient for non-linear ones. The operational characteristics (sensitivity, specificity and correctness) of the logistic models were evaluated using ROC diagrams. The prognostic models building was carried out using neural network modeling. The genetic analysis method was used to select the most significant factor characteristics. In all types of comparative statistical analysis, the significance of differences was taken at $p < 0.05$. For statistical processing of the data obtained, Statistica 10 software package (StatSoft, Inc., USA) was applied.

Results and Discussion

At the previous study's stages, we have analyzed the microbial biocenosis indicators, vagina colonial resistance, immune system and hormonal regulation system during the vaginal dysbiosis development and BV [17, 583–595; 18, 103–7; 19, 84–90]. The formation of a single pathological hormonal-immune system, which is formed under conditions of vaginal dysbiosis and supports its development, has been shown. Such a system included the formation of local and systemic immunodeficiency and a number of hormonal disorders.

The task of this work was to identify the most informative indicators that objectively reflect the state of the pathological process and the severity of dysbiosis. The NBI (variable Y) was considered as the resulting sign, while in the case of normocenosis, the variable Y acquired the value Y = 0, and in the case of dysbiosis – Y = 1. The analysis was carried out for the examination results of 298 female patients, 53 of whom were diagnosed with normocenosis, and 245 of whom were diagnosed with dysbiosis. As factor signs, 58 indicators were subjected the initial analysis (Table 1).

Table 1. – Input signs of the initial analysis of the vagina colonial resistance, immune system and hormonal regulation system indicators

X1	Age	X20	IL10	X39	CD22+
X2	Day MC	X21	TNFα	X40	LPA
Indicators in VS:		X22	TGF-1β	X41	I$_{LPA}$
X3	IgM	X23	pH	X42	CIR

X4	IgA	Indicators in blood:		X33	C3
X5	IgG	X24	FS	X44	C4
X6	IgG$_2$	X25	LT	X45	γ-INF
X7	sIgA	X26	E$_2$	X46	IL1β
X8	Lysozyme	X27	PG	X47	IL2
X9	LPA	X28	TS	X48	IL4
X10	I$_{LPA}$	X29	C	X49	IL6
X11	IC	X30	PL	X50	IL8
X12	C3	X31	fT$_3$	X51	IL10
X13	C4,	X32	fT$_4$	X52	TNFα
X14	γ-INF	X33	Lc	X53	TGF-1β
X15	IL1β	X34	CD16+	X54	IgM
X16	IL2	X35	CD3+	X55	IgA
X17	IL4	X36	CD4+	X56	IgG
X18	IL6	X37	CD8+	X57	IgG$_2$
X19	IL8	X38	IPI	X58	sIgA

Notes: *MC – menstrual cycle; VS – vaginal secretion*

To check the quality of the model forecasting, all observations (using a random number generator) were divided into three sets: training one (used to calculate the model parameters, 248 cases), control one (used to control model re-training, 20 cases) and confirming set (used to check the model adequacy when new data forecasting, 30 cases) [16, 208].

On a complete set of 58 factor signs, a linear neural network model was built and trained. The model sensitivity built on the full set of factor signs on the training set was 99.4% (95% CI 97.6% –100%), specificity – 100% (95% CI 97.7% –100%), on the confirming set the model sensitivity was 100% (95% VI 88.8% –100%), specificity 100% (95% VI 87.3% – –100%). The sensitivity and specificity on the training and supporting sets were not statistically significantly different (p = 0.15 and p > 0.99, respectively, when compared by the χ² criterion), which indicates the adequacy of the constructed model.

To identify factors that are most associated with the dysbiosis risk according to NBI, a selection of significant signs was performed using the genetic ALT algorithm method. As a result, three factor signs were selected: blood levels of IL2 (X47), IL4 (X48) and TNFα (X52).

On the selected set of three factor signs a linear neural network model was built and trained. The linear neural network model sensitivity built on three factor signs on the training set was 80.5% (95% VI 74.1% –86.2%), specificity 82.1% (95% VI 73.1% – –89, 6%), for the confirming set, the model sensitivity was 81.3% (95% VI 57.1% –96.7%), specificity 92.9% (95% VI 71.9% –100%). The sensitivity and specificity in the training and supporting sets were not statistically significantly different (p = 0.80 and p = 0.54, respectively, when compared by the χ² criterion), which proved the adequacy of this model.

To identify the possible nonlinear factor signs relationships with the risk of dysbiosis development according to NBI, a nonlinear neural network model (such as a multilayer perceptron) forecasting (model architecture is shown in Fig. 1) was also built on the selected set of signs.
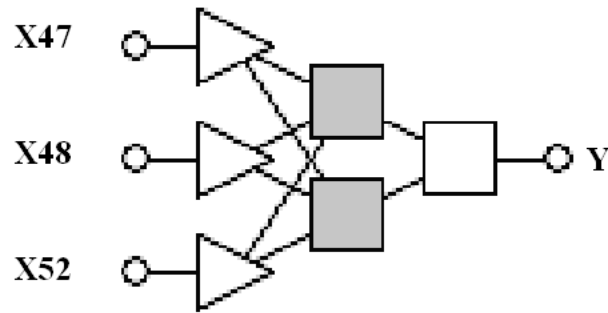
Figure 1. The architecture of the nonlinear neural network model for predicting the risk of dysbiosis development according to NBI (triangles indicate the neurons of the input layer, gray squares indicate the neurons of the hidden layer, and a white square indicate the neuron of the output layer)

Upon optimization of the model acceptance / rejection threshold, the following was obtained: the sensitivity of this model on the training set was 100% (95% VI 98.8% –100%), specificity 69.0% (95% VI 58.6% –78, 6%), for the confirming set the model sensitivity was 100% (95% VI 88.8% –100%), specificity 85.7% (95% VI 60.8% 99, 0%). The sensitivity and specificity on the training and test sets were not statistically significantly different (p> 0.99 and p = 0.34, respectively, when compared by the χ^2 criterion), which indicated the adequacy of the built model.

To assess the models prognostic characteristics, the method of constructing operating characteristic curves (ROC curves – Receiver Operating Characteristic Curve) of models (Fig. 2) was applied.
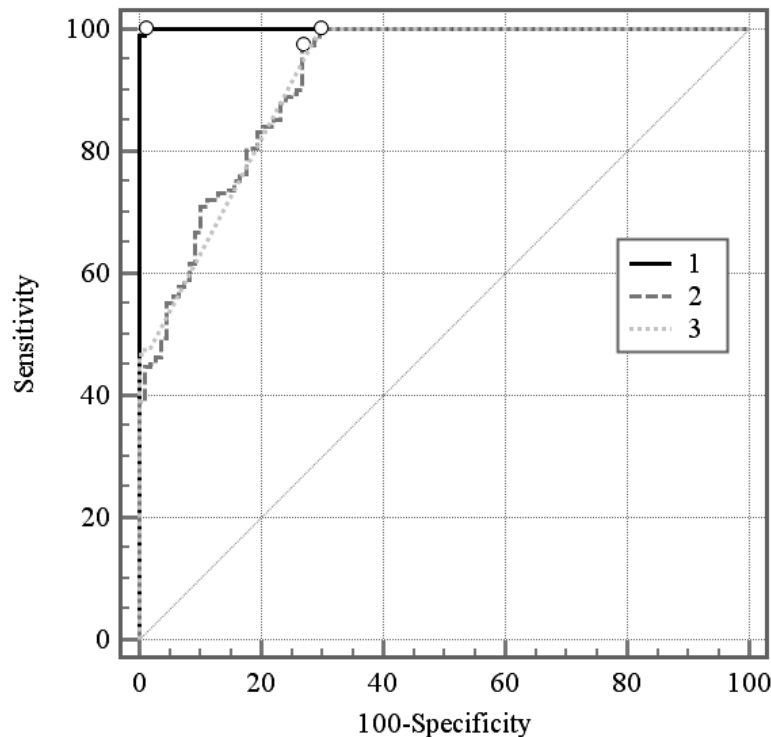

Figure 2. ROC-curves of the dysbiosis risk predicting models according to NBI
1 – Model built on all 58 factor signs; 2 – Linear neural network model, built on three selected factor signs, 3 – Nonlinear neural network model, built on three factor signs

When conducting the analysis, the area under the ROC curve of the linear neural network model built on all 58 factor signs, AUC1 = 1.00 (95% VI 0.99–1.00), was statistically significantly (p <0.001) different from 0.5. The area under the ROC curve of the linear neural network model built on three marked factor signs AUC2 = 0.92 (95% VI 0.88–0.95) was statistically significantly (p <0.001) different from 0.5. The area under the ROC curve of the nonlinear neural network model built on three marked factor signs AUC3 = 0.92 (95% VI 0.88–0.95), was statistically significantly (p <0.001) different from 0.5. Since the quality indicators of the linear and nonlinear models did not actually differ, a simpler linear model was left for the further analysis.

To identify the strength and direction of the three selected factor signs effect, the logistic regression model was analyzed; the model turned out to be adequate (χ^2 = 221.4 at p <0.001). The coefficients analysis results are shown in (Table 2).

Table 2. – Coefficients of the three-factor model for predicting the risk of dysbiosis development according to normobiota indicator (logistic regression model)

Factorial sign	The forecasting model coefficients values, b ± m	Differences significance level from 0	OR (95% CI OR)
X47	0.20±0.07	0.002*	1.22 (1.08–1.39)
X48	−0.31±0.17	0.070	–
X52	0.10±0.03	0.001*	1.11 (1.04–1.18)

Notes: OR – odds ratio; CI – confidence interval

From the logistic regression model coefficients analysis, it follows that the risk of dysbiosis development according to NBI increases statistically significantly (p = 0.002) with the IL2 level increase in the blood (OR = 1.22; 95% VI 1.08–1.39, per unit; ng/ml). The dysbiosis development increase risk (p = 0.001) according to NBI was also established with the increase in the level of TNFα in the blood (OR = 1.11; 95% VI 1.04–1.18, per unit; ng/ml).

Earlier, we found [17, 18] that the level of all proinflammatory cytokines in the blood, which include IL2 and TNFα, was increasing with dysbiosis degree increase, and reached a maximum in BV (when compared with normocenosis in 3.0 and 3, 6 times, respectively; p <0.001). At the same time, it was shown that the activation of the "inteleukin cascade" was both systemic and local in nature, and the systemic (in terms of levels increase) turned out to be 1.5–2 times higher.

In the blood, the level of anti-inflammatory cytokine – IL4 decreased in subgroups in accordance with the dysbiosis stage, which was maximally expressed in BV (5.5 times) [17]. In general, the level of pro-inflammatory cytokines, as opposed to proinflammatory ones, with the BV development decreased sharply, and not only in the blood, but also in the vaginal secretion. Maybe that's why the significance level of differences from the 0 coefficient of the logistic regression model for the factor sign X48 (IL4) turned out to be statistically insignificant (p = 0.07; see Table 2).

The main reason for the BV development is the formation of local immunodeficiency, which reduces the vaginal secretions colonization resistance, antimicrobial substances impaired secretion and providing local immune defense [4, 6]. In addition to local, immunodeficiency with BV also acquires a systemic character [8].

With bacterial dysbiosis, increased levels of proinflammatory interleukins are found in the vaginal secretion [9], which correlates with the increase in the number of Gardnerella Vaginalis and Mycoplasma hominis [10]. According to data [20], *Gardnerella vaginalis* is able to reduce the cytokine-inhibiting function of the dendritic cells of the vaginal mucosa, which leads to the atypical weak inflammatory

response. Moreover, BV-associated bacteria *Megasphaera elsdenii* and *Prevotella timonensis* induce dendritic cell maturation and increase proinflammatory cytokine levels [21]. *Prevotella timonensis* causes the immune response development mainly by the cellular type, as it promotes the differentiation of type 1 T-helpers (Th1). *Prevotella* also activates the type 2 Toll-like receptor, which leads to the production of Th17-polarized cytokines by antigen-presenting cells, including IL-23 and IL-1, and also stimulates the recruitment of neutrophils [22, 363–374].

Thus, the BV-associated microflora affects the cytokine-producing function of dendritic cells through the NF-κB signaling pathway and lymphocyte recruitment due to activation of pro-inflammatory cytokine products [23, 965–76]. On the other hand, *in vitro* studies have shown that pro-inflammatory cytokines in high concentrations characteristic of BV stimulate the growth of opportunistic microorganisms [24, 75–78].

All these facts explained the presence of a significant relationship of the three-factor model participants for dysbiosis development risk predicting according to NBI (IL2, IL4, and TNFα). It should be noted that almost all of these properties, to one degree or another, were inherent in other indicators of the immune system during the vaginal dysbiosis development. Moreover, it is obvious that, as prognostic indicators, one could expect the involvement of effector factors of colonial vaginal resistance, which directly bind or destroy bacterial antigens – lysozyme, complement components, sIgA, LPA, CD8 +, CD16 +, and others that took part in this research (see Table 1). The establishment of individual cytokines as prognostic factors to a certain extent is an unexpected fact.

From our point of view, this situation is explained by the formation of a pathological hormonal-immune system, which is formed under the conditions of vaginal dysbiosis progression and supports its development. That is why the "interleukin cascade" indicators, which objectively reflect regulatory violations, come to the fore, and BV, accordingly, can be considered as a dysregulatory pathology. It is a violation of regulatory systems that causes the progression of dysbiosis and its transition to BV.

In this regard, one can give the study results [25], in which the concept of the role of axis functioning disorder of the hormonal-microbiome-immune system in BV was formulated. According to the facts established by us, the reflection of such violations revealed cytokine factors – the content in the blood of IL2, IL4 and TNFα.

Conclusions

1. By applying the neural network analysis using the genetic ALT algorithm, three factors that are most associated with the risk of dysbiosis were selected: blood levels of IL2, IL4 and TNFα.

2. The risk of dysbiosis development due to NBI was statistically significant (p = 0.002) increased with the increase in blood IL2 content (OR = 1.22; 95% VI 1.08–1.39, per unit; ng / ml). The increase (p = 0.001) in the risk of dysbiosis development according to NBI was also established with the increase in the level of TNFα in the blood (OR = 1.11; 95% VI 1.04–1.18, per unit; ng/ml).

3. With the dysbiosis development, a pathological regulatory hormonal-immune system is formed, which contributes to its progression. In accordance with this, BV can be considered as a dysregulatory pathology, and the revealed "interleukin cascade" indicators are its markers.

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