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The Immune Profile of the Endometrium in the "Uterine Factor" of Infertility

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Abstract

Background: This study aimed to investigate the endometrial characteristics (pathomorphological and immunological) of women with infertility.

Methods and Results: Data from an immunohistochemical study of endometrial biopsies (TNF-α, IL-10, GM-CSF, CXCL16, BCA1, TGF-β1) collected during the "implantation window" and microbiota studied by real-time polymerase chain reaction in 171 patients (21 women with unexplained infertility, 36 - chronic endometritis, 74 - tubal-peritoneal infertility, 22 - external genital endometriosis, 8 - "thin" endometrium, and 10 healthy fertile women from the comparison group) were analyzed to identify molecular signatures. Chronic endometritis was verified morphologically and immunohistochemically.

Each group revealed different immune endometrial phenotypes. The basis of the "normal" phenotype was a controlled immune inflammation and a *Lactobacillus*-dominant microbiota (LDM) type. In contrast to the comparison group, in the group with the phenotype of chronic inflammation, an excessive immune response (overexpression of TNF-α, GM-CSF, CXCL16, BCA1, and a decrease in IL-10 and TGF-β1 in glandular epithelium and stroma) was determined on the background of non-*Lactobacillus*-dominated microbiota (NLDM) type (63.3%) (P<0.001). The peculiar feature of a dysplastic phenotype was a "poor" immune response, with maximal TGF-β1 overexpression (P<0.001) and a NLDM type (47.1%). We determined an excessive immune response in the proliferative endometrial phenotype (GM-CSF overexpression by 1.2 times in the glandular epithelium and stroma [P<0.001 in both cases] and a decrease in IL-10 by 1.6 times in the glandular epithelium and 1.2 times in the stroma [P<0.001 in both cases]). Uterine microbiome disorders were detected less frequently than in patients with the inflammation phenotype (31.6%) (P=0.01). In the phenotype with impaired immune status, there was a decrease in GM-CSF, BCA1, CXCL16, TNF-α, and IL-10 markers in both endometrial compartments (P<0.001) with a LDM type (81.2%).

Conclusion. The molecular signatures of the endometrium are due to the heterogeneity of immune factors and microbiota. Aberrant expression of immune factors may contribute to the formation of a microenvironment unfavorable for blastocyst implantation.**(International Journal of Biomedicine. 2023;13(4):255-260.)**

Keywords: infertility • implantation window • molecular phenotype • cytokines • endometrium

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Abbreviations

CE, chronic endometritis; **EP**, endometrial polyp; **EGE**, external genital endometriosis; **H-score**, Histo-score; **LDM**, *Lactobacillus*-dominated microbiota; **NLDM**, non-*Lactobacillus*-dominated microbiota; **RT-PCR**, real-time polymerase chain reaction; **TPI**, tubal-peritoneal infertility; **UI**, unexplained infertility.

Introduction

Over the past three decades, progress has been made in understanding the mechanisms of interaction between the endometrium and a genetically and immunologically distinct embryo. The immunological dialogue during the "implantation window" occurs under conditions of decidual transformation of fibroblast-like cells of the endometrial stroma.(1) The number, type, and activity of immune cells in intercellular signaling during endometrial remodeling, decidualization and implantation of blastocysts is regulated by ovarian steroid hormones, 17ß-estradiol and progesterone.(2) The receptive endometrium during the "implantation window" is capable of expressing cytokines, chemokines, growth factors, and adhesion molecules that contribute to the creation of an inflammatory environment and trophoblast migration.(3) The heterogeneity of data on the features of the immune system regulation leading to infertility and implantation failures is associated with complex interactions of molecular mediators during the "implantation window."

Most studies are limited to stating a violation of the optimal proinflammatory immune environment for implantation; however, the role of the microbiota in the reactions of the interaction of the endometrium-local immunity system is poorly understood.(4) The development of gene sequencing technology made it possible to determine the microbial composition of the uterus, whose functional interactions with the endometrium are different in physiological status and diseases.(5) Most studies distinguish "Lactobacillus-dominated microbiota" (LDM) type (>90% of lactobacilli) and "non-*Lactobacillus*-dominated microbiota" (NLDM) (<90% of lactobacilli, >10% of other bacteria).(6) The molecular mechanisms of the influence of microbial diversity on the blastocyst implantation process remain unclear due to the paucity and inconsistency of data on the regulation of endometrial remodeling and receptivity.(7)

This study aimed to investigate the endometrial characteristics (pathomorphological and immunological) of women with infertility.

Material and Methods

We performed a prospective examination of 171 women of reproductive age with infertility, including after ineffective attempts of in vitro fertilization. The following groups were identified: 21 patients with unexplained infertility (UI) (Group UI), 36 patients with chronic endometritis (CE) (Group CE), 74 patients with tubal-peritoneal infertility (TPI) (Group TPI), 22 patients with external genital endometriosis (EGE) (Group EGE), and 8 patients with "thin" endometrium (TE) (Group TE). The comparison group consisted of 10 healthy fertile women.

Criteria for inclusion in the research were age from 25 to 40, verified CE (morphologically and immunohistochemically (CD 138+)), TPI, infertility on the EGE background, and the woman's informed consent for participation in research.

The examination of women included an assessment of complaints, anamnesis, general and gynecological examination, and standard laboratory examination (clinical and biochemical blood analysis, general urine analysis, and hemostasiogram).

With sonographic signs of CE, endometrial polyp (PE), and endometrial hyperplasia, hysteroscopy with biopsy sampling for morphological examination was performed on Days 7-9 of the menstrual cycle).

In the phase of the "implantation window" (on Days 20-22 of the menstrual cycle), 6-8 days after the peak of ovulation), aspiration Pipelle biopsy of the endometrium was performed.

Immunohistochemical data were crucial for distinguishing molecular phenotypes of the endometrium. Pathomorphological and immunohistochemical examination of the endometrium was performed according to the standard procedure. The obtained biopsies were fixed with a 10% buffered formalin solution for 24 hours, followed by standard histological wiring and paraffin-embedding procedures. Histological sections with a thickness of 4 microns were made using Sakura rotary microscopes and stained with hematoxylin and eosin. The study of the preparations was carried out using a light microscope with an increase from ×50 to ×1000.

Immunohistochemical examination of the endometrium was performed in the "implantation window" phase (luteinizing hormone peak (+ 7)) to assess the expression of cytokines, chemokines, growth factors: TNF-α, IL10, GM-CSF, and CXCL16 in the epithelium of the glands and stroma, BCA1 in the glandular epithelium, and TGF-β in the stroma. The analysis of the results was carried out considering the number of stained cells and the intensity of their staining. H-score was calculated according to the formula: H-score = $\Sigma(Pi \times i)$, where *Pi* is the percentage of stained cells for each intensity (from 0% to 100%), *i* is the intensity of staining with a value of 0 (no evidence of staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). This score, therefore, is in the range of 0 to 300. The analysis of the results of the study with antibodies to TGF-β1 was performed only in the endometrial stroma by a semi-quantitative method by assessing the number of positive cells, regardless of the intensity of staining. Data was interpreted as follows: 0 (no positive stromal cells), 1+ (cell count of ≤ 24%), 2+ (cell count from 25% to 49%), and 3+ (cell count of ≥ 50%). The preparations were studied using a Leica DMLB light microscope with a standard set of optics.

The proliferative activity of the endometrium was assessed based on the expression of Ki-67 nuclear protein in epithelial cells and stroma.

The material was taken from the uterine cavity for microbiological examination with a double-cavity catheter for embryo transfer after the cervix was treated with a swab soaked in chlorhexidine solution. The exclusion of contamination of the material by microorganisms from the vagina and cervical canal was achieved by extending the inner part of the catheter into the uterine cavity, after which it was immersed in the outer part, then the system was removed. Endometrial samples were examined by RT-PCR (Femoflor 16 tests, NPO 50 DNA Technology LLC (Russia)) to assess the content of lactobacilli, opportunistic pathogens (*Mycoplasma hominis* and *Ureaplasma urealyticum* + *Ureaplasma parvum*) and pathogenic microorganisms (*Mycoplasma genitalium*) in genome-equivalent units (GE/ml) on the IQ5 Multicolor Real-Time PCR Detection System of BIO-RAD (USA). The microbial load was considered positive

when microorganisms in the samples were detected in an amount of more than 10^3 GE/ml.

The study was carried out in accordance with the Helsinki Declaration of the World Medical Association and approved by the Ethics Committee of the Medical Institute at the Peoples' Friendship University of Russia (RUDN University).

Statistical analysis was performed using the statistical software package SPSS version 22.0 (SPSS Inc, Armonk, NY: IBM Corp). The normality of the distribution of continuous variables was tested by the Shapiro-Wilk test. The results are presented as median (Me) and interquartile range (IQR [Q1; Q3]). The Mann-Whitney U test and Kruskal-Wallis test were used, respectively, to compare differences between 2 and 3 or more independent groups. Group comparisons with respect to categorical variables were performed using Pearson's chi-squared ($\chi 2$) test with Yates correction or, alternatively, Fisher's exact test when expected cell counts were less than 5. A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

The average age of women with infertility in the groups did not significantly differ: 31.6 (25.2;32.8) years in Group UI, 33.6 (28.2; 37.2) years in Group CE, 32.8 (27.6;36.4) years in Group TPI, 33.4 (29.2;37.2) years in Group EGE, and 31.2 (26.2; 35.4) years in Group TE. The average age of women in the comparison group was 30.6 (28.5; 36.6) years.

The basis for identifying endometrial phenotypes was immunohistochemical studies, considering the data of hysteroscopy and pathomorphological examination. Molecular signatures of the endometrium were detected: the impaired immune status phenotype (n=10) and dysplastic phenotype (n=11) in Group UI; the proliferative phenotype (n=12), chronic inflammation phenotype (n=20), "normal" phenotype (n=12) (indicators are identical to those in the comparison group), dysplastic phenotype (n=18), and impaired immune status phenotype (n=12) in Group TPI; the impaired immune status phenotype (n=10) and proliferative phenotype (n=12) in Group EGE; the chronic inflammation phenotype (n=10), proliferative phenotype (n=8), proliferative phenotype in combination with endometrial polyp (EP) (n=8), and dysplastic phenotype (n=10) in Group CE; and dysplastic phenotype (n=8) in Group TE. The indicators of healthy fertile women (the comparison group, n=10) were chosen as the reference.

Grouping of the selected variants showed that the basis of the "uterine factor" of infertility were the following phenotypes: impaired immune status phenotype, proliferative phenotype, dysplastic phenotype, chronic inflammation phenotype, and "normal" endometrium phenotype. The expression profile of markers in which is presented in Figures 1-10.

Women with a "normal" endometrial phenotype were distinguished by the balanced secretion of cytokines with moderate activation of the inflammatory molecular network modulated by stroma cells to control implantation, migration and invasion of the trophoblast. The proinflammatory Th1-immune response is considered as the activity of biologically active substances necessary for blastocyst implantation.[8] The leading role in controlling the network of immunoregulatory molecules is associated with TNF-α overexpression, the development of a local inflammatory response, and the induction of tolerant properties of dendritic cells.[9]

We believe that the predominance of the LDM type in the endometrium should be considered from the standpoint of participation in limiting the inflammatory "immune response" and activating the mechanism of immunological tolerance in the presence of Treg-dominant in the Treg/Th17 ratio.[10] These immune-microbial interactions serve as a criterion for the likelihood of blastocyst implantation. Microbial homeostasis in the endometrium might form not only the resistance to colonization by opportunistic flora, but also the ability to express genes that affect the adequate level of receptors for sex steroid hormones in the "implantation window" phase.[11]

The morphological basis of the dysplastic phenotype of the endometrium was dystrophic-atrophic changes. Immunoregulation disorders in the dysplastic phenotype consisted of a "poor immune response" combined with depletion or blocking of energy substrates. Disorders in the adequate preparation of the endometrium during the "implantation window" are due to a marked decrease in expression, in contrast to the comparison group, both in the glandular compartment of the endometrium (GM-CSF – 3.7 times, TNF-α – twice, IL-10 – 1.8 times, CXCL16 – twice, BCA1 – 2.6 times) and in the stroma (GM-CSF – 2.7 times, TNF-α – 4.6 times, IL-10 – 2.9 times, CXCL16 – 3.3 times, with a maximum TGF-β1). It is reported that the cause of implantation failures may be premature "aging" of the endometrium due to immune "stresses" and inflammatory damage.[12] TGF-β1 overexpression with activation of the nuclear factor signaling pathway NF-kB, TGF-β1/Smad3/Smad7 is believed to be one of the reasons for the formation of intrauterine synechiae.[13] Disorders of cellular metabolism in the dysplastic phenotype in the presence of NLDM type (47.1%) are likely to be revealed as a consequence of exposure to toxic metabolites caused by the persistence of low-immunogenic infections.

The disorders in the histological dating of the endometrium in 21.9% of women with a phenotype of impaired immune status indicate the criticality of taking into account the markers of "maturity," especially in IVF/IVF-ICSI protocols for synchronization with the developing embryo. The imbalances in the endometrial cytokine cascades and absence of proinflammatory Th1 shift during the implantation window (the expression of GM-CSF, BCA1, CXCL16 decreased by 1.4 times, TNF-α – by 1.3 times, IL-10 – by 1.2 times in the glandular epithelium; in the stroma, the expression of GM-CSF, TNF-α, CXCL16 decreased by 1.3 times, IL-10 – by 1.2 times) disrupt the ability of stroma cells to decidualize. Implantation failures are associated with the impairment in immune tolerance caused by a decrease in the number and function of Treg cells.[14]

The phenotype of chronic inflammation was determined by the excess expression of proinflammatory cytokines in the epithelium of the endometrial glands in comparison with anti-inflammatory ones (an increase in TNF–α by 1.1 times, GM-CSF, CXCL16, BCA1 by 1.2 times, a decrease in IL-10 by 2 times). In the endometrial stroma, the expression level of GM-CSF, TNF-α, CXCL16 was significantly higher than in the control by 1.2 times, and IL-10 was lower by 1.8 times, and the level of TGF-β was the lowest in comparison with other groups.

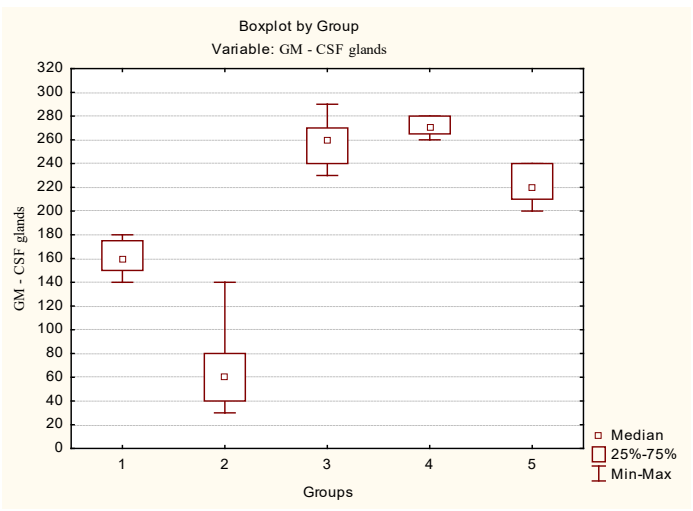

Fig. 1. *Expression of GM-CSF in glandular epithelium.*

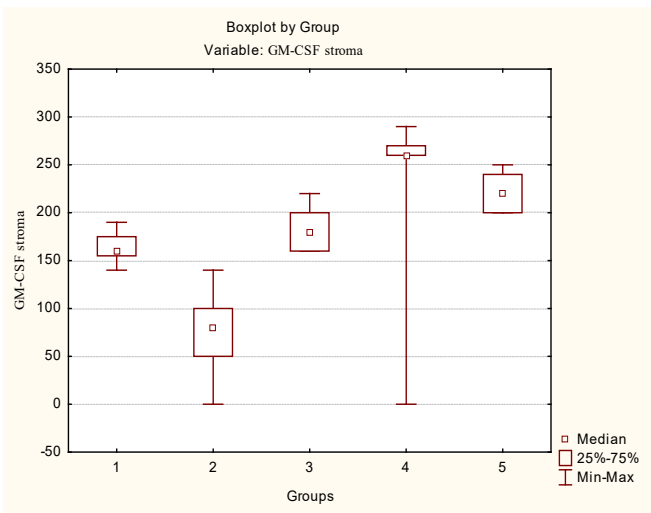

Fig. 2. *Expression of GM-CSF in endometrial stroma.*

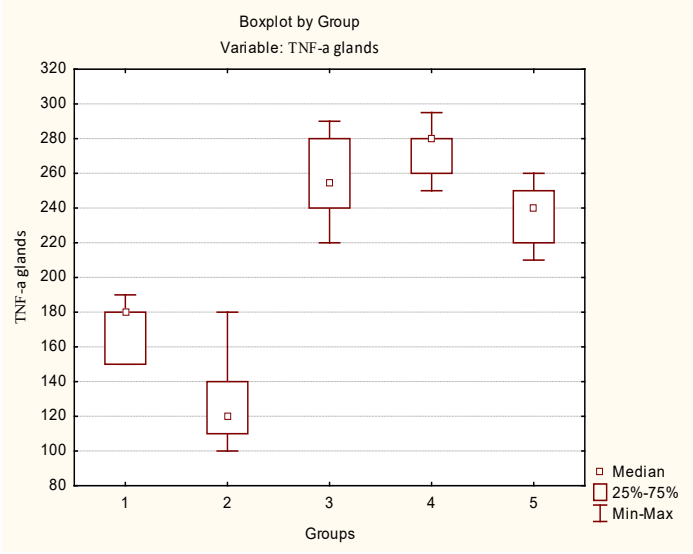

Fig. 3. *Expression of TNF-α in glandular epithelium.*

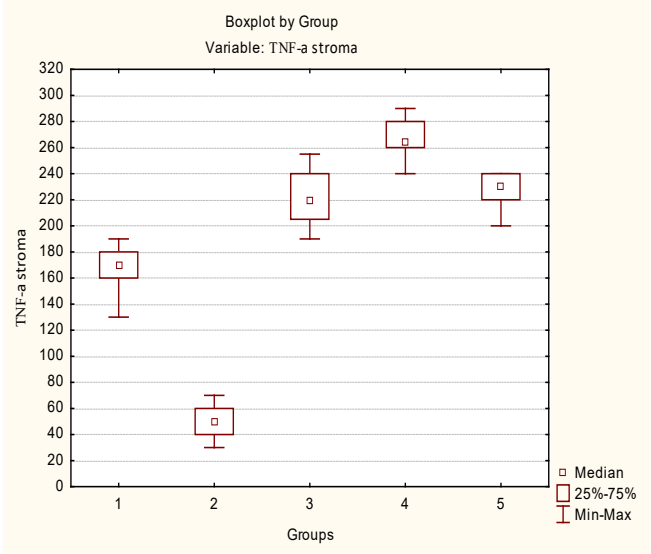

Fig. 4. *Expression of TNF-α in endometrial stroma.*

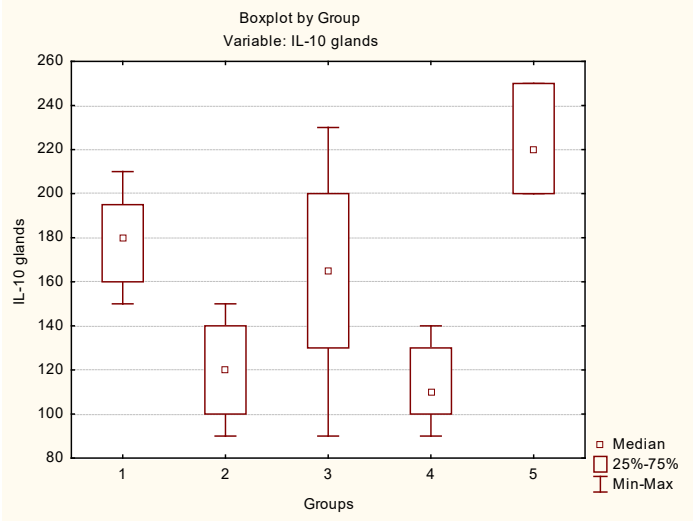

Fig. 5. *Expression of IL-10 in glandular epithelium.*

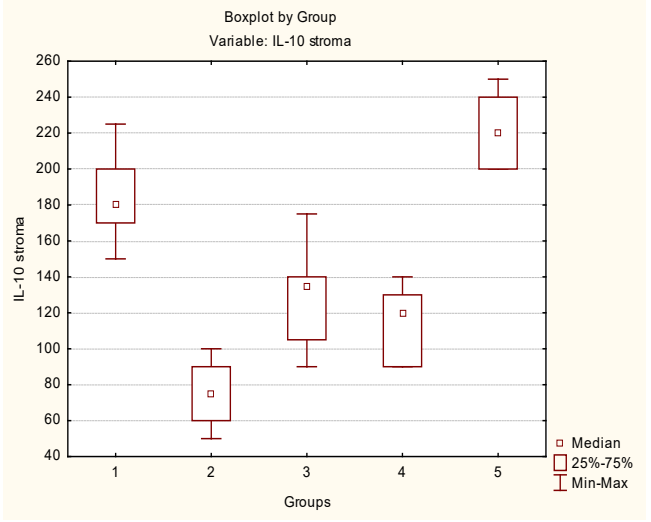

Fig. 6. *Expression of IL-10 in endometrial stroma.*

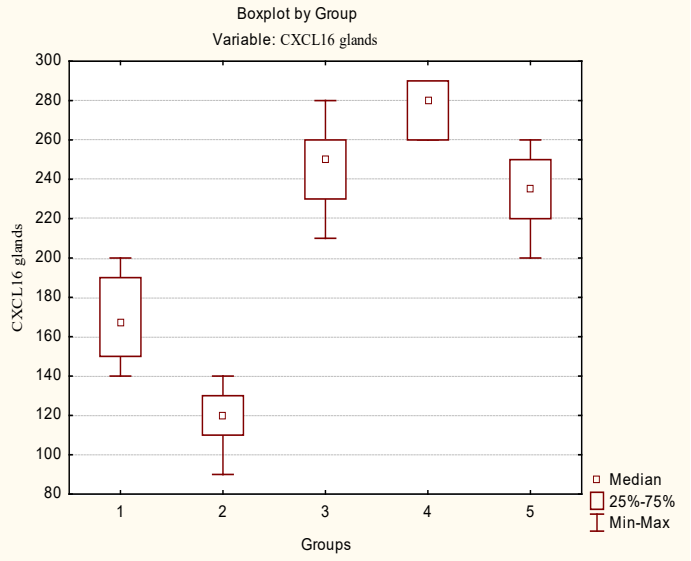

Fig. 7. *Expression of CXCL16 in glandular epithelium.*

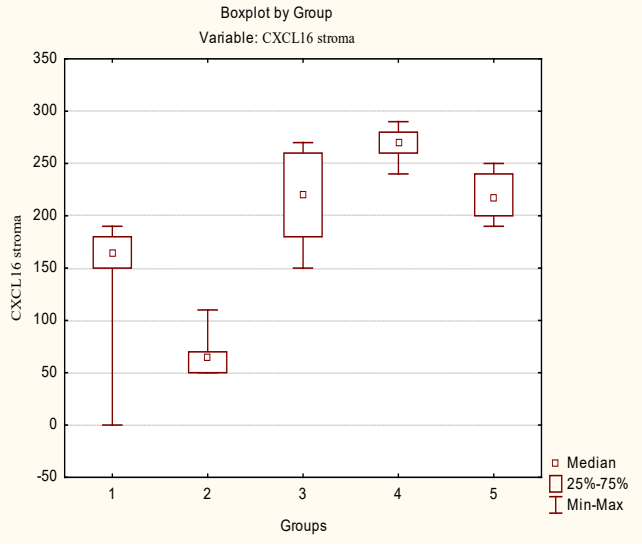

Fig. 8. *Expression of CXCL16 in endometrial stroma.*

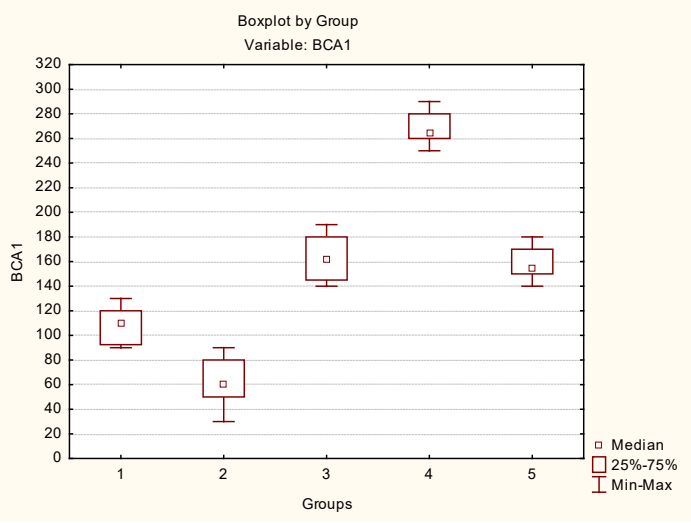

Fig. 9. *Expression of BCA1 in glandular epithelium.*

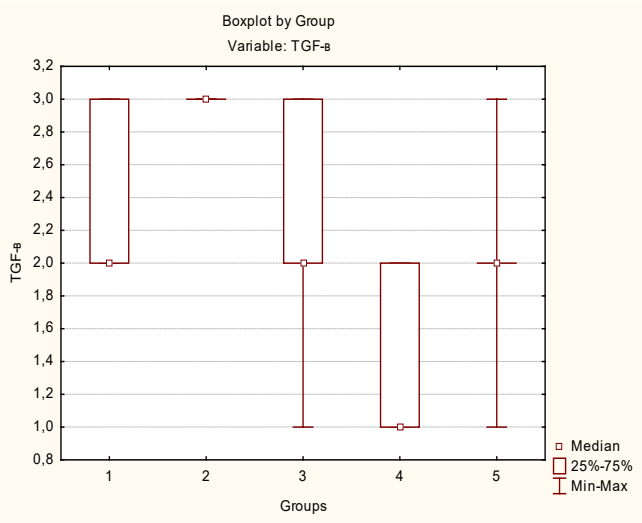

Fig. 10. *Expression of TGF-β in endometrial stroma.*

Figures 1-10. *Groups: 1 – impaired immune status phenotype, 2 – dysplastic phenotype, 3 – proliferative phenotype, 4 – chronic inflammation phenotype, 5 – "normal" endometrium phenotype*

The abnormal immune microenvironment of the "inflamed" endometrium is associated with the introduction of pathogenic bacteria, the main component of whose membrane (lipopolysaccharide) mediates an increase in the level of chemokine CXCL13 (BCA1), inflammation, and recruitment into the stroma of the B cell pool.[15] The infiltration of the stroma by plasma cells leads to changes in the architectonics and dysfunction of the endometrium. A decrease in the expression of TGF-β and IL-10 in CE is believed to be the cause of a quantitative or functional deficiency of the anti-inflammatory clone of Treg cells on the background of an inflammatory stroma reaction, fibrosis, and implantation failures.[16]

The dysbiotic profile of the endometrium in 47.6% of infertile women with CE appears to cause unfavorable molecular mechanisms for implantation. The impairment in microbial homeostasis in CE (the prevalence of a NLDM type in 63.3%, as well as the excessive growth of *Gardnerella vaginalis*, *Ureaplasma* spp. and mixes of *Atopobium vaginae*/*Enterobacteriaceae* in the absence of a lactobacilli bacteria) probably stimulates the overexpression of proinflammatory cytokines that create a microenvironment aggressive for blastocyst implantation. We believe that the revealed heterogeneity of the composition of the uterine microbiome in women with CE explains the heterogeneity of data on reproductive outcomes – from infertility, recurrent implantation failures and miscarriages to successful delivery. Our data complement the ideas about the breakdown of the mechanisms of adaptation to the constant exposure to microbes in CE and, as a result, the inadequacy of the immune response and the "vicious circle" of chronic inflammation.

The molecular profile of women with a proliferative endometrial phenotype was characterized by the immunomodulatory activity of GM-CSF (increased expression

by 1.2 times) on the background of moderate overexpression of TNF-α, CXCL16 (1.1 times) and a decrease in IL-10 (1.3 times) in the glandular epithelium. In the endometrial stroma, significant differences from the comparison group were revealed only in relation to GM-CSF – by 1.2 times and IL-10 – by 1.6 times**.** The imbalance in local cytokine production confirms the participation in the formation of a proliferative phenotype of the endometrium not only of proinflammatory cytokines (GM-CSF, TNF-α, CXCL16), the overexpression of which is associated with an increase in cytotoxic T cells, but also impairments in local steroidogenesis.

With a proliferative endometrial phenotype, disorders of the uterine microbiome were less common than with chronic inflammation (NLDM type - 31.6%). A decrease in lactobacilli level with a relative increase in the levels of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Bacteroides*, *E. coli,* and *Bacteroides fragilis* was observed in hyperplastic processes of the endometrium.[17] The participation of microbiota and immune-inflammatory "networks" in the genesis of focal endometrial hyperplasia is proposed to be considered through an increase in the activity of the β-glucuronidase enzyme in the presence of certain bacteria, followed by an increase in the level of local estrogens.

Conclusion

CE was verified in 57.1% of women. In the regulation of complex mechanisms of implantation, the participation of molecular interactions of microbiota (LDM and NLDM types) and immunocompetent mediators (cytokines, chemokines, growth factors), predictive of impaired decidual transformation and expression of genes involved in the regulation of endometrial receptivity, has been revealed.

Competing Interests

The authors declare that they have no competing interests

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